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Syngnathid fishes: Biology, ecology, physiology, conservation and innovative rearing techniques

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Syngnathid fishes: Biology, ecology, physiology, conservation and innovative rearing techniques

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Editorial: Syngnathid fishes: biology, ecology, physiology, conservation and innovative rearing techniques

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Editorial on the Research Topic
Syngnathid fishes: biology, ecology, physiology, conservation and innovative rearing techniques

Syngnathids (a bony fish family that includes seahorses, pipefishes and seadragons) have unique morphological and biological traits that include fused jaws, an exoskeleton and male parental care (Ahnesjö and Craig, 2011). They are mostly found in shallow coastal areas, and their unconventional life history renders them particularly vulnerable to population depletion (Foster and Vincent, 2004). Although some species have been listed as Vulnerable, Endangered, and even Critically Endangered by the IUCN Red List of Threatened Species, many are classified as Data Deficient (DD) due to insufficient information (IUCN, 2020; Pollom et al., 2021). Therefore, further research is needed to understand their distribution, population trends and main threats. A significant portion of the research on syngnathids is relatively recent (Cohen et al., 2017; Segaran et al., 2023). This Research Topic provides 12 original research and review contributions that provide an overview of current trends in research related to the biology, physiology, ecology and conservation of syngnathids.

We have gained considerable insights into syngnathid evolutionary innovation, particularly concerning the evolution of male pregnancy (Lin et al., 2016; Roth et al., 2020). The review by Parker et al. sheds light on the unique modification of the syngnathid immune system and its associations with physiology and reproduction, and highlights future research objectives.

Climate change forces species to extend their physiological tolerance limits, or undergo range shifts. Efforts to minimize future biodiversity losses should consider: (1) species' distributions correlated with environmental conditions (Mascaró et al., 2016; Hernández-Urcera et al., 2021; Pierri et al., 2022; Borges et al., 2023), (2) adaptive mechanisms responding to environmental changes (Aurélio et al., 2013; Faleiro et al., 2015; Qin et al., 2018; Carneiro et al., 2021; Carneiro et al., 2022; Gomes et al., 2023), and (3) prediction and modeling of future environmental scenarios for species distribution.

Most syngnathids inhabit variable environments and face anthropogenic threats, resulting in habitat loss and population declines. Of particular concern is the use of dried syngnathids in Traditional Chinese Medicine (TCM) (Vincent et al., 2011). Seahorses, in particular, are believed to be nutritionally beneficial for humans, and Cabral et al. focused on the nutritional value of cultured *Hippocampus hippocampus*. The relative fatty acid composition was similar to that of other seahorse species valued in TCM, with males exhibiting a more suitable profile for human consumption.

Information on stressors is required for estimating future distributional shifts under a global warming scenario. Current knowledge on threats is limited (McCauley et al., 2015), but attempts have been made to estimate its cumulative human impacts. High-accuracy models predicted that 5 of 17 DD seahorses are threatened, and indicated that bycatch and pollution are the best predictors of threat category (Zhang and Vincent, 2019). Silveira et al. evaluated bycatch captures of *H. patagonicus* in trawling fisheries in Brazil, and reported annual incidental captures exceeding 2 million seahorses (6 individuals/day/vessel). Conservation management at a fine scale should consider local ecological knowledge and social perceptions of human communities that depend on wild syngnathid resources, especially for data-poor species. Fontelles Ternes et al. investigated the perception of seahorse-watching operators regarding abundance, local threats, and conservation insights in Brazilian mangrove ecosystems. All survey participants considered seahorses to be threatened (55% highlighted declines in abundance) and proposed environmental surveillance, zoning and education as the main conservation actions to limit the further decline of seahorse populations.

Shallow coastal areas, such as estuaries and lagoons, receive chemicals that influence the reproduction of aquatic animals. Long-term exposure to low levels of endocrine-disrupting chemicals can cause population collapse. When studying the brood pouch transcriptome in male Syngnathus scovelli during synthetic estrogen exposure, Rose et al. (2023) observed female-specific ornamentation, and identified candidate brood pouch genes as future biomarkers. Changes in iridescence, an important sexual signal in several pipefish species (Rosenqvist and Berglund, 2011), may disrupt sexual selection (Sárria et al., 2011). With an algorithm, Tosto et al. aimed to detect geographical variation in female ornamentation of *S. scovelli*, indicating that external factors such as chemicals could affect the strength of sexually selected iridescence signals.

The effects of global warming were experimentally investigated in adult *H. guttulatus* by Costa et al. In agreement with previous findings in juveniles (Planas et al., 2012), no increased growth gain was identified under extreme temperature increases (24°C), despite higher activity and food consumption. Although thermal stress at extreme temperatures could not be confirmed, Del Vecchio et al. (2022) reported that prolonged exposure of *H. erectus* to even sublethal temperatures resulted in the accumulation of injurious effects, resulting in cell death. Furthermore, Aurélio et al. (2013) reported great resilience to heat stress in adult *H. guttulatus*. However, early life history stages displayed greater thermal sensitivity with potential cascading consequences for their growth and survival. Faleiro et al. (2015) suggested that future ocean changes might further threaten seahorse conservation as a consequence of the combined effects of warming and acidification. Monteiro et al. identified areas of environmental suitability for European syngnathids over the next century. Particularly dramatic changes are predicted within semi-enclosed seas (e.g., the Baltic, Mediterranean and Black Sea), resulting in the northward range expansions.

The distribution of syngnathids is linked to vegetation cover, which determines feeding ecology (Moreau and Vincent, 2004; Teske et al., 2007; Borges et al., 2023). The interaction and competition between sympatric species might select for behavioral changes, as shown experimentally for European seahorses by Spatafora et al. (2023). The authors concluded that an increased density of the seahorse *H. guttulatus* resulting from greater habitat availability affected the behavior of *H. hippocampus*, reducing its activity, and modifying the distributions of both species. Most syngnathids feed on small prey, mainly crustaceans, but show local trophic adaptations, depending on both prey availability and snout characteristics (Manning et al., 2019). Although gut content analysis is useful for assessing dietary regimes, the analysis of stable isotopes provides potentially more precise information on the contribution of food sources to the diet (Peterson and Fry, 1987). However, Planas reported variability of estimates in the reconstruction of the dietary regime in *S. acus*, depending on the time lag between the sampling of both potential dietary resources and target fishes. Tamara et al. elucidated the diet of *H. guttulatus* using a non-invasive DNA metabarcoding technique, improving our understanding of habitat-specific prey selection. Diet composition differed between habitats, as did trophic flexibility, which may be essential for the resilience of this species. Due to some limitations of metabarcoding compared to metagenomics because not all prey species may be detectable using the former, Serite et al. compared both techniques to assess the diversity of prey items in two estuarine pipefishes, *S. watermeyeri* and *S. temminckii*. In addition to determining that dietary competition is limited because each pipefish has a preferred type of prey, their findings suggest that metagenomics may, in some cases, outperform metabarcoding.

Syngnathids face a range of challenges and threats. This Research Topic of research and review articles provides valuable insights, and highlights key areas for future research. Although interesting advances have been made in the knowledge of syngnathids' biology and ecology, much research is still needed to safeguard these fascinating creatures. Advancing our knowledge of syngnathids requires generating data by means of a multidisciplinary approach encompassing several key areas of research that should be prioritized in the future: population assessment, distribution, habitat use and niche partitioning, bycatch mitigation, molecular research (particularly genomics), species interactions, habitat protection and restoration, climate change impact and resilience, aquaculture and husbandry, conservation and community engagement, and legal protection. Further advances on these topics are fundamental prerequisites for formulating effective and rational conservation strategies for the family Syngnathidae.

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References

Ahnesjö, I., and Craig, J. F. (2011). The biology of Syngnathidae: pipefishes, seadragons and seahorses. *J. Fish Biol.* 78, 1597–1602. doi: 10.1111/j.1095-8649.2011.03008.x

Aurélio, M., Faleiro, F., Lopes, V. M., Pires, V., Lopes, A. R., Pimentel, M. S., et al. (2013). Physiological and behavioral responses of temperate seahorses (*Hippocampus guttulatus*) to environmental warming. *Mar. Biol.* 160, 2663–2670. doi: 10.1007/s00227-013-2259-8

Borges, A. K. M., Alves, R. R. N., and Oliveira, T. P. R. (2023). Mapping seahorses in a Brazilian estuary: mangrove structures as key predictors for distribution and habitat preference. *PeerJ* 11, e15730. doi: 10.7717/peerj.15730

Carneiro, M. D., García-Mesa, S., Sampaio, L. A., and Planas, M. (2021). Primary, secondary, and tertiary stress responses of juvenile seahorse *Hippocampus reidi* exposed to acute acid stress in brackish and seawater. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 255, 110592. doi: 10.1016/j.cbpb.2021.110592

Carneiro, M. D. D., García-Mesa, S., Sampaio, L. A., and Planas, M. (2022). Implications of salinity and acidic environments on fitness and oxidative stress parameters in early developing seahorses *Hippocampus reidi*. *Animals* 12, 3227. doi: 10.3390/ani12223227

Cohen, F. P. A., Valenti, W. C., Planas, M., and Calado, R. (2017). Seahorse aquaculture, biology and conservation: knowledge gaps and research opportunities. *Rev. Fish. Sci. Aquac.* 25, 100–111. doi: 10.1080/23308249.2016.1237469

Del Vecchio, G., Galindo-Sánchez, C. E., Tripp-Valdez, M. A., López-Landavery, E. A., Rosas, C., and Mascaró, M. (2022). Transcriptomic response in thermally challenged seahorses Hippocampus erectus: The effect of magnitude and rate of temperature change. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 262, 110771. doi: 10.1016/j.cbpb.2022.110771

Faleiro, F., Baptista, M., Santos, C., Aurélio, M. L., Pimentel, M., Pegado, M. R., et al. (2015). Seahorses under a changing ocean: the impact of warming and acidification on the behaviour and physiology of a poor-swimming bony-armoured fish. *Conserv. Physiol.* 3, 1–77. doi: 10.1093/conphys/cov009

Foster, S. A., and Vincent, A. C. (2004). Life history and ecology of seahorses: implications for conservation and management. *J. Fish Biol.* 65, 1–61. doi: 10.1111/j.0022-1112.2004.00429.x

Gomes, M., Lopes, V. M., Mai, M. J., Paula, J. R., Bispo, R., Batista, H., et al. (2023). Impacts of acute hypoxia on the short-snouted seahorse metabolism and behaviour. *Sci. Tot. Environ.* 904, 166893. doi: 10.1016/j.scitotenv.2023.166893

Hernández-Urcera, J., Murillo, F. J., Regueira, M., Cabanellas-Reboredo, M., and Planas, M. (2021). Preferential habitats prediction in syngnathids using species distribution models. *Mar. Environ. Res.* 172, 105488. doi: 10.1016/j.marenvres.2021.105488

IUCN (2020) *2020 IUCN red List of Threatened Species (Online)*. Available at: http://www.iucnredlist.org/.

Lin, Q., Fan, S., Zhang, Y., Xu, M., Zhang, H., Yang, Y., et al. (2016). The seahorse genome and the evolution of its specialized morphology. *Nature* 540, 395. doi: 10.1038/nature20595

Manning, C. G., Foster, S. J., and Vincent, A. C. J. (2019). A review of the diets and feeding behaviours of a family of biologically diverse marine fishes (Family Syngnathidae). *Rev. Fish Biol. Fish.* 29, 197–221. doi: 10.1007/s11160-019-09549-z

Mascaró, M., Amaral-Ruiz, M., Huipe-Zamora, I., Martínez-Moreno, G., Simões, N., and Rosas, C. (2016). Thermal tolerance and phenotypic plasticity in juvenile *Hippocampus erectus* Perr : effect of acute and chronic exposure to contrasting temperatures. *J. Exp. Mar. Biol. Eco.* 483, 112–119. doi: 10.1016/j.jembe.2016.07.005

McCauley, D. J., Pinsky, M. L., Palumbi, S. R., Estes, J. A., Joyce, F. H., and Warner, R. R. (2015). Marine defaunation: animal loss in the global ocean. *Science* 347, 1255641. doi: 10.1126/science.1255641

Moreau, M.-A., and Vincent, A. C. J. (2004). Social structure and space use in a wild population of the Australian short-headed seahorse *Hippocampus breviceps* Peters 1869. *Mar. Freshw. Res.* 55, 231–239. doi: 10.1071/MF03159

Peterson, B. J., and Fry, B. (1987). Stable isotopes in ecosystem studies. *Annu. Rev. Ecol. Syst.* 18, 293–320. doi: 10.1146/annurev.es.18.110187.001453

Pierri, C., Lazic, T., Gristina, M., Corriero, G., and Sinopoli, M. (2022). Large-scale distribution of the European seahorses (*Hippocampus* Rafinesque 1810): a systematic review. *Biology* 11, 325. doi: 10.3390/biology11020325

Planas, M., Blanco, A., Chamorro, A., Valladares, S., and Pintado, J. (2012). Temperature-induced changes of growth and survival in the early development of the seahorse *Hippocampus guttulatus*. *J. Exp. Mar. Biol. Ecol.* 438, 154–162. doi: 10.1016/j.jembe.2012.10.003

Pollom, R., Ralph, G., Pollock, C., and Vincent, A. (2021). Global extinction risk for seahorses, pipefishes and their near relatives (Syngnathiformes). *Oryx* 55, 497–506. doi: 10.1017/S0030605320000782

Qin, G., Johnson, C., Zhang, Y., Zhang, H., Yin, J., Miller, G., et al. (2018). Temperature-induced physiological stress and reproductive characteristics of the migratory seahorse *Hippocampus erectus* during a thermal stress simulation. *Biol. Open* 7, bio032888. doi: 10.1242/bio.032888

Rosenqvist, G., and Berglund, A. (2011). Sexual signals and mating patterns in Syngnathidae. *J. Fish Biol.* 78, 1647–1661. doi: 10.1111/j.1095-8649.2011.02972.x

Roth, O., Solbakken, M. H., Tørresen, O. K., Bayer, T., Matschiner, M., Baalsrud, H. T., et al. (2020). Evolution of male pregnancy associated with remodeling of canonical vertebrate immunity in seahorses and pipefishes. *Proc. Natl. Acad. Sci. U.S.A* 117, 9431–9439. doi: 10.1073/pnas.1916251117

Sárria, M. P., Santos, M. M., Reis-Henriques, M. A., Vieira, N. M., and Monteiro, N. M. (2011). The unpredictable effects of mixtures of androgenic and estrogenic chemicals on fish early life. *Environ. Intern.* 37, 418–424. doi: 10.1016/j.envint.2010.11.004

Segaran, T. C., Aouissi, H. A., Noor, M. I. M., Wahid, M. E. A., Lananan, F., Petrisor, A.-I., et al. (2023). Assessing the state of seahorse research through scientometric analysis: an update. *Rev. Fish Biol. Fisheries.* 33, 1237–62. doi: 10.1007/s11160-023-09794-3

Teske, P. R., Lockyear, J. F., Hecht, T., and Kaiser, H. (2007). Does the endangered Knysna seahorse, *Hippocampus capensis*, have a preference for aquatic vegetation type, cover or height? *Afr. Zool.* 42, 23–30. doi: 10.1080/15627020.2007.11407373

Vincent, A. C. J., Foster, S. J., and Koldewey, H. J. (2011). Conservation and management of seahorses and other Syngnathidae. *J. Fish Biol.* 78, 1681–1724. doi: 10.1111/j.1095-8649.2011.03003.x

Zhang, X., and Vincent, A. C. J. (2019). Using cumulative human-impact models to reveal global threat patterns for seahorses. *Conserv. Biol.* 33, 1380–1391. doi: 10.1111/cobi.13325

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Was that my meal? Uncertainty from source sampling period in diet reconstruction based on stable isotopes in a syngnathid fish

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Stable isotopes can be used to reconstruct diet in fishes, an approach that is particularly useful in threatened or endangered species where lethal sampling needs to be avoided To apply this method stable isotopes need to be measured both in the consumers and dietary sources As turnover rates may largely differ across tissues, the reconstruction should consider the uncertainty associated with sampling timing for both consumers and dietary sources. The present study investigated for the first time the application of a time lag for the estimation of the trophic position and the dietary regime in the greater pipefish *Syngnathus acus* (Family Syngnathidae). For that, two isotopic ($\delta^{13}C$ and $\delta^{15}N$) data sets from dorsal fin-clipped tissues were used, including values in potential sources sampled (a) concomitantly with consumers (Unshifted approach) or (b) in the season sampled preceding consumer sampling (Shifted approach). The results attained indicate that the trophic position of the species was not affected (< 0.6% change) by shifting when analyzed for the annual dataset (global assessment). However, the trophic level estimated in the shifted treatment was ca. 6% higher than in the unshifted approach when the analysis was performed using a seasonal dataset (seasonal assessment including exclusively specimens collected in summer). Regarding the reconstruction of the dietary regime and in comparison to the unshifted approach, the results attained with shifted sampling revealed a higher preference for amphipods (43.7%), isopods (19.5%) and mysidaceans (12.1%), and a reduction in the reconstructed abundance for harpacticoid copepods (5.1%). These findings were similar to those attained for specimens and food web components collected in the same sampling period (seasonal dataset), both in females and males. This study demonstrates (1) the variability of estimates in the reconstruction of diet regimes depending on the data time shifting applied, and (2) the importance of considering adequate time lags in the assessment for syngnathid fishes.

KEYWORDS

Syngnathidae, pipefish, *Syngnathus acus*, trophic niche, dietary regime, stable isotopes, time lag

Introduction

The trophic position and dietary regimes of animalscan be analyzed following several approaches, including gut content and fecal material analyses (Hynes, 1950; Hyslop, 1980; Jobling, 1987; Baker et al., 2013), faecal metabarcoding and metagenomics (Barnett et al., 2010; Valentini et al., 2009; Kodama et al., 2020), fatty acid signature analysis (Iverson et al., 2004; Xu et al., 2020), or stable isotope analysis (SIA) (DeNiro and Epstein, 1981; Post, 2002; Caut et al., 2009; Boecklen et al., 2011; McMahon et al., 2011),. The latter is particularly interesting in studies involving vulnerable species, where non-lethal sampling should be achieved, but implies the analysis of target specimens, and their potential dietary resources and baseline resources which are the base of the food web (e.g., particulate organic matter, primary producers, and consumers). Assessments of temporal variation in diets are important for the understanding of the trophic ecology of fishes. Seasonal variation in the relative contribution of dietary resources should be reflected in the isotopic fingerprint of fish fin or muscle tissues.

The advantage of using clips of dorsal fin for SIA is that it is a non-lethal technique, which is highly recommended for vulnerable and endangered species (Sanderson et al., 2009; Valladares and Planas, 2012; Busst et al., 2015). In syngnathids and other fish species, the dorsal fin clips can be used as surrogates of muscle tissues, which provide mid-term information on the composition of the diet (Valladares and Planas, 2012; Valladares et al., 2016). Different tissues respond differently to dietary shifts due to dissimilarities in tissue turnover rates (Tieszen et al., 1983; Madigan et al., 2012; Busst et al., 2015; Vander Zanden et al., 2015). Consequently, isotopic values in fishes do not necessarily mirror their recent dietary regimes. Since different animal tissues incorporate stable isotopes at different rates or in different fractions, the dietary shifts will be reflected differently in tissues (Tieszen et al., 1983; Hobson and Clark, 1992; Dalerum and Angerbjörn, 2005; Jardine et al., 2017). The most straightforward isotopic approach for assessing temporal diet variation is to compare samples from the same type of tissue that has been sampled over time (Dalerum and Angerbjörn, 2005). This approach provides either long (bone, connective tissue, teeth), medium (muscle, scales, fins) or short-term (liver, blood) dietary variation (Tieszen et al., 1983; Madigan et al., 2012; Busst et al., 2015; Vander Zanden et al., 2015). Blood data typically provide the most reliable interpretations for recent diet and migration studies in fishes, whereas white muscle, scales and fins show medium-scale estimates (Guelinckx et al., 2007; Thomas and Crowther, 2015; Davis and Pineda-Munoz, 2016). Hence, available knowledge on the incorporation and turnover rates of stable isotopes in tissues must be considered.

In ecological and experimental studies, the inference of resource usage through stable isotopes is typically estimated from samples of prey and consumers taken concomitantly or over a short time interval. This procedure is often a result of sampling limitations in the field (i.e., time and cost) but might introduce methodological caveat depending on the consumer and the type of tissue used as reference material. Inferring dietary regimes for a fish population from samples collected at different times (i.e., repeated sampling of a proxy over time), concomitantly with source and baseline sampling, might lead to quantitative biases and misrepresentations of diet composition estimates (Possamai et al., 2021). In trophic studies, it is important that the sampling regime effectively captures the temporal and spatial isotopic variation in food sources and target organisms. One of the main uncertainties, when using Bayesian mixing models in diet reconstruction from isotopic data refers, to the periods over which the tissues of interest were synthesized. The consumer tissues have the chance to incorporate and then reflect their isotopic signatures with a delay depending on tissue turnover rates (Hesslein et al., 1993; Martinez del Rio et al., 2009; Wolf et al., 2009). This delay is known as time lag, which also varies depending on the species. The period over which the diet is to be estimated and what tissues best reflect this period are of pivotal importance (Phillips et al., 2014). Time lags are unknown for most species, and they should not be neglected in trophic analyses from isotopic values, especially when assessing seasonal changes (Possamai et al., 2021).

The present study highlights for the first time in syngnathids (Family Syngnathidae) the importance of applying time lags to both isotopic baselines and dietary resources. Incorporating appropriate time lags into stable isotope analyses in trophic studies are important. The time lag applied in the study approximates the isotopic turnover time expected for food assimilation and the incorporation in tissues of small fishes (see Possamai et al., 2021 and references therein).

Isotopic data from wild populations of syngnathids are limited and large isotopic datasets from annual monitoring are almost lacking. Four syngnathid species (*Hippocampus guttulatus*, *Hippocampus hippocampus*, *Entelurus aequoreus* and *Syngnathus acus*) have been collected in Cíes Archipelago (Piñeiro-Corbeira et al., 2021; Planas et al., 2021). Those species co-occur on some areas in Cíes Archipelago, and display similar trophic features (Piñeiro-Corbeira et al., 2021). However, a seasonal monitoring for two consecutive years only provided a large set of isotopic data for *Syngnathus acus* Linnaeus, 1758. Hence, this pipefish was selected in this study as representative of syngnathid populations in Cíes Archipelago.

The great pipefish *S. acus* is the largest European syngnathid. Although the knowledge on the ecology of this species is partially known, its ecology and trophic characteristics have been recently studied for the population inhabiting Cíes Archipelago (Atlantic Islands National Park, NW Iberian Peninsula) (2021; Planas et al., 2020a; Hernández-Urcera et al., 2021; Piñeiro-Corbeira et al., 2021; Planas, 2022). The population inhabiting Cíes

Archipelago consists almost exclusively of large adults that migrate by the end of the breeding season, which is typically from mid-spring to summer. The species is a dietary specialist fish foraging mostly near the substrate. This pipefish partially shifts its diet relative to prey availability (Piñeiro-Corbeira et al., 2021; Planas, 2022). Previous studies reported that *S. acus* is a selective but flexible predatory fish displaying high annual and seasonal trophic plasticity (Planas, 2022), foraging preferentially on amphipods, and, to a lesser extent, on carideans, copepods, isopods, and mysidaceans, in decreasing order. The seasonal fluctuating characteristics of their preferred habitat, including temperature regime, development of the macroalgal beds, structure of epifaunal assemblages, as well as migratory events (Piñeiro-Corbeira et al., 2021) have a strong effect on the availability and characteristics of the dietary resources, which is reflected on their stable isotope profiles (Planas, 2022).

This study considered the aforementioned concerns and potential isotopic mismatches due to different temporal lags between producers and consumers. The investigation evaluated the application of a time lag in the incorporation of stable isotope values between dietary resources and consumers (*Syngnathus acus*) to enhance (1) trophic position estimates and (2) the reconstruction of relative proportions of dietary resources in the species. Previous studies in the syngnathid *H. guttulatus* concluded that the isotopic effect of a shift in the dietary regime of adults would be reflected with a delay of about 3 months in either fin tissues or fish larvae (Planas et al., 2020a; Planas et al., 2020b). Since similar studies in other syngnathids are lacking, this study assumes that *S. acus* display similar tissue turnover rates.

Materials and methods

Study sites

This study was conducted in Cíes Archipelago (42°13'N, 8° 54'W) off Galicia (Spain) in the Atlantic Islands Marine National Park (NW Iberian Peninsula, Europe) (Figure 1) (see Fernández et al., 2020 for more information). Based on seaweed cover, substrate characteristics, and exposure level to open water (Piñeiro-Corbeira et al., 2021; Planas et al., 2021; Hernández-Urcera et al., 2021), three subtidal sites (A, B, and C; 2–15 m depth) (Figure 1) located on the east coast of Cíes Archipelago were surveyed (two visual censuses per site and season) in spring, summer, and autumn in 2017 and 2018. The sites were positioned near the coastline on rocky bottoms frequently interrupted by sandy patches. The surveys monitored the occurrence of the greater pipefish *S. acus*. Sighted specimens were captured for further sampling and the depth and location of the fish collection were annotated. Specific details on sampling methodology are provided by Planas (2022).

Fish collection and sampling

Syngnathus acus specimens (93 females and 55 males) were hand-caught, transferred to numbered plastic bags, and moved to a support boat. Subsequently, the fish were anaesthetized with a solution of Ethyl 3-aminobenzoate methane sulfonate (MS-222; 0.1 g L-1; Sigma-Aldrich Co., USA), morphologically identified, weighed (W, g), measured for standard length (SL, cm), and marked subcutaneously using visible implant fluorescent elastomers (VIFE; Northwest Marine Technology Inc., USA). A small portion of the posterior end of the dorsal fin was clipped (Valladares and Planas, 2012), transferred to screw-capped tubes containing 95% ethanol, and conserved at 4°C for stable isotope analysis (SIA). The sex of sampled fishes was determined using the trunk shape and pregnancy. The presence of previous marks (recapture events) was also annotated whenever possible. After sampling, all fishes were transferred and maintained in buckets containing fresh seawater until equilibrium was restored and normal fish activity and respiration were achieved. Fish were subsequently released at the capture site (usually 2-3 h after initial capture).

Samples of potential prey (epifauna and mysidaceans) for *S. acus* and the bivalve *Musculus costulatus* as source baseline were collected concomitantly with fish collections for further identification and stable isotope analysis (Piñeiro-Corbeira et al., 2021).

Stable isotopes analysis

For isotopic ($\delta^{13}C$, $\delta^{15}N$) and total C and N analyses, the samples were rinsed with distilled water, oven dried at 60°C for 24 h before being ground into a fine powder in a mortar and pestle, transferred to tin capsules, and weighted (± 1 µg). Ground and dried samples were analyzed at SAI (Servizos de Apoio á Investigación, University of A Coruña, Spain) by continuous-flow isotope ratio mass spectrometry using a FlashEA1112 elemental analyzer (Thermo Finnigan, Italy) coupled to a Delta Plus mass spectrometer (FinniganMat, Germany) through a Conflo II interface. Isotopic values are expressed as permil (‰) in delta notation relative to VPDB (Vienna Pee Dee Belemnite) and atmospheric air. As part of an analytical batch run, a set of international reference materials for $\delta^{15}N$ values (IAEA-N-1, IAEA-N-2, and USGS25) and $\delta^{13}C$ values (NBS 22, IAEA-CH-6, and USGS24) were analyzed. The precision (standard deviation) of the analysis of the laboratory standard (acetanilide) for $\delta^{13}C$ and $\delta^{15}N$ was ± 0.15‰. The isotopic analysis procedure fulfils the requirements of the ISO 9001 standard.

Due to the low lipid content in fin samples of fishes (< 5% lipids, C:N < 3.56) (Post, 2002; Post et al., 2007), normalization

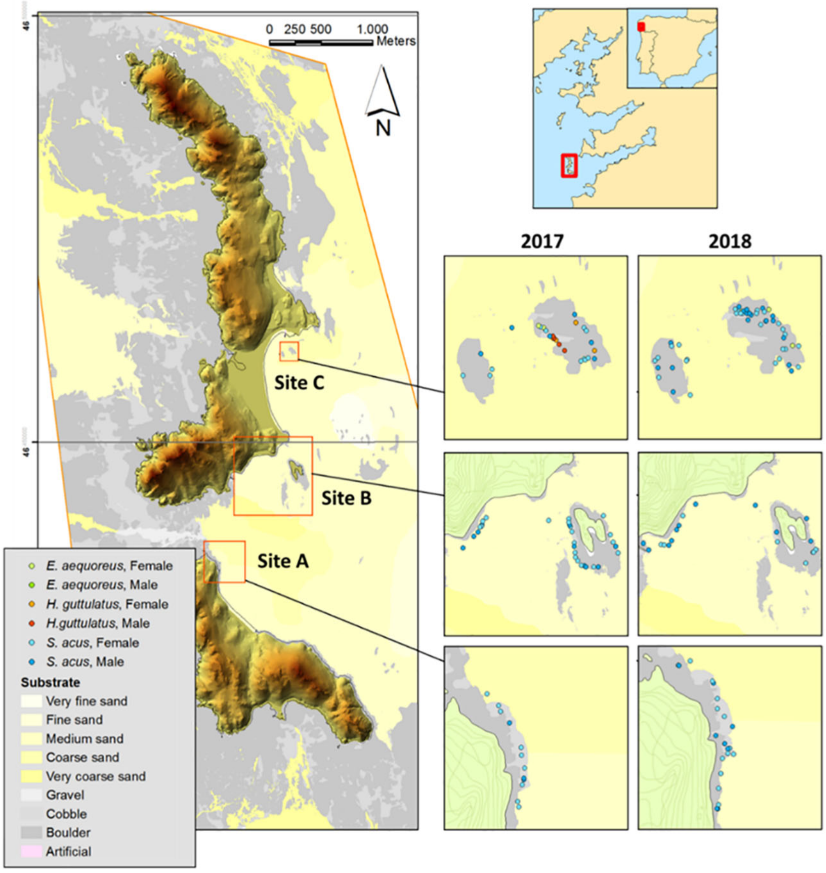

FIGURE 1
Sites (A), (B), and (C) surveyed for *Syngnathus acus* monitoring in Cíes Archipelago (Galicia, NW Iberian Peninsula) in spring, summer, and autumn (2017–2018). Substrate characteristics and syngnathids captured (*S. acus*, *Entelurus aequoreus*, and *Hippocampus guttulatus)* are shown.

for lipid correction was not necessary (Valladares and Planas, 2012) Since C:N values in several epifaunal groups revealed a lipid content higher than 5%, specific conversion factors constructed for lipid normalization were applied (conversion factors provided by Piñeiro-Corbeira et al., 2021). Additionally, some epifaunal groups were acidified before SIA (Piñeiro-Corbeira et al., 2021) by adding dilute (10%) HCl drop-by-drop, until CO_2 release was no longer observed (Jaschinski et al., 2008; Vafeiadou et al., 2013).

Data analyses

Data and statistical analyses were conducted in R v.3.6.1 (R Core Team, 2019). All means are reported with standard deviations. A P-value threshold of 0.05 was considered significant in all statistical analyses. The graphics were constructed using ggplot2 v3.3.0 (Wickham et al., 2020) and lattice v0.20–41 (Sarkar et al., 2020) packages.

The relative contributions of prey to the diet of *S. acus* were estimated considering the average isotopic values of potential prey in (a) the same season as consumer sampling (Unshifted) and (b) the previous season (a 3-month interval) of consumer sampling (Shifted). For consumers, two approaches were considered (Figure 2): (a) Global Assessment – including pipefish specimens collected for the entire monitoring period (summer and autumn in 2017 and spring, summer and autumn in 2018), and (b) Seasonal assessment – including specimens collected in summer (2017 and 2018) when the fish were growing. The annual assessment provided global information for a long period (2 years), so that the effect of dietary changes over a limited period (i.e., a season) might not be revealed in the analysis of the annual dataset. On the contrary, the seasonal assessment would reflect explicitly changes over a short period. In the present study, summer was selected for the seasonal analysis, as there were data available for summer-2017 and summer-2018 as well as spring-2017 and spring-2018, which were necessary for the shifted and unshifted approaches.

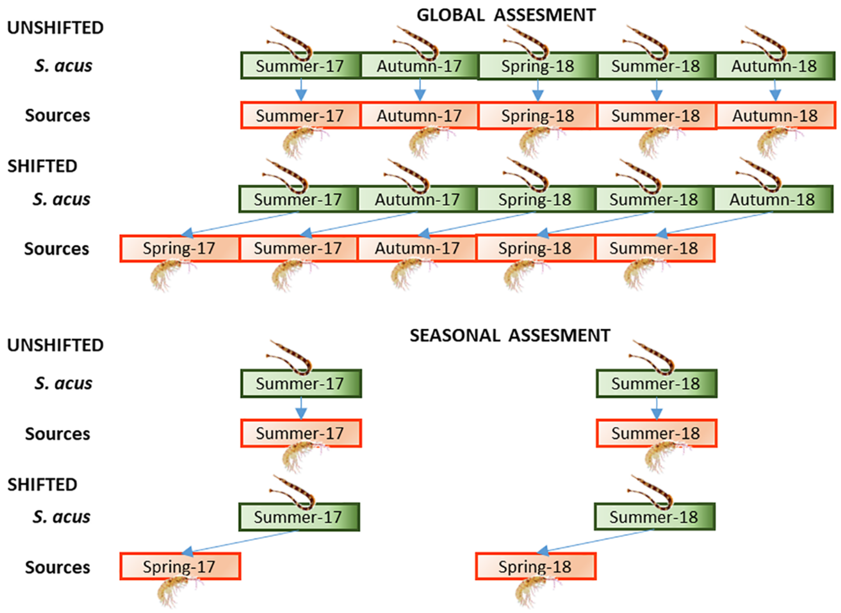

FIGURE 2

Approaches followed for the assessment of the dietary regime in *S. acus*. Global assessment – specimens collected for the entire monitoring period (summer and autumn in 2017 and spring, summer and autumn in 2018). Seasonal assessment – specimens collected in summer (2017 and 2018). Treatments: average isotopic values of potential sources sampled **(A)** concomitantly with consumers (Unshifted), or **(B)** in the previous season of consumer sampling (Shifted).

Aiming to enhance diet contribution estimates, the shifted approach applied source isotopic data from the previous season of consumer sampling (see Gristina et al., 2017). The choice of baselines is crucial in trophic position estimates using stable isotope data (Post, 2002; Hussey et al., 2014; Possamai et al., 2021). In the global assessment, seasonal δ^{13}C and δ^{15}N values in *M. costulatus* (Risso, 1826) were used as baselines for each sampling period, whereas δ^{13}C and δ^{15}N values in summer (unshifted approach) or spring (shifted approach) were used as the isotopic baseline.

Fish trophic position (TP) was estimated from the δ^{13}C and δ^{15}N values using tRophicPosition v. 0.7.7 (Quezada-Romegialli et al., 2018), an R package incorporating a Bayesian model for the calculation of consumer TP at the population level. Experimentally derived TDF (Trophic Discriminant Factors) values (2.5 for δ^{13}C; 3.9 for δ^{15}N) for syngnathids were applied (Planas et al., 2020a). δ^{13}C and δ^{15}N values in the bivalve *Musculus costulatus* (i.e., whole individuals due to their small size, ca. 1-2 mm) were used as trophic baselines (TP = 2) in calculations to estimate TP (Planas et al., 2020a; Piñeiro-Corbeira et al., 2021). In the global assessment, all the *M. costulatus* were used, but only the bivalves from the season in question were used in the seasonal comparison.

Isotopic niche areas and pairwise niche overlap in *S. acus* were assessed using a δ^{13}C and δ^{15}N bi-plot as multidimensional niche indicator data. The isotopic niche region was defined as the joint probability density function of the multidimensional niche indicators, at a probability alpha of 95%. The package NicheRover v.1.1.0 was used to assess differences in trophic niche features and to estimate trophic overlaps (Lysy et al., 2014). The method is insensitive to sample size and incorporates statistical uncertainty using Bayesian methods (Swanson et al., 2015).

From the isotopic values in selected potential prey (amphipods, harpacticoid copepods, carideans, isopods, and mysidaceans) (see Planas et al., 2020a for isotopic data) and in the consumer *S. acus*, the dietary regimes were estimated using Bayesian Mixing Models (SIMM) (Stock and Semmens, 2016), using the MixSIAR package v.3.1.12 (Stock et al., 2018). The SIMM procedure is fully described in Planas et al. (2020a). Briefly, SIMM polygons were constructed with isotopic signatures adjusted for experimentally derived TDFs (2.5‰ for δ^{13}C; 3.9‰ for δ^{15}N) (Planas et al., 2020a) to determine the proportion of consumers that were included inside the mixing polygon bound by all potential sources. Five pipefish specimens with a low probability (< 5%) of being positioned inside the mixing polygon were not included in the subsequent Bayesian models (Smith et al., 2013). The models were run with Markov chain Monte Carlo (MCMC) parameters of three chains of 300,000 iterations, and a burn-in phase of 200,000 (long run). The model included individuals as a random effect, and one error term (process error). Convergence and diagnostic statistics were evaluated using both Gelman–Rubin and Geweke tests. Bayesian model outputs are reported as median ± 95% confidence interval.

Inter-sexual comparisons for trophic position and dietary regimes were run based on previous findings that reported differences in isotopic signatures and niche areas occupied by males and females (Planas, 2022).

Results

Sample and isotopic data descriptors

The average size and weight values in *S. acus* specimens were 34.4 ± 7.0 cm SL (range: 16.4–49.8 cm) and 33.8 ± 20.4 g (range: 2.8–102.7 g), respectively. The size and weight were higher in males (36.1 ± 7.0 cm SL; 41.3 ± 22.7 g) than in females (33.3 ± 6.8 cm SL; 29.1 ± 17.3 g). Adult specimens larger than 30 cm SL accounted for 77% of the total captures.

Average isotopic values in *S. acus* specimens were -15.4 ± 0.5‰ for δ^{13}C (-15.5 ± 0.4‰ in males and -15.3 ± 0.5‰ in females) and 11.1 ± 0.6‰ for δ^{15}N (11.3 ± 0.5‰ in males and 10.9 ± 0.6‰ in females) (Table 1). Isotopic values were maximal in autumn for δ^{13}C and in spring for δ^{15}N.

Trophic niche and position

The estimated isotopic niche area was broader in females (5.04 ± 0.63) than in males (3.63 ± 0.55) (Figure 3). The isotopic niche overlap analysis revealed that the posterior probability (*a* = 0.95) that a female will be included within the niche of males is 80.7%. However, the isotopic trophic niche of males was markedly overlapped by that of females (93.7% overlap).

The global baseline (*M. costulatus*) for the whole study was -17.7± 0.1‰ for δ^{13}C and 5.3 ± 0.3‰ for δ^{15}N. The unshifted approach including all analyzed pipefish revealed that the trophic level of *S. acus* specimens was 3.40 ± 0.06, being lower in females (3.36 ± 0.05) than in males (3.47 ± 0.06) (P<0.001) (Table 2). Those levels were almost identical to those attained with the shifting approach (< 0.6% change). However, in the seasonal approach, the trophic level in the shifted treatment was 6% higher than in unshifted sampling.

Diet reconstruction

The isotopic values, corrected for trophic discrimination, of individual consumers (2.5‰ for δ^{13}C; 3.9‰ for δ^{15}N), and the means and standard deviations of isotopic values of five candidate dietary sources (Amphipoda, Copepoda, Caridea, Isopoda and Mysidacea) are depicted in Figure 4. Only five out of 115 specimens were positioned outside the 95% confidence interval and they were not included in further analyses.

The estimated relative contributions of dietary items from the unshifted treatment were higher for amphipods (30.9%) and copepods (24.8%), followed by carideans (14.3%) and isopods (16.9%). The contribution of mysidaceans was much lower (8.7%) (Figures 5, S1). The analysis carried out with shifted data revealed an overall increased contribution of amphipods (43.7%), isopods (19.5%) and mysidaceans (12.1%), and a reduction in the contribution percent for copepods (5.10%). Regarding temporal changes (from summer 2017 to autumn

TABLE 1 Isotopic values (δ^{13}C and δ^{15}N; mean ± sd) in *Syngnathus acus* specimens collected in Cíes Archipelago from summer-2017 to autumn-2018.

			δ^{13}C (‰)		δ^{15}N (‰)	
Period	**Gender**	**N**	**Mean**	**s.d.**	**Mean**	**s.d.**
Whole study	Pooled	115	-15.4	0.5	11.1	0.6
	Males	44	-15.5	0.4	11.3	0.5
	Females	71	-15.3	0.5	10.9	0.6
Summer-2017	Females	17	-15.7	0.4	10.9	0.5
Summer-2017	Males	6	-15.8	0.4	11.0	0.3
Autumn-2017	Females	8	-15.3	0.5	10.9	0.6
Autumn-2017	Males	4	-15.1	0.2	10.9	0.4
Spring-2018	Females	18	-15.6	0.3	11.3	0.5
Spring-2018	Males	20	-15.6	0.4	11.6	0.4
Summer-2018	Females	24	-14.9	0.3	10.5	0.7
Summer-2018	Males	14	-15.2	0.2	11.1	0.3
Autumn-2018	Females	4	-14.4	0.3	11.3	0.3
Autumn-2018	Males	0	–	–	–	–

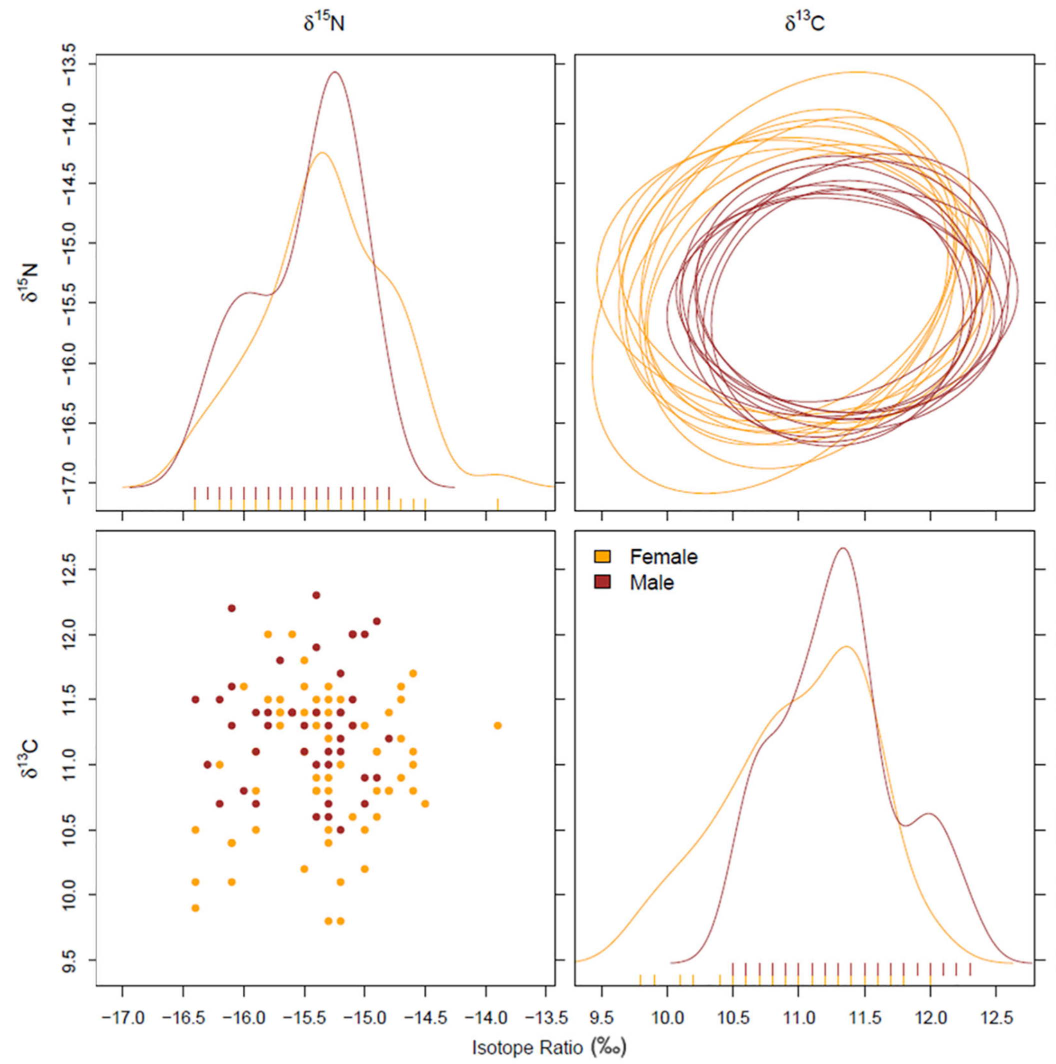

FIGURE 3
Global assessment – Niche plots (95% alpha) (A - $\delta^{15}N$; D - $\delta^{13}C$), density distributions **(B)**, and raw data **(C)** for each pairwise combination of isotope ($\delta^{13}C$ and $\delta^{15}N$) data for *S. acus* males and females.

TABLE 2 Trophic position (mean ± sd) in *Syngnathus acus* specimens collected in 2017 and 2018 in Cíes Archipelago.

	Pooled			n	Females			n	Males			n	P
Global assessment													
Unshifted	3.40	±	0.06	115	3.36	±	0.05	71	3.47	±	0.06	44	<0.001
Shifted	3.41	±	0.06	115	3.38	±	0.06	71	3.48	±	0.06	44	<0.001
Seasonal assessment													
Unshifted	3.25	±	0.08	61	3.23	±	0.07	41	3.32	±	0.07	20	<0.001
Shifted	3.38	±	0.07	61	3.35	±	0.08	41	3.44	±	0.09	20	<0.001

Global assessment: specimens collected in 2017 (summer and autumn) and 2018 (spring, summer and autumn). Seasonal assessment: specimens collected in summer 2017 and summer 2018. Treatments: average isotopic values of potential prey sampled concomitantly with consumers (Unshifted) or in the previous season of consumer sampling (Shifted). P: significance of t-test comparing females and males.

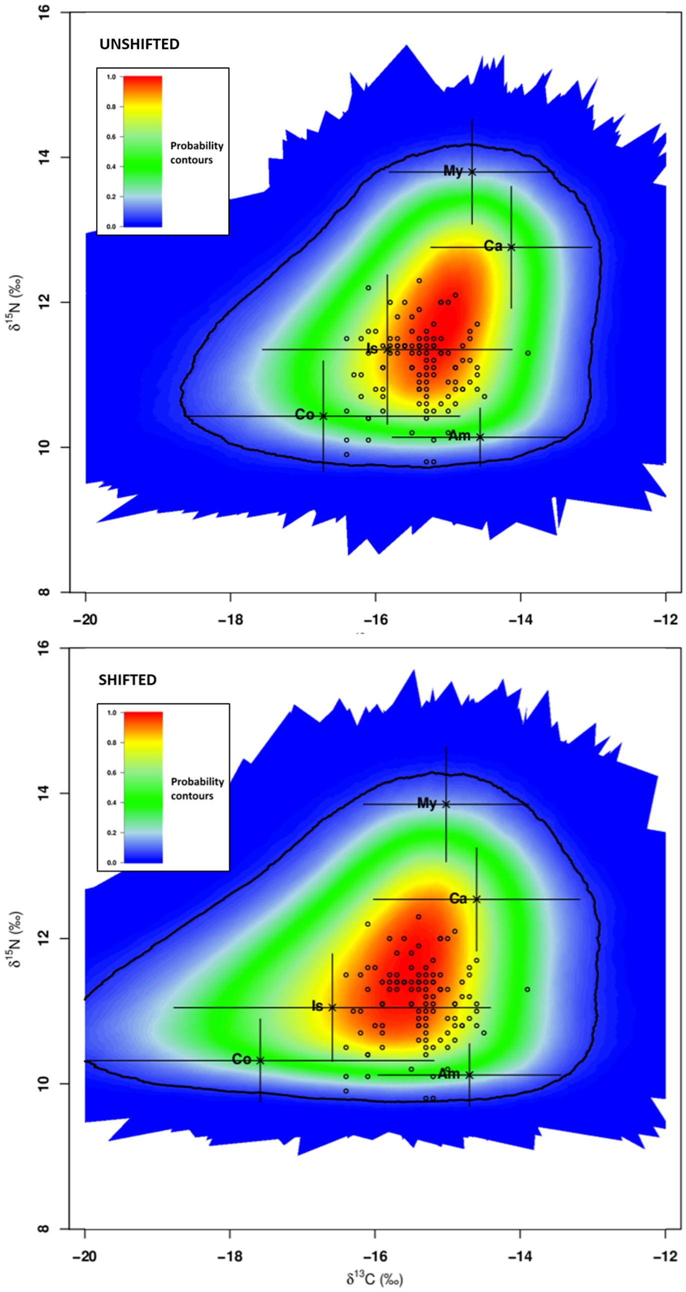

FIGURE 4
Global assessment - SIMM polygon for *Syngnathus acus* (n=110; 2017 and 2018 surveys) relative to average δ^{13}C and δ^{15}N signatures of five potential prey sources (mean ± sd; black crosses) adjusted for TDF values and consumers (*S. acus*; open circles; five outliers not included). Treatments: average isotopic values of potential prey sampled concomitantly with consumers (Unshifted) or in the previous season of consumer sampling (Shifted). The coloured region represents the 95% confidence interval. Probability contours are at the 5% level. Sources: Am - Amphipoda, Co - Copepoda, Ca - Caridea, Is – Isopoda, and My - Mysidacea.

2018), the estimated relative contributions of copepods to the mixture were consistently higher in unshifted data (20.5 ± 9.0 percentage points). The estimates for other taxa were highly variable across sampling periods (Figure S2). Amphipods and isopods were the most affected by shifting but did not show any trend.

Regarding the seasonal assessment from summer-2017 and summer-2018 specimens, the comparison between unshifted

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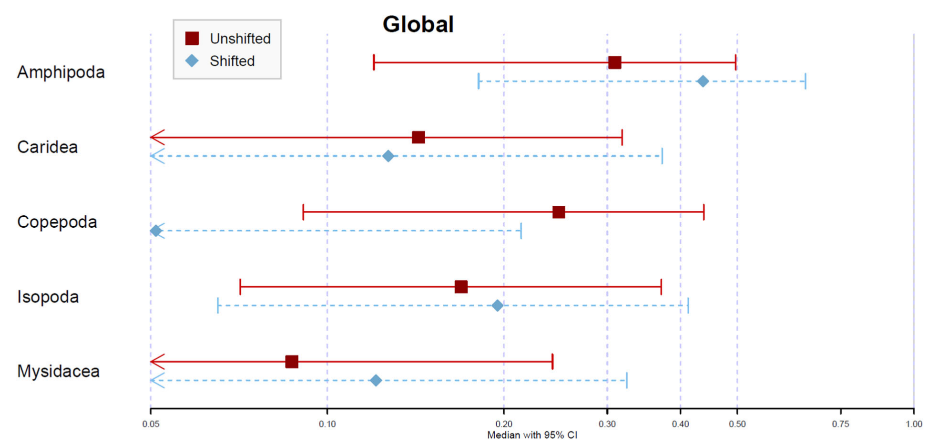

FIGURE 5
Global assessment – Average posterior proportional contribution for *S. acus* as estimated by Bayesian Stable Isotope Mixing Model (SIMM). Analyses based on isotopic (δ^{13}C and δ^{15}N) data for dorsal fin tissues of 110 fishes sampled in Cíes Archipelago in 2017 (summer and autumn) and 2018 (spring, summer and autumn). Treatments: average isotopic values of potential sources sampled concomitantly with consumers (Unshifted) or in the previous season of consumer sampling (Shifted). Symbols represent the median contribution of each key source with 95% (lines) credible intervals.

and shifted treatments showed similar effects to those for the global assessment (Figures 6 and S3). The contribution of amphipods to the diet of *S. acus* was lower in unshifted data (39.0% in females and 23.5% in males) compared to shifted data (54.5% in females and 39.7% in males) in both females and males. Comparatively, copepods contribution was higher with unshifted data (28.1% in females and 34.2% in males) than with shifted data (7.6% in females and 10.7% in males). The assessment of both summer seasons (2017 and 2018) provided results highly similar to those attained with pooled data (Figures S3, S4) but the credible intervals and inter-sex differences for the most contributing source (amphipods) were reduced using shifted data.

Discussion

This study investigated (a) the seasonal variability of potential dietary resources for *S. acus* in Cíes Archipelago (i.e., reflected in isotopic fluctuations both in resources and baseline organisms), and (b) the use of tissues with moderate turnover rates (i.e., dorsal fin as a proxy of muscle tissue). The results attained highly recommend the application of a time lag in the reconstruction of the diet in *S. acus*, especially when assessing trophic changes in the short- or mid-term (from weeks to months). The shifted approach revealed that the species mainly feed on a narrower range of taxa than previously reported.

This study pointed out significant differences for some trophic features in the pipefish *Syngnathus acus* depending on the time lag applied for the assessment of the resources contributing to their biomass. The use of time lags is extremely important but rarely evaluated or applied in trophic studies (Possamai et al., 2021; Ballutaud et al., 2022). The time lag applied in the present study was based on several assumptions (Planas et al., 2020a). Tissue turnover rates and isotopic equilibrium data after a diet shift in syngnathids are only available for the seahorse *Hippocampus guttulatus* (a species co-occurring with *S. acus* in Cíes Archipelago). Hence, that information was applied to *S. acus* in the present study. Previous unpublished isotopic data (S. Valladares et al.) in wild *H. guttulatus* maintained in captivity for nearly 2 yr at IIM-CSIC facilities showed that the isotopic equilibrium with a new diet was achieved in less than 5 months. Also, Thomas and Crowther (2015) pointed out that consumers are considered to have achieved an isotopic steady-state with a new diet after ca. 3.5 half-lives (about 4 months). Considering this information and the typical shape of the curve fitted to isotopic data following a diet shift (e.g., Hesslein et al., 1993), a time lag of 3 months (i.e., one season) seems to be realistic.

Seasonality determines changes in the structure of seaweed assemblages as well as in the epifauna that supports many species, including syngnathids (Seed et al., 1981; Winkler et al., 2017). In Cíes Archipelago, *S. acus* preferentially occupies rocky areas dominated by the fucal *Gondolaria baccata* and to a lesser extent by *Codium* spp. (Planas et al., 2021). These perennial seaweeds are ideal habitats for pipefishes and other syngnathids, recovering their biomass in spring-summer while recovering from autumn-winter adverse conditions (Piñeiro-Corbeira et al., 2021). The seasonal changes in macroalgae communities are accompanied by fluctuations in epifaunal assemblages. *G. baccata* provides complex branched secondary structures with high species richness and diversity, especially in summer

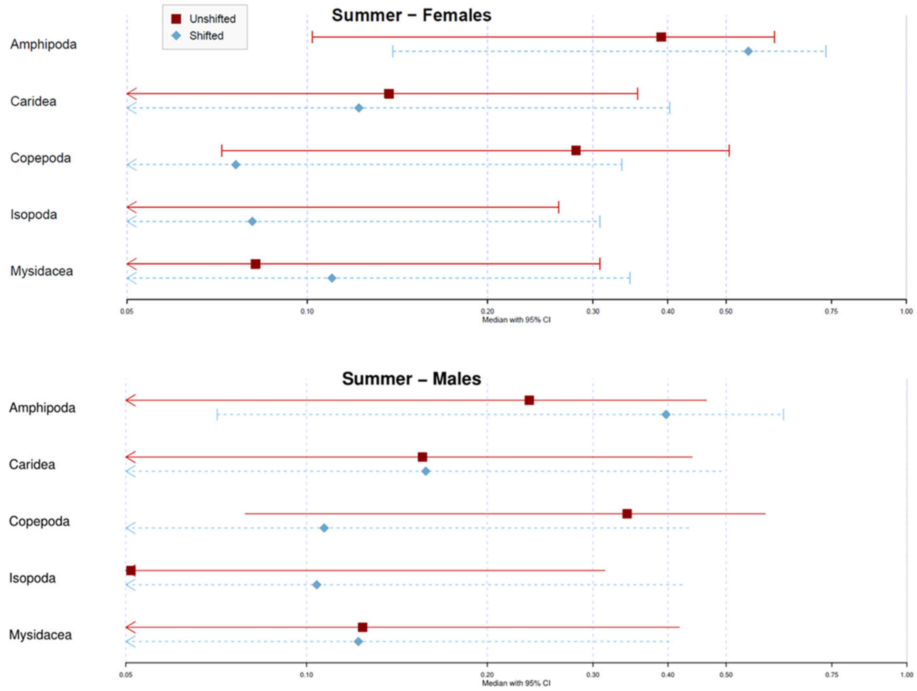

FIGURE 6
Seasonal assessment - Posterior proportional contribution for *Syngnathus acus* as estimated by Bayesian Stable Isotope Mixing Model (SIMM). Analyses based on isotopic (δ^{13}C and δ^{15}N) data for dorsal fin tissues of specimens sampled on Cíes Archipelago in summer 2017 and summer 2018 (Pooled samples). Treatments: average isotopic values of potential prey sampled concomitantly with consumers (Unshifted) or in the previous season of consumer sampling (Shifted). Symbols represent the median contribution of each key source with 95% (lines) credible intervals.

compared to autumn and spring (Viejo, 1999). Hence, the diversity and abundances of potential dietary sources for *S. acus* fluctuate seasonally. Gammaridae, Corophidae, and Amphilochidae are amphipods that show high seasonal variability in Cíes, especially in summer and autumn (Piñeiro-Corbeira et al., 2021). Numerically, harpacticoids are the dominant epifauna associated with seaweeds in Cíes, but their relative abundances decrease markedly in autumn. However, the relative abundance of amphipods is notably high, especially in *G. baccata*. In terms of biomass and energy content and compared to copepods or other small-sized prey, foraging upon amphipods would be highly advantageous to large pipefish such as those from Cíes Archipelago. This statement is supported by the results attained in this study. Even though copepods might be numerically dominant in some periods in the guts of the Aegen Sea specimens (Taşkavak et al., 2010), their high contribution to the diet might rely on their small size (range: 7.3 – 23.2 cm SL) compared to *S. acus* from Cíes Archipelago (range: 16.4–49.8 cm SL; mean: 34.4 ± 7.0 cm SL). Unfortunately, young or small specimens were not present in Cíes Archipelago, impeding further comparisons with those from the Aegean Sea or to analyze ontogenic patterns in *S. acus* lifespan.

Syngnathus acus is a secondary consumer that forages primarily upon epifaunal organisms associated with seaweeds (Piñeiro-Corbeira et al., 2021). This is a common feature in many syngnathids (Manning et al., 2019), which is imposed by their tiny mouth at the tip of a long, but narrow snout (de Lussanet and Muller, 2007). In the global assessment, the unshifted and shifted approaches provided similar overall trophic positions for *S. acus* (3.40 and 3.41, respectively). In the seasonal approach, however, the analysis performed revealed differences in trophic position when using shifted data, revealing a slight but significant increase (6%) in the trophic position compared to unshifted data (3.38 ± 0.07 and 3.25 ± 0.08, respectively). Nevertheless, the isotopic space occupied by the fish was similar in both approaches.

Amphipods are the most preferred prey for many syngnathids (Manning et al., 2019). Previous assessments performed with data of dietary resources sampled concomitantly with consumers (unshifted approach in this study) reported a high contribution of amphipods and a substantial role of copepods in the dietary regime of *S. acus* (Planas et al., 2020a; Planas, 2022). The increased predation upon amphipods also seems to be common in other areas (e.g., Aegean Sea), as inferred from gut content analysis (Gürkan and Taşkavak, 2019; Gürkan et al., 2021).

The application of isotopic time lags can enhance the estimation of baseline δ^{15}N values and subsequent estimates

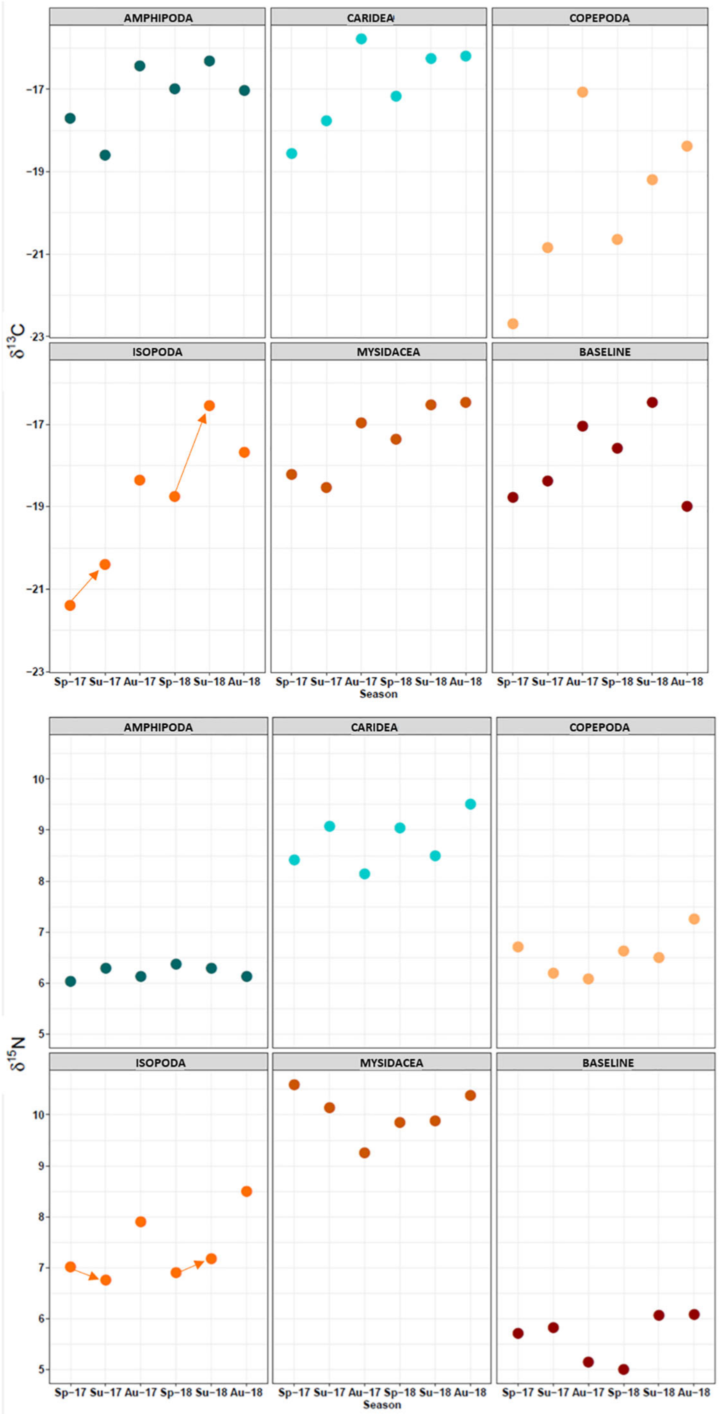

FIGURE 7
Seasonal changes in $\delta^{13}C$ and $\delta^{15}N$ values for dietary resources. Arrows: Change from spring to summer values for isopods.

of consumer trophic position (Possamai et al., 2021). In the present study, a marked effect of time lag was evident in the reconstruction of the dietary regime in *S. acus*. Hence, the use of time lags in the incorporation of stable isotopes between sources and consumers should provide more accurate modelling results, especially when comparing tissues with medium or slow turnover rates that have been sampled over time.

Globally (annual assessment), the relative contributions of copepods and, to a lesser extent, amphipods to the biomass of *S. acus* were particularly affected by the application of a time lag. With the unshifted approach, amphipods and copepods were under- and markedly higher overestimated, respectively (29% and 386%, respectively) compared to the shifted approach. The relative contributions of mysidaceans and isopods were also underestimated (28% and 13%, respectively) by the unshifted approach. These differential results seemed to rely largely on seasonal changes in δ^{13}C values, which were more fluctuating than δ^{15}N (Figure 7). In most taxa, δ^{13}C signatures were lower in spring compared to autumn. However, that trend was not followed by the baseline organism *M. costulatus*, which showed relatively large annual and seasonal variability. The results highlight the importance of characterizing baseline δ^{13}C accurately, whenever possible. The effect of the time lag on amphipods and copepods was also noticed in the seasonal approach, especially for copepods (270% underestimation in the unshifted approach). Hence, stable isotope data suggest that *S. acus* specimens at the time of capture had likely foraged upon a lower proportion of copepods than that resulting from the unshifted approach. This discrepancy has been highlighted in studies comparing gut content analyses and isotopic data (Hadwen et al., 2007).

Regarding the potential sources of organic matter for both sexes, the effect of seasonal shifting on dietary reconstruction seemed to affect males and females in a similar manner. This finding was expected considering that *S. acus* population in Cíes was mostly represented by mature specimens and that trophic differences between sexes were mainly due to the presence of nonovigerous females rather than of mature specimens (Planas, 2022). In contrast to immature females, the high dietary similarity between males (mature and immature) and mature females would reveal long residence periods in the same habitat while feeding on similar resources.

Conclusion

This isotopic study has implications regarding the importance of sources of organic matter to tissues of the pipefish *S. acus*. The study highlights for the first time the importance of applying time lags to both isotopic baseline and sources in the diet reconstruction of syngnathids, particularly in *S. acus*. While it is important to consider that the choice of what animal tissue to analyze may influence inferences of organic matter sources, selecting the appropriate time lag will enhance estimates accuracy. To evaluate diet from tissues with medium-time turnover rates and to detect any seasonal shifts in food habits, it is highly recommended whenever possible that those factors be explicitly evaluated considering the target organism. The experimental approach used in this study (i.e., fin tissue as a proxy; time lag: 2-3 months) could be applied to similar studies in other temperate syngnathid species when specific data are unknown.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Planas, M. (2022), “Data: Ecological traits and trophic plasticity in the greater pipefish *Syngnathus acus* in NW Iberian Peninsula”, Mendeley Data, V1, doi: 10.17632/kf3tn3d9j8.1).

Ethics statement

Fish capture, handling, and sampling were conducted in compliance with all bioethics standards on animal experimentation of the Spanish Government (R.D. 1201/2005, 10th October) and the Regional Government Xunta de Galicia (Reference REGA ES360570202001/16/FUN/BIOL.AN/MPO02).

Author contributions

MP: Funding acquisition, Project administration, conceptualization, methodology, stable isotopes, data curation, formal analysis, visualization, writing- original draft preparation, writing - review and editing.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Baker, R., Buckland, A., and Sheaves, M. (2013). Fish gut content analysis: robust measures of diet composition. *Fish. Fish.* 15, 170–177. doi: 10.1111/faf.12026

Ballutaud, M., Travers-Trolet, M., Marchal, P., Dubois, S. F., Giraldo, C., Parnell, A. C., et al. (2022). Inferences to estimate consumer's diet using stable isotopes: Insights from a dynamic mixing model. *PloS One* 17 (2), e0263454. doi: 10.1371/journal.pone.0263454

Barnett, A., Redd, K. S., Frusher, S. D., Stevens, J. D., and Semmens, J. M. (2010). Non-lethal method to obtain stomach samples from a large marine predator and the use of DNA analysis to improve dietary information. *J. Exp. Mar. Biol. Ecol.* 393, 188–192. doi: 10.1016/j.jembe.2010.07.022

Boecklen, W. J., Yarnes, C. T., Cook, B. A., and James, A. C. (2011). On the use of stable isotopes in trophic ecology. *Annu. Rev. Ecol. Evol. Syst.* 42, 411–440. doi: 10.1146/annurev-ecolsys-102209-144726

Busst, G., Basic, T., and Britton, J. R. (2015). Stable isotope signatures and trophic-step fractionation factors of fish tissues collected as non-lethal surrogates of dorsal muscle. *Rapid Commun. Mass Spectrom.* 29, 1535–1544. doi: 10.1002/rcm.7247

Caut, S., Angulo, E., and Courchamp, F. (2009). Variation in discrimination factors (Delta n-15 and delta c-13): the effect of diet isotopic values and applications for diet reconstruction. *J. Appl. Ecol.* 46, 443–453. doi: 10.1111/j.1365-2664.2009.01620.x

Dalerum, F., and Angerbjörn, A. (2005). Resolving temporal variation in vertebrate diets using naturally occurring stable isotopes. *Oecologia* 144, 647–658. doi: 10.1007/s00442-005-0118-0

Davis, M., and Pineda-Munoz, S. (2016). The temporal scale of diet and dietary proxies. *Ecol. Evol.* 6, 1883–1897. doi: 10.1002/ece3.2054

de Lussanet, M. H. C., and Muller, M. (2007). The smaller your mouth, the longer your snout: Predicting the snout length of *Syngnathus acus*, *Centriscus scutatus* and other pipette feeders. *J. R. Soc Interface* 4, 561–573. doi: 10.1098/rsif.2006.0201

DeNiro, M. J., and Epstein, S. (1981). Influence of diet on the distribution of nitrogen isotopes in animals. *Geochim. Cosmochim. Acta* 45, 341–351. doi: 10.1016/0016-7037(81)90244-1

Fernández, E., Barañano, C., Alejo, I., Barreiro, R., Bellas, J., Besada, V., et al. (2020). *Islas cíes: Un ecosistema en la frontera* (Vigo: Concello de Vigo).

Gristina, M., Bertrandino, S., Cardone, F., Mentino, D., Corriero, G., and Scillitani, G. (2017). Skin filament recovery after clipping in *Hippocampus guttulatus*: behavioural and histological aspects. *Aquat. Biol.* 26, 149–157. doi: 10.3354/ab00680

Guelinckx, J., Maes, J., Van Den Driessche, P., Geysen, B., Dehairs, F., and Ollevier, F. (2007). Changes in d^{13}C and d^{15}N in different tissues of juvenile sand goby *Pomatoschistus minutus*: a laboratory diet-switch experiment. *Mar. Ecol. Pro. Ser.* 341, 205–215. doi: 10.3354/meps341205

Gürkan, Ş., Innal, D., and Gulle, I. (2021). Monitoring of the trophic ecology of pipefish species (*Syngnathus abaster*, *Syngnathus acus*) in an alluvial lake habitat (Lake bafa, Turkey). *Oceanol. Hydrobiol. Stud.* 50, 24–32. doi: 10.2478/oandhs-2021-0003

Gürkan, Ş., and Taşkavak, E. (2019). The relationships between gut length and prey preference of three pipefish (*Syngnathus acus*, *Syngnathus typhle*, *Nerophis ophidion* linnaeus 1758) species distributed in Aegean Sea, Turkey. *Iran. J. Fish. Sci.* 18, 1093–1100. doi: 10.22092/ijfs.2019.118277

Hadwen, W. L., Russell, G. L., and Arthington, A. H. (2007). Gut content- and stable isotope-derived diets of four commercially and recreationally important fish species in two intermittently open estuaries. *Mar. Fresh. Res.* 58, 363–375. doi: 10.1071/MF06157

Hernández-Urcera, J., Murillo, F. J., Regueira, M., Cabanellas-Reboredo, M., and Planas, M. (2021). Preferential habitats prediction in syngnathids using species distribution models. *Mar. Environ. Res.* 172, 105488. doi: 10.1016/j.marenvres.2021.105488

Hesslein, R. H., Hallard, K. A., and Ramal, P. (1993). Replacement of sulfur, carbon,and nitrogen in tissue of growing broad whitefish (*Coregonus nasus*) inresponse to a change in diet traced by δ^{34}S, δ^{13}C, and δ^{15}N. *Can. J. Fish. Aquat. Sci.* 50, 2071–2076. doi: 10.1139/f93-230

Hobson, K. A., and Clark, R. G. (1992). Assessing avian diets using stable isotopes I: turnover of ^{13}C in tissues. *Condor* 94, 181–188. doi: 10.2307/1368807

Hussey, N. E., Macneil, M. A., McMeans, B. C., Olin, J. A., Dudley, S. F., Cliff, G., et al. (2014). Rescaling the trophic structure of marine food webs. *Ecol. Lett.* 17 (2), 239–250. doi: 10.1111/ele.12226

Hynes, H. B. N. (1950). The food of fresh-water sticklebacks (*Gasterosteus aculeatus* and *Pygosteus pungitius*), with a review of methods used in studies of the food of fishes. *J. Anim. Ecol.* 19, 36–58. doi: 10.2307/1570

Hyslop, E. J. (1980). Stomach contents analysis: a review of methods and their application. *J. Fish. Biol.* 17, 411–429. doi: 10.1111/j.1095-8649.1980.tb02775.x

Iverson, S. J., Field, C., Don Bowen, W., and Blanchard, W. (2004). Quantitative fatty acid signature analysis: a new method of estimating predator diets. *Ecol. Monogr.* 74, 211–235. doi: 10.1890/02-4105

Jardine, T. D., Hobson, K. A., and Soto, D. X. (2017). "Introduction to stable isotopes in food webs," in *Food forensics: Stable isotopes as a guide to authenticity and origin*. Eds. J. F. Carter and L. A. Chesson (Boca Ratón: CRC Press).

Jaschinski, S., Hansen, T., and Sommer, U. (2008). Effects of acidification in multiple stable isotope analyses. *Limnol. Oceanogr. Methods* 6, 12–15. doi: 10.4319/lom.2008.6.12

Jobling, M. (1987). Marine mammal faeces samples as indicators of prey importance - a source of error in bioenergetics studies. *Sarsia* 72, 255–260. doi: 10.1080/00364827.1987.10419722

Kodama, T., Hirai, J., Tawa, A., Ishihara, T., and Ohshimo, S. (2020). Feeding habits of the pacific bluefin tuna (Thunnus orientalis) larvae in two nursery grounds based on morphological and metagenomic analyses. *Deep Sea Res. Pt. II* 175, 104745. doi: 10.1016/j.dsr2.2020.104745

Lysy, M., Stasko, A. D., and Swanson, H. K. (2014). "nicheROVER: (Niche) (R)egion and Niche(Over)lap metrics for multidimensional ecological niches," in *R package version 1.1.0*. Available at: https://CRAN.R-project.org/package=nicheROVER.

Madigan, D. J., Litvin, S. Y., Popp, B. N., Carlisle, A. B., Farwell, C. J., and Block, B. A. (2012). Tissue turnover rates and isotopic trophic discrimination factors in the endothermic teleost, pacific bluefin tuna (*Thunnus orientalis*). *PloS One* 7, e49220. doi: 10.1371/journal.pone.0049220

Manning, C. G., Foster, S. J., and Vincent, A. C. J. (2019). A review of the diets and feeding behaviours of a family of biologically diverse marine fishes (Family syngnathidae). *Rev. Fish. Biol. Fish.* 29, 197–221. doi: 10.1007/s11160-019-09549-z

Martinez del Rio, C., Wolf, N., Carleton, S. A., and Gannes, L. Z. (2009). Isotopic ecology ten years after a call for more laboratory experiments. *Biol. Rev.* 8491–111. doi: 10.1111/j.1469-185X.2008.00064.x

McMahon, K. W., Fogel, M. L., Johnson, B. J., Houghton, L. A., and Thorrold, S. R. (2011). A new method to reconstruct fish diet and movement patterns from δ^{13}C values in otolith amino acids. *Can. J. Fish. Aquat. Sci.* 68, 1330–1340. doi: 10.1139/f2011-070

Phillips, D. L., Inger, R., Bearhop, S., Jackson, A. L., Moore, J. W., Parnell, A. C., et al. (2014). Best practices for use of stable isotope mixing models in food web studies. *Can. J. Zool.* 835, 823–835. doi: 10.1139/cjz-2014-0127

Piñeiro-Corbeira, C., Iglesias, L., Nogueira, R., Campos, S., Jiménez, A., Regueira, M., et al. (2021). Structure and trophic niches in mobile epifauna assemblages associated with seaweeds and habitats of syngnathid fishes in cíes archipelago (Atlantic islands marine national park, NW Iberia). *Front. Mar. Sci.* 8. doi: 10.3389/fmars.2021.773367

Planas, M. (2022). Ecological traits and trophic plasticity in the greater pipefish *Syngnathus acus* in the NW Iberian peninsula. *Biology* 11, 712. doi: 10.3390/biology11050712

Planas, M., Chamorro, A., Paltrinieri, A., Campos, S., Nedelec, K. , and Hernández–Urcera, J. (2020a). Effect of diet on breeders and inheritance in syngnathids: Application of isotopic experimentally derived data to field studies. *Mar. Ecol. Prog. Ser.* 650, 107–123. doi: 10.3354/meps13315

Planas, M., Olivotto, I., González, M. J., Laurà, R. , and Zarantoniello, M. (2020b). A multidisciplinary experimental study of the effects of breeders diet on newborn seahorses (*Hippocampus guttulatus*). *Front. Mar. Sci.* 7. doi: 10.3389/fmars.2020.00638

Planas, M., Piñeiro-Corbeira, C., Bouza, C., Castejón-Silvo, I., Vera, M., Regueira, M., et al. (2021). A multidisciplinary approach to identify priority areas for the monitoring of a vulnerable family of fishes in Spanish marine national parks. *BMC Ecol. Evol.* 21, 4. doi: 10.1186/s12862-020-01743-z

Possamai, B., Hoeinghaus, D. J., and Garcia, A. M. (2021). Shifting baselines: Integrating ecological and isotopic time lags improves trophic position estimates in aquatic consumers. *Mar. Ecol. Prog. Ser.* 666, 19–30. doi: 10.3354/meps13682

Post, D. M. (2002). Using stable isotopes to estimate trophic position: models, methods, and assumptions. *Ecology* 83, 703–718. doi: 10.2307/3071875

Post, D. M., Layman, C. A., Arrington, G., Takimoto, J., Quatrocchi, J., and Montaña, C. G. (2007). Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analyses. *Oecologia* 152, 179–189. doi: 10.1007/s00442-006-0630-x

Quezada-Romegialli, C., Jackson, A. L., Hayden, B., Kahilainen, K. K., Lopes, C., and Harrod, C. (2018). tRophicPosition, an r package for the Bayesian estimation of trophic position from consumer stable isotope ratios. *Methods Ecol. Evol.* 9, 1592–1599. doi: 10.1111/2041-210X.13009

R Core Team (2019). *R: A language and environment for statistical computing* (Vienna, Austria: R Foundation for Statistical Computing). Available at: https://www.R-project.org.

Sanderson, B. L., Tran, C. D., Coe, H. J., Pelekis, V., Steel, E. A., and Reichert, W. L. (2009). Nonlethal sampling of fish caudal fins yields valuable stable isotope data for threatened and endangered fishes. *Trans. Am. Fish. Soc* 138, 1166–1177. doi: 10.1577/T08-086.1

Sarkar, D., Andrews, F., Wright, K., Klepeis, N., and Murrell, P. (2020) *Lattice: Trellis graphics for r*. Available at: https://cran.r-project.org/web/packages/lattice/index.html.

Seed, R., Elliott, M. N., Boaden, P. J. S., and O'Connor, R. J. (1981). The composition and seasonal changes amongst the epifaunal associted with *Fucus serratus* l. @ in strangford lough, northern Ireland. *Cah. Biol. Mar.* 22, 243–266.

Smith, J. A., Mazumder, D., Suthers, I. M., and Taylor, M. D. (2013). To fit or not to fit: Evaluating stable isotope mixing models using simulated mixing polygons. *Methods Ecol. Evol.* 4, 612–618. doi: 10.1111/2041-210X.12048

Stock, B. C., Jackson, A. L., Ward, E. J., Parnell, A. C., Phillips, D. L., and Semmens, B. X. (2018). Analyzing mixing systems using a new generation of Bayesian tracer mixing models. *PeerJ* 6, e5096. doi: 10.7717/peerj.5096

Stock, B., and Semmens, B. (2016) *MixSIAR GUI user manual. version 3*. Available at: https://github.com/brianstock/MixSIAR.

Swanson, H. K., Lysy, M., Power, M., Stasko, A. D., Johnson, J. D., and Reist, J. D. (2015). A new probabilistic method for quantifying n-dimensional ecological niches and niche overlap. *Ecology* 96, 318–324. doi: 10.1890/14-0235.1

Taşkavak, E., Gürkan, Ş., Severa, T. M., Akalına, S. , and Özaydına, O. (2010). Gut contents and feeding habits of the great pipefish, *Syngnathus acus* linnaeus 1758, in izmir bay (Aegean Sea, Turkey). *Zool. Middle East* 50, 75–82. doi: 10.1080/09397140.2010.10638414

Thomas, S. M., and Crowther, T. W. (2015). Predicting rates of isotopic turnover across the animal kingdom: a synthesis of existing data. *J. Anim. Ecol.* 84, 861–870. doi: 10.1111/1365-2656.12326

Tieszen, L. L., Boutton, T. W., Tesdahl, K. G., and Slade, N. A. (1983). Fractionation and turnover of stable carbon isotopes in animal tissues: implications for δ^{13}C analysis of diet. *Oecologia* 57, 32–37. doi: 10.1007/BF00379558

Vafeiadou, A. M., Adão, H., De Troch, M., and Moens, T. (2013). Sample acidification effects on carbon and nitrogen stable isotope ratios of macrofauna from a zostera noltii bed. *Mar. Freshw. Res.* 64, 741–745. doi: 10.1071/MF12169

Valentini, A., Pompanon, F., and Taberlet, P. (2009). DNA Barcoding for ecologists. *Trends Ecol. Evol.* 24, 110–117. doi: 10.1016/j.tree.2008.09.011

Valladares, S., and Planas, M. (2012). Non-lethal dorsal fin sampling for stable isotope analysis in seahorses. *Aquat. Ecol.* 46, 363–370. doi: 10.1007/s10452-012-9407-y

Valladares, S., Soto, D. X., and Planas, M. (2016). Dietary composition of endangered seahorses determined by stable isotope analysis. *Mar. Fresh Res.* 68, 831–839. doi: 10.1071/MF16013

Vander Zanden, M. J., Clayton, M. K., Moody, E. K., Solomon, C. T., and Weidel, B. C. (2015). Stable isotope turnover and half-life in animal tissues: a literature synthesis. *PloS One* 10, e0116182. doi: 10.1371/journal.pone.0116182

Viejo, R. M. (1999). Mobile epifauna inhabiting the invasive *Sargassum muticum* and two local seaweeds in northern Spain. *Aquat. Bot.* 64, 131–149. doi: 10.1016/S0304-3770(99)00011-X

Wickham, H., Chang, W., Henry, L., Pedersen, T. L., Takahashi, K., Wilke, C., et al. (2020) *Ggplot2: Create elegant data visualisations using the grammar of graphics*. Available at: https://CRAN.R-project.org/package=ggplot2.

Winkler, N. S., Pérez-Matus, A., Villena, A. A., and Thiel, M. (2017). Seasonal variation in epifaunal communities associated with giant kelp (*Macrocystis pyrifera*) at an upwelling-dominated site. *Austral Ecol.* 42, 132–144. doi: 10.1111/aec.12407

Wolf, N., Carleton, S. A., and Martínez del Rio, C. (2009). Ten years of experimental animal isotopic ecology. *Funct. Ecol.* 23, 17–26. doi: 10.1111/j.1365-2435.2008.01529.x

Xu, H., Turchini, G. M., Francis, D. S., Liang, M., Mock, T. S., Rombenso, A., et al. (2020). Are fish what they eat? a fatty acid's perspective. *Prog. Lipid Res.* 80, 101064. doi: 10.1016/j.plipres.2020.101064

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Genome rearrangements, male pregnancy and immunological tolerance – the curious case of the syngnathid immune system

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The syngnathid fish group (seahorses, pipefishes and seadragons) is a fascinating lineage associated with an array of evolutionary peculiarities that include diverse morphologies and their unique male pregnancy. These oddities also extend to their immune systems, with a growing body of research highlighting a range of intriguing immunological characteristics and genomic rearrangements, which pose questions regarding their evolutionary history and immune strategies. The functional loss of the major histocompatibility complex class II pathway (MHC II) in the *Syngnathus* genus and related pathway components in the seahorse (*Hippocampus*) were two discoveries that initially piqued interest. This sparked discussions concerning immune capabilities, possible facilitative roles in advanced male pregnancy evolution through means of evoking immunological tolerance, as well as a general re-evaluation of how we interpret vertebrate immunological plasticity. Experimental approaches have attempted to clarify further the impact of immune repertoire loss on the efficacy of the syngnathid immune response, specificities regarding the pathways in play during pregnancy as well as the concept of immunological inheritance. The first characterization of the immune cell repertoire of *Syngnathus typhle* using scRNA-seq represents the latest step to understanding the immune dynamics of these enigmatic fish. This report serves as a review for the immunological insights into the fascinating syngnathid fish group; encompassing their evolutionary history, immune cell populations, links to male pregnancy, and sex specificity, in addition to highlighting future research opportunities in need of investigation.

KEYWORDS

syngnathidae, immunity, male pregnancy, review, seahorse, pipefish, genome, evolution

1 Introduction

Facilitated by the evolution of self-non-self recognition mechanisms, the immune system provides crucial protection against harmful pathogens as well as maintaining immune homeostasis. Over time, the immune system has evolved into a diverse set of specific, rapid and modulatory pathways facilitated by multifunctional cell types and chemical signals. Innate immunity represents the first line of defence, a rapid, generally non-specific response that initiates antigen-presentation, inflammation and activation of the complement and adaptive immune systems (Medzhitov and Janeway, 2000). The adaptive immune system provides highly specific responses upon pathogen re-exposure and is chief orchestrator of immune memory (Bonilla and Oettgen, 2010). Assisted by the major histocompatibility complexes (MHC I and II), antigen presentation processes must equally be able to determine self from non-self in order to avoid autoimmune related responses (Ljunggren and Kärre, 1990; Edwards and Hedrick, 1998). Forms of the evolutionary conserved innate immunity are found in all vertebrates and most invertebrates, evolving prior to the adaptive branch, which first emanated in primordial jawed vertebrates and has since become a hallmark of vertebrate evolution (Flajnik and Kasahara, 2010). Adaptive immune components are well conserved among gnathostomes from sharks to mammals, and cases of genomic immune system plasticity were deemed rare among this lineage. This has since been refuted with marine species such as the elephant shark (Venkatesh et al., 2014) and coelacanth (Amemiya et al., 2013), as well as teleost fishes including anglerfish (Dubin et al., 2019; Swann et al., 2020), Gadiformes (Star et al., 2011; Malmstrøm et al., 2016) and several representatives of syngnathids (Haase et al., 2013; Roth et al., 2020) exhibiting remarkable cases of adaptive immune system remodelling. These cases raise questions concerning the conventions of vertebrate immunity and the underlying requirements for a functional immune system.

One group in particular that has attracted significant interest is the syngnathid fish group comprising seahorses, pipefishes, pipehorses and seadragons (Herald, 1959; Dawson, 1986). The bizarre and diverse morphologies held among syngnathid teleosts are emblematic of their peculiar evolutionary path, having also evolved the sole instance of male pregnancy in the animal kingdom (Stölting and Wilson, 2007). Recent discoveries have highlighted the occurrence of adaptive immune system remodelling in some pipefish and seahorse species, giving rise to a convoluted and drastically alternative set of immune defences (Bahr and Wilson, 2011; Haase et al., 2013; Roth et al., 2020). Alongside and succeeding these studies, research has delved further into the molecular underpinnings that shaped these enigmatic fish defences; exploring the links with male pregnancy evolution, immunological tolerance, transgenerational immune priming and alternative immune strategies (Figure 1) (Roth et al., 2012; Whittington et al., 2015; Beemelmanns and Roth, 2016a; Keller and Roth, 2020; Whittington and Friesen, 2020; Parker et al., 2022). This review attempts to summarise the growing body of research concerning the syngnathid immune system, its evolution, and associations with other facets of their physiology and reproduction.

2 Genomics of the immune system

The last decade has seen the release of several high quality syngnathid genomes. Most of the studies focused around the genetics underlying syngnathid unique morphology and male pregnancy evolution (Small et al., 2016; Roth et al., 2020; Zhang et al., 2020; He et al., 2021; Li et al., 2021; Qu et al., 2021; Small et al., 2022). The genomes were fundamental to linking the loss of particular genes to a number of morphological traits such as the loss of mineralized teeth (loss of P/Q-rich *scpp* genes), pelvic fins (loss of *tbx4*) and ribs (HOX gene losses) (Lin et al., 2016; Small et al., 2016; Zhang et al., 2020; Qu et al., 2021). This research also paved the way towards the realization that the genetics of male pregnancy are highly complex. The diversity of intricate brooding structures, such as the *Hippocampus* pouch and *Syngnathus* skin folds, as well as behaviour and immune system adaptations make it difficult to disentangle the evolutionary path and genetic foundations of male pregnancy. Nonetheless, some advancements in the understanding of male pregnancy evolution have been made. For example, syngnathid genome studies reported expansions of patristacin (*pastn*) genes, metalloproteases that are involved in egg hatching (Small et al., 2016; Lin et al., 2016). This, together with pregnancy-related expression patterns suggests a role of *pastn* genes in male pregnancy evolution. The immune gene repertoire of syngnathids also appears to be modified when compared to other teleost fish, showing expansions and contractions of certain crucial gene families and pathways, as well as the loss of some key adaptive immune system components (Roth et al., 2020).

2.1 The major histocompatibility complex

The major histocompatibility complex (MHC) represents a group of adaptive immune system genes. In tetrapods and chondrichthyes, the MHC genes are closely linked on the same chromosomal region. However, in teleosts this region is split between different chromosomes

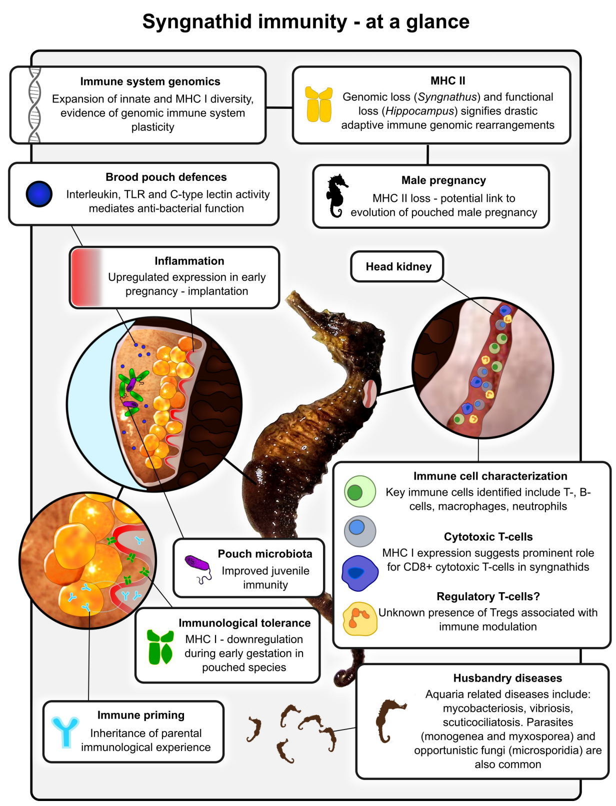

FIGURE 1
Schematic diagram highlighting some of the key immunological characteristics and research findings associated with syngnathid fishes.

and lacks defined synteny. The MHC is usually divided into two major classes that represent two arms of the adaptive immune system - I and II. The MHC class I pathway is responsible for protection against viruses and malfunctioning cells, while the MHC class II pathway defends against extracellular threats, like bacteria and parasites. Both are also crucial mediators of self-non-self recognition and maintenance of self-tolerance (Cooper and Alder, 2006; Neefjes et al., 2011; Grimholt, 2016; Roth et al., 2020). The genes that encode MHC class I and II molecules are present in multiple copies within a genome and often are highly polymorphic. These genes are further grouped into classical and non-classical subsets, depending on the structure and binding capacities of the encoded proteins. The latter is often deduced based on homology. Classical molecules are highly expressed and polymorphic; they bind peptide antigens and present them to immune cells. Non-classical molecules vary in their roles, from binding of non-peptide antigens to accessory molecules (Dijkstra et al., 2013; Grimholt et al., 2015; Grimholt, 2016). Five MHC class I lineages exist in teleosts - U, Z, S, L, and P, with all classical MHC I molecules in teleosts belonging to the U lineage (Grimholt et al., 2015; Grimholt, 2016). Teleost MHC class II molecules are divided into three lineages - A, B and E, with classical teleost MHC II molecules belonging to the A lineage, which seem to be present in all species (with a few exceptions) (Dijkstra et al., 2013; Grimholt, 2016).

Following the report in cod (Star et al., 2011) it was discovered that the pipefish *Syngnathus typhle* lacks expression of the *mhc II* α/β, *cd4* and the MHC class II transactivator (*ciita*) genes, and express a truncated version of MHC II invariant chain gene (*cd74*) (Haase et al., 2013). Consequently, it was hypothesized that like cod, *S. typhle* had lost the MHC II adaptive immune system component. The loss of these genes along with the activation induced cytidine deaminase (*aicda*) gene was then confirmed with genome studies, first in *Syngnathus scovelli* (Small et al., 2016) and then in other *Syngnathus* species (Roth et al., 2020). To date, there are only four vertebrate clades that have lost the conventional MHC class II pathway. The *Callorhinchus milii* (elephant shark) genome lacks *cd4* and related transcription factors, but contains polymorphic MHC II genes (Venkatesh et al., 2014). The *Syngnathus* genus, the entire Gadiformes order, *Lophius piscatorius* and some ceratioid species, all appear to have lost key MHC II pathway genes rendering it non-functional (Star et al., 2011; Haase et al., 2013; Malmstrøm et al., 2016; Small et al., 2016; Dubin et al., 2019; Swann et al., 2020; Roth et al., 2020).

The *Hippocampus* species have modifications in the autoimmune regulator (*aire*), *cd74*, and *ciita* genes, which also suggests an altered adaptive immune system. CIITA regulates the expression of MHC I and MHC II genes in antigen-presenting cells, and AIRE is responsible for negative selection on self-recognizing T cells. Genes encoding for AIRE and CIITA in *Hippocampus* have highly divergent exons compared to other teleost species, while CD74 has modifications in the CLIP (class II-associated invariant chain peptide) region (Roth et al., 2020). In the mammalian adaptive immune system, the CLIP region blocks the peptide-binding groove of a classical MHC class II molecule until it reaches the MHC class II compartment (MIIC) of the late endosome. The CLIP then is removed from the peptide-binding groove through the interaction with the non-classical MHC II DM molecule, allowing other peptides (e.g. antigens) to bind to it. In mammals, both the CD74 with intact CLIP and the MHC II DM are crucial for normal MHC II pathway function. Curiously, all teleosts appear to lack the aforementioned MHC II DM molecule (Neefjes et al., 2011; Dijkstra et al., 2013). To date the mechanism with which CLIP dissociates from the MHC II peptide-binding groove in teleosts is unknown. Dijkstra and colleagues suggested that the accessory molecule might not be needed at all, since some MHC II molecules bind CLIP with low affinity and that CLIP was observed to disassociate rapidly at low pH, or other non-classical MHC II molecules could functionally replace the DM (Dijkstra et al., 2013). However, the fact that CD74 with intact CLIP is preserved in most sequenced teleost species suggests that either it is homologous in function to mammalian CD74, and thus species that lost it have a modified MHC II pathway, or it has functions outside of the pathway or even the immune system.

In syngnathids, the MHC I pathway also appears to be modified. When compared to Syngnathiformes without male pregnancy, species with pregnancy (Nerophinae, *Hippocampus, and Syngnathus*) were estimated to have an expansion of genes encoding the MHC I U lineage molecules (Roth et al., 2020), though another study reports smaller numbers in some *Syngnathus* and *Hippocampus* species (11/30 vs 7/11) (Qu et al., 2021). Surprisingly, these expanded genes form distinct clusters on a gene tree. Such clustering might represent sub- or neofunctionalization, serving as an adaptation to male pregnancy and a compensatory mechanism for the MHC II loss, where certain MHC I lineages specialise on the cross-presentation pathway or perform an entirely different immune function. Similar patterns of MHC I expansions and clustering can be observed within the Gadiformes order (Star et al., 2011; Malmstrøm et al., 2016).

2.2 Alternative immune pathways

Despite high metabolic costs, the gnathostome adaptive immune system offers a tremendous advantage in a form of highly specific immune responses and immune memory. The key components of the system are highly conserved throughout the gnathostome lineage and thus it seems highly unusual for a taxon to lose a core part of its functionality (Flajnik, 2018).

Since the first report in cod there was a debate concerning the reasons and mechanisms of the MHC II loss in certain teleost clades. Two broad scenarios have been proposed so far. The first scenario simply proposes that the MHC II pathway is dispensable in teleosts and was lost through genetic drift. The second suggests that the loss was mediated by directional selection. The authors then proposed two hypotheses for the second scenario: the metabolic shift hypothesis and the functional shift hypothesis (Star and Jentoft, 2012). Though originally discussed for cod, these hypotheses can be applied to all clades that have lost the MHC II pathway.

The metabolic shift hypothesis describes a situation where under particular environmental conditions the metabolic gains of losing the MHC II pathway would outweigh the protection it provides. The mutations that hamper expression would then be favoured, resulting in the gradual loss of the pathway and its core genes. Here the loss of MHC II pathway is independent of any other potential modifications to the immune system (e.g. expansion of innate immune receptors). On the contrary, the functional shift hypothesis suggests that certain environments could favour the development of alternative adaptive or innate immune pathways that make the MHC II pathway redundant.

Selection on the pathway would then be relaxed and through genetic drift, it would slowly degenerate. In a functional shift hypothesis, the emergence of these alternative immune pathways would predate or occur concurrently with the loss of MHC II. Thus, if the hypothesis is supported, in the species that lost MHC II pathway we can expect to observe an alternative immune gene profile (e.g. *via* MHC I cross-presentation). It is important to note that these hypotheses are not mutually exclusive and could act on the immune system simultaneously (Star and Jentoft, 2012).

Since then evidence supporting the metabolic cost hypothesis was obtained in cod-like fishes demonstrating that the loss of MHC II predated expansions of MHC I genes, hinting that the latter might be a compensatory response rather than a competing alternative immune strategy (Malmstrøm et al., 2016). The Atlantic cod also shows a distinct Toll-like receptor (TLR) profile. TLRs represent a large family of Pattern Recognition Receptors (PRRs) and are one of the key components of the innate system (Brubaker et al., 2015). The expansion of *tlr7*, *8*, *9*, *22* and *25*, and contractions of *tlr2* and *5* in cod is hypothesized to be a consequence of the MHC II loss and greater reliance on the innate immune system (Solbakken et al., 2016).

In syngnathids, only one genome study has mentioned the TLR repertoire and related pathways (He et al., 2021). The researchers reported species-specific contractions of TLR 2, 4 and 5 cascades in *Hippocampus abdominalis* using Gene ontology (GO) analysis approach. The expanded genes that belong to the aforementioned GO terms are *mapk14a*, *mapk3*, *s100b*, *tab2*, *ikbkg*, *peli1b*, *irak1* and *dusp3a* (He et al., 2021). While these genes do indeed belong to the TLR signalling pathways, the results have to be treated with caution as they represent only a small fraction of the GO term size (~50 genes) and at least some of these genes also belong to other pathways that are unrelated to the immune system. Strangely enough, the TLR10 cascade term is not mentioned in the manuscript, but it also appears to be enriched because of the same eight genes. However, the TLR1, TLR2, and TLR9 related genes that appear to be contracted within the whole Syngnathidae lineage were only referred to in the supplementary materials. *Tlr18* is reported to be expanded in the ancestor of Syngnathidae, yet no additional information is provided (i.e. copy number information) (He et al., 2021). In turn, investigating the diversity of TLRs in syngnathids is crucial as sub/neofunctionalization of the expanded TLR lineages could serve as an alternative to the lost MHC II pathway.

A number of genome studies have used similar approaches, casting a wide net and looking at enriched GO terms or expanded protein families, rather than at selected individual genes or groups of genes (Small et al., 2016; Zhang et al., 2020; Qu et al., 2021; Small et al., 2022). Such an approach helps to look at a broader picture of syngnathid genome evolution, but cannot substitute a more detailed investigation of immune system components. In order to trace the immune system evolution alongside the male pregnancy gradient a thorough genome scan of all sequenced syngnathids is needed. The genes encoding innate and adaptive immune system receptors, co-receptors, accessory, regulatory and signalling molecules should be evaluated for presence-absence and copy number variations. So far, only a fraction of immune pathways have been evaluated.

In the genomes of the leafy seadragon (*Phycodurus eques*) and "weedy" seadragon (*Phyllopteryx taeniolatus*) seven gene families related to innate immunity experienced contractions, among them contractions in tripartite motif-containing (TRIM), immune-associated nucleotide-binding (IAN), and mannose receptor (MRC) gene families were identified, confirming previous reports in syngnathids (Small et al., 2022). The TRIM family of proteins are involved in many cellular processes, including within the immune system. Expansions of certain genes encoding for TRIM sub-families have been described in teleosts and shown to have strong antiviral activities (Ozato et al., 2008; Van Der Aa et al., 2009; Langevin et al., 2017). Mannose receptor family (C-type lectin superfamily) is a multifunctional protein family with roles within adaptive and innate immune systems (Vasta et al., 2011). Interestingly, a Manado pipefish (*Microphis manadensis*) genome study also reports contractions within nine C-type lectin-domain containing families (Zhang et al., 2020). The IAN/GTPase of the immunity-associated protein (IAN/GIMAP) family genes were shown to be upregulated in zebrafish during viral infections (Balla et al., 2020). GIMAP and GIMAP-like genes have also been identified in invertebrates and hypothesized to have immune functions (Weiss et al., 2013; Milan et al., 2018; Lu et al., 2020; Limoges et al., 2021). The *gimap4* gene was shown to be upregulated during pouch development and late pregnancy in *S. typhle*, which is suggested to suppress lymphocyte maturation and proliferation protecting the eggs (Roth et al., 2020). Despite the overall IAN/GIMAP family contraction, *gimap4* seems to remain intact throughout the Syngnathidae lineage (Small et al., 2022). In addition to the aforementioned C-type lectin family contraction, contractions of NACHT-domain and immunoglobulin-domain containing families in *M. manadensis* have been reported (Zhang et al., 2020).

Alternative immune strategies could develop not only *via* expansions/contractions of certain receptor molecules, but also *via* modifications of regulatory pathways. Interleukins are a group of short protein cytokines that represent promising candidates for such investigation, owing to their important involvement in innate and adaptive immune system regulation (Secombes et al., 2011).

3 Male pregnancy

3.1 Immunological tolerance

The evolution of the unique male pregnancy can only be attributed to the syngnathid fish group, of which there are a number of varying brooding strategies and physiological adaptations, some of which are similar to female amniotes (Stölting and Wilson, 2007; Whittington and Friesen, 2020). Syngnathid brooding forms range from simplified external egg-attaching integument tissue (Nerophinae), egg-retaining inverted skin flap extensions, to the advanced fully enclosed marsupium-like brood pouches with placenta-like structures (Wilson et al., 2001; Carcupino et al., 2002; Ripley et al., 2010). These brooding differences between closely related species allow for the examination of evolutionary change and the potential drivers or crucial adaptations that culminate in advanced forms of pregnancy. In turn, pregnancy evolution is heavily linked with the evolution of the adaptive immune system, and therefore syngnathids provide scope to understanding immune system evolution and its relevance within the realm of pregnancy. Currently, it is generally accepted that the expansion of a number of vertebrate systems accommodating organism physical growth, likely coincided with adaptive immune system evolution (Kasahara, 2000).

The evolution of gestation requires specialized morphological and immune gene expression changes (Moffett-King, 2002; Zenclussen et al., 2006; Hedlund et al., 2009). However, the co-evolution of gestation and the immune system creates a dilemma, regarding the avoidance of embryonic rejection *via* immune modulation and still maintaining maternal immune vigilance (La Rocca et al., 2014). In mammals, these problems have been solved through gene expression changes during pregnancy and at its onset, specific immune cell activities and specialized uterine/placental tissues (Moffett and Loke, 2006; Hedlund et al., 2009). In a general sense, immunological function in syngnathids is found to be disparate between pregnant and non-gravid individuals (Small et al., 2016; Roth et al., 2020; Parker et al., 2022). Similar suppression of the adaptive immune system has been noted in syngnathid pregnancy. This is through the diversity downregulation of MHC I genes and the functional (*Hippocampus* spp.) and complete genomic loss of MHC II (*Syngnathus* spp.), which appears a striking potential solution to immune regulation in pouched syngnathids when compared with the less drastic gene downregulation (Roth et al., 2020; Parker et al., 2022). MHC I pathway related downregulation was found to occur specifically during early gestation in syngnathids with a defined brood pouch, contrary to pouchless species (Parker et al., 2022). Immunological activity differences between brooding strategies have also been observed in *S. typhle* (inverted brood pouch), which exhibited a greater immune investment during pregnancy compared with *Nerophis ophidion* (pouchless) (Keller and Roth, 2020). These reports suggest that the evolution of the more 'intimate' brooding strategies required the coevolution of immune suppressive measures to accommodate the progeny.

Following mammalian coitus, sperm containing seminal fluid enters the female reproductive tract (Poiani, 2006). Seminal plasma is enriched with signalling molecules that have been shown to influence successful pregnancy establishment and implantation (Robertson et al., 2011; Robertson et al., 2013). It is also posited to act as an immunological tolerance primer for the receiving female to avoid embryo rejection (Tremellen and Robertson, 1999; Robertson et al., 2018). Mucus-like fluid has been reported to surround deposited syngnathid eggs (Carcupino et al., 1997; Watanabe, 1999), however, its significance or function is unknown. Whittington and Friesen (2020) have posited that the fluid could be a female equivalent to seminal fluid that potentially influences the onset and immunological homeostasis of male pregnancy. This is a deeply interesting concept that should be explored in more depth in the future.

3.2 Brood pouch defences

Unlike mammals, pouched syngnathids have had to overcome another immunological quandary when it comes to internal gestation relating to the inner pouches and progeny exposure to environmental water and pathogens (Fiedler, 1954; Whittington and Friesen, 2020; Parker et al., 2022). Balancing the activity of immune system defences and concurrent immunological tolerance measures is a challenging proposition and is yet to be fully understood. Immunological function during male pregnancy is found to be disparate to non-gravid individuals (Small et al., 2016; Roth et al., 2020; Parker et al., 2022), with immunological factor concentrations at their greatest during pregnancy in seahorses (Lin et al., 2017). Bacterial activity and growth is thought to be facilitated in the sealed brood pouch particularly during the later gestation stages (Whittington and Friesen, 2020) and are likely a key driver of these immune disparities between pregnancy statuses. Uterine flushing in some gestating shark species is believed to assist with gas exchange and waste disposal around the time of parturition (Burger, 1967; Evans et al., 1982; Tomita et al., 2016; Tomita et al., 2017). A consequential upregulation of immune processes is also suggested to counter the influx of harmful pathogens (Sunyem and Vooren, 1997; Ellis and Otway, 2011; Buddle et al., 2020). Related upregulated expression of immune genes during parturition have also been described in seahorses with studies advocating the occurrence of similar brood pouch flushing (Whittington et al., 2015; Parker et al., 2022). Brood pouch flushing and its role in immune defence is an intriguing concept that will require further experimental studies.

A number of specific immunological defence components within syngnathid brooding structures have been proposed. Transcriptomic evidence of interleukin release (Whittington et al., 2015; Jiang et al., 2022), TLR gene expression (Whittington et al., 2015; Zhang et al., 2019; Wu et al., 2021) and C-type lectin activity (Melamed et al., 2005; Small et al., 2013; Whittington et al., 2015) in syngnathid brood pouches pertain to anti-bacterial function. The co-option of the antifungal *hepcidin* genes in seahorses is believed to have dampened their antimicrobial potential in the brood pouch to assist with immune homeostasis, but could still play a minor defence role against pouch dwelling pathogens (Whittington et al., 2015; Xiao et al., 2022). Bulk RNA-seq studies have reported the upregulated expression of genes coding for phospholipase sPLA2-IB and the macroglobulin A2M in the brood pouch during pregnancy in *Hippocampus* species (Wu et al., 2021; Parker et al., 2022; Xiao et al., 2022). Both are suggested to provide antimicrobial assistance, however, their presence and specific function within the brood pouch would benefit from further functional experimentation. Retinoic acid has a number of physiological functions, many of which revolve around immune system regulation (Larange and Cheroutre, 2016). In turn, retinoic acid concentration stability was suggested to be important for avoiding oxidative stress during male pregnancy (Li et al., 2020). Prolactin has been shown to influence growth, skin secretion regulation and immunological function in teleost fishes (Páll et al., 2004; Richards et al., 2009). It has also been isolated in the seahorse brood pouch during pregnancy and is thought to contribute to pouch osmoregulation; however, its potential immunological role is yet to be properly defined (Boisseau, 1967; Patron et al., 2008; Scobell and MacKenzie, 2011; Whittington and Wilson, 2013; Clarke and Bern, 2012). Upregulated seahorse pouch-derived genes with implicated immune roles are regularly identified, however, a clear understanding of the functional relationships connecting most of the aforementioned components is still lacking. In time, condensing molecular and gene expression findings into a comprehensible network should help discern pregnancy immune modulation processes from pathogen protective measures.

3.3 Inflammation

The inflammatory function of the uterine tissues during early mammalian pregnancy is associated with tissue swelling which supports egg implantation (Mor and Abrahams, 2002; Dekel et al., 2010; Chavan et al., 2017). The fleshiness of mature male syngnathid brood pouches has been observed, in preparation for the deposition of eggs (Harlin-Cognato et al., 2006; Whittington and Friesen, 2020; Parker et al., 2022). These visual observations were recently corroborated by the upregulation of inflammation-related genes during the early stages of gestation in syngnathids of external, inverted brooding and advanced brooding forms (Parker et al., 2022), and during pouch development (Roth et al., 2020). Inflammatory pathway induction stimulates immune cell recruitment, which consequently causes cytokine release leading to tissue reshaping/remodelling (Granot et al., 2012). Therefore, it is conceivable that the extension of tissue folds in the syngnathid pouch is influenced by inflammatory pathways, and in turn aids egg immersion in pouched syngnathids. The molecular triggers for this inflammation are unknown; however, it could in part be induced by a seminal-like substance coating the deposited eggs. Seminal fluid is known to trigger inflammation upon entering the female reproductive tract in mammals (Robertson, 2005). A similar function may be found in syngnathids with female egg-coating fluid serving as the stimulant, however, this would require extensive work to clarify. Inflammation exhibited in the integument tissue of pouchless syngnathid species such as *N. ophidion* is suggested to be representative of the evolutionary root of this form of egg retention assistance (Parker et al., 2022). Similarities drawn with reproductive strategies in ricefishes (Hilgers et al., 2022), support the idea of inflammation assisting with the instigation of evolutionary modification and tissue specialisation. A number of brooding strategies have evolved within the lineage that occupy morphological gaps between the inverted dual skin-flapped pouch of *S. typhle* and the pouchless *N. ophidion*. For example, *Stigmatopora* pipefish species have pouch extensions akin to *S. typhle*, but without complete egg envelopment, while *Doryrhamphus dactyliophorus* have evolved thinner membranous egg capsules to retain the growing embryos (Wilson et al., 2001). Exploring the expression profiles of such phylogenetic representatives could provide a clearer understanding of the influence of inflammatory processes on the evolution of brood retention.

4 Sex specificity

Across a number of species, distinct differences have been documented concerning the immune capabilities of the respective sexes, with males generally possessing a less efficient immune system compared with females (Hamilton, 1948; Møller et al., 1998; Kurtz et al., 2000; Falagas et al., 2007; Roth et al., 2008; Abdullah et al., 2012). In humans, this evolutionary disparity is in part associated with female pregnancy, with an increase in immune potential linked to the parent providing the highest degree of investment (Rolff, 2002). Therefore, the strength of parental immunity appears to depend on the life-history strategy; however, within the syngnathid lineage changes in sex roles and the degrees of parental investment vary depending on the species, rendering the immunological activity and concept of sex-role reversal difficult to disentangle. Despite this, in some cases of induced parental care, sex role reversal in syngnathids appears to have potentially led to distinct sexual immune dimorphism, with males adopting the role with greatest immunological and parental responsibility, while females are tasked with attracting mates. Studies on *Hippocampus comes* and *S. typhle* support this difference, with paternal immune response efficiencies appearing greater than in females (Roth et al., 2011; Lin et al., 2016a). These reports also suggest that competition for mates reduced immunity and that the adoption of parental care during pregnancy likely has a positive effect on the parent's immunity. Experimental exposure to water contaminants further support this sex distinction with immunocompetence in males greatly exceeding that of females (Jiang et al., 2019).

Hormones represent a complicated but compelling set of factors charged with mediating many important steps in mammalian pregnancy. Hormonal dynamics are intrinsically different between males and females in humans, and endocrine processes dictate immune shifts in females during pregnancy (Robinson and Klein, 2012). A number of endocrine-related studies in syngnathids have been conducted to date, highlighting their importance in syngnathid pregnancy, parturition and pouch development (Boisseau, 1967; Mayer et al., 1993; Ripley and Foran, 2010; Scobell and MacKenzie, 2011; Whittington and Wilson, 2013; Paul et al., 2020; Dudley et al., 2021). Others have identified sexual dimorphism in the pipefish liver, suggesting that estrogen in pipefish regulates reproductive physiology similarly to fish without reversed sex roles (Rose et al., 2015). However, the significance of sex specific hormonal activity in the realm of syngnathid male pregnancy, sex role reversal and specifically immune function still remains relatively unknown, but is an interesting proposition for future investigation.

5 Immune priming

5.1 Transgenerational immune priming

Transgenerational immune priming (TGIP) describes the transfer of parentally derived immunological experience to the progeny (Grindstaff et al., 2003). TGIP has a crucial influence on offspring survival and *via* the maternal line is a phenomenon well reported across the animal kingdom (Roth et al., 2018). The unique male pregnancy provided the mechanistic opportunity for a transfer of maternal experience *via* the egg in combination with a transfer of paternal experiences provided *via* the paternal brood pouch during male pregnancy (Roth et al., 2012; Beemelmanns and Roth, 2016a). The parental investment dynamics are found to be asymmetric with maternal immune benefits only persisting during the early life stages, compared with the paternal immune influence which was suggested to be long-lasting (Roth et al., 2012; Beemelmanns and Roth, 2016a). In addition, maternal priming is suggested to benefit the offspring's adaptive immune system, while the paternal influence rather influences the innate branch (Roth et al., 2012; Beemelmanns and Roth, 2016a). This is in contrast to the investment dynamics of conventional mammalian sex roles and is one of the very few instances of paternally derived TGIP in the animal kingdom. Sex-specific grandparental immune priming has also been determined in concert with the male pregnancy system, with F2 offspring benefitting from the immunological experiences of the grandparents (Beemelmanns and Roth, 2017). Both these reports further support the influence of TGIP on the co-evolutionary arms race between pathogens and their hosts and that sex-role reversal still maintains the typical immune priming customary to mammals where the female primarily supports the offspring. Under changing environmental conditions, when parents are exposed to an additional environmental stressor (i.e., a temperature shift), the transfer of immunity from parents to offspring is hampered, implying that trans-generational plasticity reaches its limits when multiple stressors occur during the parental generation and offspring environments become unpredictable (Roth and Landis, 2017). TGIP might be influenced or partly maintained by a specific community of maternal and paternal microbes (Beemelmanns et al., 2019).

5.2 Brood pouch microbiota

Host-associated microbiota are integral for a number of physiological processes, including nutritional uptake, development and immunity; colonizing vulnerable regions such as the skin and gut in many vertebrates (Robinson et al., 2010; Hooper et al., 2012; Hacquard et al., 2015). Recently there has been a surge in research relating to the composition, evolutionary characteristics and function of the syngnathid brood pouch microbiota (Beemelmanns and Roth, 2016b; Beemelmanns et al., 2019; Wagner, 2019). It has been observed that upon immune system activation in pregnant males, there is an upward turn in microbial community richness (Beemelmanns et al., 2019). This is proposed to coincide with larval mouth opening and consequent microbial colonisation of the progeny. The establishment of a cohesive, functional microbiome is widely recognized as a crucial player in immune system development and efficiency (Gómez and Balcázar, 2008; Belkaid and Hand, 2014). Pouch microbial community changes during pregnancy, environmental influences, as well as diversity differences between pouch types and species are all topics that would benefit from further investigation. These along with future functional experimentation should improve the understanding of the functional relationships that exist between microbes, male pregnancy and immunity.

6 Immune cells

6.1 Syngnathid immune organs

The major immune organ and the first to develop in syngnathids is the head kidney (Tort et al., 2003; Falk-Petersen, 2005). Splenic presence and functionality among syngnathids is largely unsubstantiated with no spleen identifications in pipefish, while in seahorses its presence is contentious, with a number of contrasting reports (Matsunaga and Rahman, 1998; Novelli et al., 2015; Luo et al., 2016; Ofelio et al., 2018; Wijerathna et al., 2022). In seahorses, developmental studies report the presence of a spleen during early juvenile development (Novelli et al., 2015; Ofelio et al., 2018), but adult spleens are seldom reported. This could be an indication of splenic shrinkage during adulthood, which in turn could render them functional redundant, however, this speculation would require further experimental clarification. The gut-associated lymphoid tissue (GALT) is a mucosal region located in the intestines, commonly found in humans and other animals, performing an important immunological role in the gut maintaining and developing immune cells in preparation for a response (Haley, 2017). In seahorses and pipefish, the GALT has been deduced missing or at the very least reduced to a vestigial level, with immune cells primarily stemming from the main head kidney (Matsunaga and Rahman, 1998; Roth et al., 2020). This loss was proposed to be an ancestral change in predatory activity, reducing the need for gut related immunological reserves (Matsunaga and Rahman, 1998). As with other teleost species, syngnathids likely possess gill-associated lymphoid tissue (GIALT) that offers mucus derived immunological protection from external pathogens encountered during oxygen uptake (Salinas, 2015). This is a clear sign of immunological activity in the tissue (Roth et al., 2012; Birrer et al., 2012; Luo et al., 2016). Nasopharynx-associated lymphoid tissue (NALT) and skin-associated lymphoid tissue (SALT) are equally important mucosal immune hubs, serving to protect the skin and olfactory organs, respectively (Salinas, 2015). Interestingly, research pertaining to the presence of SALT and NALT in syngnathids is yet to materialise but should be encouraged as it could hold the answers for many immune related knowledge gaps across the lineage.

6.2 Immune cell populations

The identification of immune cell types in teleost fishes in recent years has been assisted by the development of efficient cell sorting machinery and the advent of single-cell RNA sequencing methodologies (Islam et al., 2014; Chen et al., 2019). These methods are at the forefront of transcriptome research, providing a high-resolution investigative assessment of cell types that transcends traditional bulk-RNA sequencing techniques. Among the fish species that have so far undergone immune cell characterizations or specific cell isolations are zebrafish (*Danio rerio*) (Dee et al., 2016; Athanasiadis et al., 2017; Carmona et al., 2017; Tang et al., 2017; Hernández et al., 2018; Ferrero et al., 2020; Loes et al., 2021), Atlantic cod (*Gadus morhua*) (Guslund et al., 2020; Guslund et al., 2022), Atlantic salmon (*Salmo salar*) (Smith et al., 2021), Nile tilapia (*Oreochromis niloticus*) (Niu et al., 2020; Wu et al., 2021), Mexican tetra (*Astyanax mexicanus*) (Peuß et al., 2020) and rainbow trout (*Oncorhynchus mykiss*) (Perdiguero et al., 2021). Recently, the first syngnathid immune cell repertoire was characterized in *S. typhle* providing a crucial baseline for future immune cell studies within the lineage (Parker et al., 2022). This report described the presence of a number of key immune cell types and their associated gene identifiers including macrophages (*mrc1*, *mpeg1*), neutrophils (*cebpe*, *lce*, *ncf4*), B- (*iglc1*, *cd53*, *cd79b*) and T-cell lymphocytes (*cd2*, *cd3e*, *v-tcr*). Interestingly, no signs of CD4$^+$ T-cell types were observed, which is in line with the loss of MHC II in the species (Haase et al., 2013; Roth et al., 2020). However, perhaps the most interesting discoveries concerned two genes within the T-cell cluster, *ilr2rb* and *gzma*, suggesting the potential presence of regulatory T-cells (Tregs) and cytotoxic T-lymphocytes (CTLs), respectively. Immunosuppressive Treg populations and their function remain elusive in syngnathids but if their identification can be confirmed, it would be a fascinating addition to the immunological tolerance discussion surrounding the lineage. The identification of CTLs in this study, along with their prominence in another recent study in the same species, suggest that they may be at the forefront of the syngnathid immune response (Parker and Roth, 2022). Elevated CTL activity in the MHC II/CD4$^+$ cell devoid *S. typhle* could also hint at a potential compensatory measure that has evolved, however, these deductions likely require further substantiation.

Transcriptome assessments of the leafy appendages possessed by the seadragon, *P. taeniolatus*, uncovered inflammation-related gene expression, suggesting a potential immunological role (Qu et al., 2021). The defensive addition of highly upregulated MHC I gene expression in the leafy extensions supports the theory that due to the appendages being crucial to seadragon camouflage and therefore survival, there is added importance in its protection and regeneration.

7 Husbandry pathology

The demand for syngnathid species, in particular seahorses, has increased drastically over the last decade as they have become a prominent feature in traditional Chinese medicine and ornamental fish collections (Vincent, 1996). These practices rely on prolific husbandry set-ups, which in turn increase the demand for information on efficient aquaculture rearing methods (Koldewey and Martin-Smith, 2010). Syngnathid fish husbandry is associated with elevated infection risks due to excessive fish handling, lower water quality and higher stock densities than those found in the natural environment (Prosser et al., 2011). Even factors such as ambient aquaria noise are believed to induce stress and impact immunological efficiency in syngnathids (Anderson et al., 2011). Syngnathid fishes raised in aquaria systems are subjected to numerous pathogenic challenges which are often exacerbated due to imperfections in husbandry rearing conditions (Sanaye et al., 2013). Here is a brief overview of some of the more common pathogens encountered by syngnathids within the aquaria trade, and their related diseases. A more comprehensive list of harmful pathogens and health issues is also included (Table 1).

Mycobacteria related infections can lead to mycobacteriosis, a disease that reared seahorses are particularly susceptible to (Koldewey, 2005). Infection has been shown to stimulate atypical lesions on a number of organs and body parts such as the tail, spleen, liver and kidney in seahorse cultures (Bombardini et al., 2006; Balcázar et al., 2011; Fogelson et al., 2017; Fogelson et al., 2018), while infections in seadragon and pipefish species have also been cited (Bombardini et al., 2006; Bonar et al., 2013).

A number of flavobacteria strains such as *Tenacibaculum* spp., *Cellulophaga fucicola* and *Flavobacterium columnare* have all been isolated previously from pipefish, seahorse and seadragon aquaria stocks (Bombardini et al., 2006; LePage, 2012; Declercq et al., 2014; LePage et al., 2015). Targeted tissues can vary greatly among fish species, with these syngnathid studies highlighting signs of necrosis in the gills, tail, skin and muscle.

Vibrio strains are the most abundant, diverse opportunistic marine pathogens and regularly used experimentally to assess syngnathid fish immunity (Thompson et al., 2004; Birrer et al., 2012; Landis et al., 2012; Roth et al., 2012; Goehlich et al., 2021). When conditions suit, they are a common cause of disease in aquaria raised syngnathids (Alcaide et al., 2001; Balcázar et al., 2010; Balcázar et al., 2010; Wang et al., 2016). Symptoms can include lethargy, skin spots, loss of appetite and tail necrosis (Balcázar et al., 2010). *Vibrio* are also implicated in opportunistic secondary infections, with recent findings suggesting that gas bubble disease (GBD) associated with syngnathid husbandry (Lin et al., 2010; LePage et al., 2015; Zhang et al., 2015), renders fish susceptible to vibrionic invasion (Kang et al., 2022). Moreover, juvenile seahorses are particularly susceptible to *Vibrio* strains, which can lead to a number of physiological and developmental issues (Lin et al., 2016b; Shao et al., 2019).

Scuticociliates are a group of parasitic unicellular marine organisms and renowned causative agents of scuticociliatosis, another common disease among aquaria fish species such as seadragons, seahorses and pipefishes (Marcer et al., 2005; Garner et al., 2008; Rossteuscher et al., 2008; Sang et al., 2011; Bonar et al., 2013; Di Cicco et al., 2013; Ofelio et al., 2014; Armwood et al., 2021). Often scuticociliatosis leads to severe skin lesions and necrosis, while internal organs, blood vessels and gills are also regularly affected (Cheung et al., 1980; Woo and Buchmann, 2012; Bonar et al., 2013; Ofelio et al., 2014).

Monogenean flatworms are another common parasite often reported among syngnathids including many from the *Syngnathus* pipefish group (Bombardini et al., 2006; Williams et al., 2008; Vaughan et al., 2010; Paladini et al., 2010). They have been shown to parasitize a number for anatomical regions including the brood pouch, skin and gills (Williams et al., 2008; Paladini et al., 2010; Cone et al., 2013). Myxospora are microscopic parasites from the cnidaria phylum that commonly infiltrate seahorses (Vincent and Clifton-Hadley, 1989; Sears et al., 2011), seadragons (Garner et al., 2008; Bonar et al., 2013) and pipefish (Longshaw et al., 2004). The parasites often reside in the gall bladder of the infected individuals (Vincent and Clifton-Hadley, 1989; Longshaw et al., 2004; Bombardini et al., 2006).

A number of fungal pathogens including microsporidia, primarily of the genus *Glugea*, and melanized fungi, have been isolated from syngnathid fishes (Bombardini et al., 2006; Nyaoke et al., 2009). *Glugea* strains are spore-forming organisms capable of transforming infected fish cells into proliferating masses known as xenomas a key symptom of microsporidiosis (Lom and Dyková, 2005; Dyková and Lom, 2007). In *Hippocampus erectus*, *Glugea* had a particular tendency to corrupt skin and connective tissues (Blasiola, 1979; Vincent and Clifton-Hadley, 1989; Bombardini et al., 2006). Phaeohyphomycosis stems from the infection of opportunistic melanized fungi such as those from the *Exaphiala* genus, and has been documented a number of times, particularly in weedy and leafy seadragons where they had a tendency to infect vascular tissues (Nyaoke et al., 2009; Bonar et al., 2013).

Viral pathogens and their aquacultural impacts are under-researched in syngnathids, with very little information documented on the topic. Suspected viral induced lesions have been identified in *H. abdominalis* previously without conclusively identifying the specific culprit (LePage et al., 2015), while a more recent paper has isolated and characterized a

TABLE 1 Harmful pathogens identified in aquaria bred or raised syngnathid fishes.

Type	Class	Strains	Tissue	Species	Reference
Bacterial	Actinomycetia	*Mycobacterium poriferae*	K, SB	*Hippocampus erectus*	(Anderson and Petty, 2013)
		Mycobacterium syngnathidarum	O	*Syngnathoides biaculeatus*	(Fogelson et al., 2018)
			M	*Hippocampus erectus*	(Fogelson et al., 2018)
		Mycobacterium chelonae	S	Unspecified	(Koldewey, 2005)
		Mycobacterium marinum	S	Unspecified	
		Mycobacterium fortuitum	S	Unspecified	
		Mycobacterium spp.	S	*Hippocampus erectus*	(Bombardini et al., 2006)
			S	*Syngnathus typhle*	
			S	*Syngnathus scovelli*	
			S	*Syngnathoides biaculeatus*	
			SB, G	*Phycodurus eques*	(Bonar et al., 2013)
			SB, G	*Phyllopteryx taeniolatus*	
		Nocardia nova	S	*Hippocampus reidi*	(Dill et al., 2017)
		Gordonia sp.	K, SB	*Hippocampus erectus*	(Anderson and Petty, 2013)
		Tsukamurella paurometabola	K, M, S	*Hippocampus barbouri*	(Florio et al., 2004; Bombardini et al., 2006)
	Bacilli	*Bacillus subtilis*	T	*Hippocampus kuda*	(Sanaye et al., 2013)
	Chlamydiia	Chlamydiales spp.	S, G	*Phycodurus eques*	(Langdon et al., 1991; Meijer et al., 2006)
			S, G	*Syngnathus acus*	(Longshaw et al., 2004; LePage et al., 2015)
	Flavobacteriia	*Cellulophaga fucicola*	S	*Hippocampus kuda*	(LePage, 2012)
		Flavobacterium columnare	G	*Hippocampus abdominalis*	(LePage et al., 2015)
				Hippocampus kuda	
				Phyllopteryx taeniolatus	
		Tenacibaculum maritimum	S, G	*Syngnathus typhle*	(Bombardini et al., 2006)
		Tenacibaculum mesophilum	S	*Hippocampus kuda*	(LePage et al., 2012)
		Tenacibaculum aestuarii	T	*Hippocampus kuda*	(Declercq et al., 2014)
	Gammaproteobacteria	*Aeromonas* spp.	S	*Hippocampus erectus*	(Bombardini et al., 2006)
			S	*Syngnathus scovelli*	
		Pseudoalteromonas spongiae	T	*Hippocampus kuda*	(Sanaye et al., 2013)
		Vibrio harveyi	S	*Hippocampus kuda*	(Alcaide et al., 2001; Tendencia, 2004; Raj et al., 2010; Binh et al., 2016; Xie et al., 2020)
			S	*Hippocampus spinosissimus*	(Binh et al., 2016)
			S	*Hippocampus comes*	
			S, L, Mo	*Hippocampus* sp.	(Alcaide et al., 2001)

(Continued)

TABLE 1 Continued

Type	Class	Strains	Tissue	Species	Reference
			S, K	*Hippocampus erectus*	(Qin et al., 2017; Li et al., 2020)
		Vibrio alginolyticus	S	*Hippocampus kuda*	(Binh et al., 2016; Xie et al., 2020)
			S	*Hippocampus spinosissimus*	(Binh et al., 2016)
			S	*Hippocampus comes*	
			S, T	*Hippocampus guttalatus*	(Balcázar et al., 2010)
			S, T	*Hippocampus hippocampus*	
			G, K, L	*Hippocampus reidi*	(Martins et al., 2010)
			S, G	*Hippocampus erectus*	(Bombardini et al., 2006)
			S, G, K, SP, B	*Hippocampus barbouri*	(Florio et al., 2004; Bombardini et al., 2006)
			S, G	*Hippocampus abdominalis*	(Bombardini et al., 2006)
			S, G	*Hippocampus zosterae*	
			S, G	*Syngnathus scovelli*	
		Vibrio vulnificus	S, H	*Hippocampus kuda*	(Binh et al., 2016; Jiang et al., 2020)
			S	*Hippocampus spinosissimus*	(Binh et al., 2016)
			S	*Hippocampus comes*	
		Vibrio splendidus	S, T	*Hippocampus guttalatus*	(Balcázar et al., 2010)
			S, T	*Hippocampus hippocampus*	
		Vibrio fortis	I	*Hippocampus erectus*	(Wang et al., 2016)
		Vibrio tubiashii	L, K	*Hippocampus erectus*	(Shao et al., 2019)
		Vibrio rotiferianus	T	*Hippocampus erectus*	(Yang et al., 2017)
		Vibrio sp.	S	*Hippocampus kuda*	(Sanaye et al., 2013)
		Photobacterium ganghwense	T	*Hippocampus kuda*	
		Photobacteria sp.	I, L, K	*Hippocampus erectus*	(Zhang et al., 2022)
			S	*Hippocampus kuda*	(Sanaye et al., 2013)
Fungal	Chaetothyriomycetes	*Exophiala angulospora*	D, BV	*Phyllopteryx taeniolatus*	(Nyaoke et al., 2009)
			D, BV	*Phycodurus eques*	
		Exophiala aquamarina	D, BV	*Phycodurus eques*	
		Exophalia lecanii-corni	F, M, K, S, SB	*Hippocampus erectus*	(Armwood et al., 2021)
		Exophiala sp.	S, T	*Hippocampus erectus*	(Blazer and Wolke, 1979; Armwood et al., 2021)
			L	*Hippocampus* sp.	(Stoskopf, 1993)
		Melanized fungi (Unspecified)	BV	*Phycodurus eques*	(Bonar et al., 2013)
			BV	*Phyllopteryx taeniolatus*	
	Dothideomycetes	*Cladosporium* spp.	U	*Hippocampus erectus*	(Bombardini et al., 2006)

(Continued)

TABLE 1 Continued

Type	Class	Strains	Tissue	Species	Reference
			U	*Hippocampus zosterae*	
			U	*Hippocampus barbouri*	
	Microsporea	*Glugea heraldii*	C	*Hippocampus erectus*	(Blasiola, 1979; Vincent and Clifton-Hadley, 1989)
		Glugea sp.	S	*Hippocampus erectus*	(Bombardini et al., 2006)
	Sordariomycetes	*Fusarium solani*	S	*Hippocampus erectus*	(Salter et al., 2012; Brown et al., 2020)
	Terrasporidia	*Nucleospora hippocampi*	I	*Hippocampus erectus*	(Wang et al., 2022)
Parasitic	Cestoda	*Proteocephalidae sp*	I	Unspecified	(Bombardini et al., 2006)
	Chromadorea	*Anisakis simplex*	V	*Syngnathus acus*	(Longshaw et al., 2004)
	Conoidasida	*Cryptosporidium* sp.	I	*Hippocampus kuda*	(LePage et al., 2015)
		Eimeria phyllopterycis	I	*Phyllopteryx taeniolatus*	(Osborn et al., 1999; Upton et al., 2000)
		Eimeria syngnathi	I	*Syngnathus abaster*	(Yakimoff and Gousseff, 1936)
		Coccidian protozoa (undefined)	I	*Phyllopteryx taeniolatus*	(Bonar et al., 2013)
	Kinetoplastida	*Ichthyobodo*sp.	G	Unspecified	(Koldewey, 2005)
	Monogenea	*Gyrodactylus corleonis*	S, F, G	*Syngnathus typhle*	(Paladini et al., 2010)
		Gyrodactylus eyipayipi	U	*Syngnathus acus*	(Vaughan et al., 2010)
		Gyrodactylus neretum	U	*Syngnathus typhle*	(Paladini et al., 2010)
		Gyrodactylus pisculentus	S	*Syngnathus fuscus*	(Williams et al., 2008)
		Gyrodactylus shorti	U	*Syngnathus scovelli*	(Holliman, 1963)
		Gyrodactylus leptorhynchi	BP, S	*Syngnathus leptorhynchus*	(Cone et al., 2013)
		Gyrodactylus syngnathi	U	*Syngnathus rostellatus*	(Appleby, 1996)
		Gyrodactylus sp.	S	*Syngnathus typhle*	(Bombardini et al., 2006)
			S	*Syngnathus scovelli*	
	Myxozoa	*Sinuolinea phyllopteryxa*	U	*Phyllopteryx taeniolatus*	(Garner et al., 2008)
		Sphaeromyxidae	GB	*Hippocampus erectus*	(Vincent and Clifton-Hadley, 1989; Sears et al., 2011)
		Sphaeromyxa sabrezesi	U	*Hippocampus brevirostris*	(Bellomy, 1969)
			U	*Hippocampus guttulatus*	
		Ceratomyxa sp.	GB	Unspecified	(Bombardini et al., 2006)
		Myxidium incurvatum	GB	*Syngnathus acus*	(Longshaw et al., 2004)
		Kudoa quadratum	M	*Syngnathus acus*	
		Myxozoa sp.	K, BV	*Phyllopteryx taeniolatus*	(Bonar et al., 2013)
			K, BV	*Phycodurus eques*	
	Oligohymenophorea	*Philasterides dicentrarchi*	S	*Phycodurus eques*	(Rossteuscher et al., 2008)
			S	*Phyllopteryx taeniolatus*	
			V	*Hippocampus abdominalis*	(Marcer et al., 2005; Di Cicco et al., 2013)
			I	*Hippocampus kuda*	(Sang et al., 2011)

(Continued)

TABLE 1 Continued

Type	Class	Strains	Tissue	Species	Reference
		Porpostoma notatum	S	*Hippocampus hippocampus*	(Ofelio et al., 2014)
		Uronema marinum	G, M	*Hippocampus erectus*	(Cheung et al., 1980)
			BV, N, Bl, K	*Hippocampus kuda*	
		Uronema sp.	T	*Hippocampus kuda*	(Declercq et al., 2014)
		Uronema-like	E, S, L	*Phycodurus eques*	(Bombardini et al., 2006)
			E, S, L	*Phyllopteryx taeniolatus*	
			S, L	*Syngnathoides biaculeatus*	
		Scuticociliatida (no species)	D	*Phyllopteryx taeniolatus*	(Umehara et al., 2003)
			S, M	*Doryrhamphus dactyliophorus*	(Armwood et al., 2021)
			S, BV, G, M	*Phyllopteryx taeniolatus*	(Bonar et al., 2013)
			S, BV, G, M	*Phycodurus eques*	
		Miamiensis avidus	U	*Hippocampus* sp.	(Thompson and Moewus, 1964)
		Trichodina sp.	G	*Syngnathus acus*	(Longshaw et al., 2004)
	Palaeacanthocephala	*Corynosoma australe*	U	*Hippocampus* sp.	(Braicovich et al., 2005)
	Prostomatea	*Cryptocaryon irritans*	S	*Hippocampus kuda*	(Sanaye et al., 2013)
	Phyllopharyngea	*Brooklynella hostilis*	S	*Hippocampus kuda*	(Blasiola, 1983)
	Spirotrichea	*Licnophora hippocampi*	G, S	*Hippocampus trimaculatus*	(Meng and Yu, 1985)
	Trematoda	*Cryptocotyle lingua*	C	*Syngnathus acus*	(Longshaw et al., 2004)
		Dictysarca virens	K, SB	*Hippocampus erectus*	(Anderson and Petty, 2013)
		Digenea sp.	I	Unspecified	(Bombardini et al., 2006)
Viral	Magsaviricetes	*Betanodavirus*	N	*Hippocampus abdominalis*	(Chen et al., 2022)
Other		External gas-bubble disease	S	*Syngnathus schlegeli*	(Sanaye et al., 2013; Kang et al., 2022)
			S	*Hippocampus haema*	
		Gas-bubble disease	S, BP, H	*Hippocampus erectus*	(Bombardini et al., 2006; Lin et al., 2010; Sanaye et al., 2013)
			SB, I	*Hippocampus abdominalis*	(Woods, 2000; Sanaye et al., 2013)
		Fibrosarcoma	BP	*Hippocampus erectus*	(Willens et al., 2004)
		Angioma/ lymphangioma	S	*Hippocampus erectus*	(Boylan et al., 2015)
		Cardiac rhabdomyosarcoma	He	*Phyllopteryx taeniolatus*	(LePage et al., 2012)
		Pancriatic islet cell neoplasia	P	*Phyllopteryx taeniolatus*	(Bonar et al., 2013)

Infected tissues include: B (brain), Bl (bladder), BP (brood pouch), BV (blood vessel), C (connective tissue), D (disseminated), De (dermis), E (eye), F (fin), G (gills), H (head), He (heart), I (intestine), K (kidney), L (liver), M (muscle), Mo (mouth), N (neuronal), O (ovary), S (skin), SB (swim bladder), SP (spleen), T (tail), U (unspecified) and V (viscera).

new strain of virus called the seahorse nervous necrosis virus (SHNNV) (Chen et al., 2022). Extracted from the brain and eye, SHNNV is deduced to cause harmful vacuolations in the organs and based on infection experiments is more virulent among juveniles. In *H. abdominalis*, *viperin*, an antiviral related gene was identified and upregulated in intestinal and kidney tissues when exposed to infection, suggesting it could have a role in modulating syngnathid antiviral responses (Tharuka et al., 2019). These limited findings strengthen the need for further research focused on elucidating the pathology, diversity and general relevance of viruses in the syngnathid aquaculture trade.

8 Conclusion

Syngnathids are some of the most fascinating subjects for evolutionary and immunological research due to their unique male pregnancy and intriguing immunological rearrangements. Molecular based studies highlighted here provide an ideal platform for future experimental work, which should focus on understanding the functional properties and mechanisms at play and how they relate to syngnathid evolution and physiology. By sharing a recent common ancestor and exhibiting diverse brooding strategies, syngnathids are useful candidates for comparative work and interpreting the nuances of evolutionary adaptation. Understanding the intricate inner workings of syngnathid immune function, immunological tolerance and pregnancy, should provide a useful alternative perspective to model species research and could prove vital for the development of applied autoimmune and other medical related practices.

Author contributions

JP, AD and OR contributed to the conception of the manuscript. JP wrote the first draft of the manuscript, JP, AD and OR wrote sections of the manuscript. All authors contributed to the manuscript review, and read and approved the final submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Abdullah, M., Chai, P.-S., Chong, M.-Y., Tohit, E. R. M., Ramasamy, R., Pei, C. P., et al. (2012). Gender effect on in vitro lymphocyte subset levels of healthy individuals. *Cell Immunol.* 272 (2), 214–219. doi: 10.1016/j.cellimm.2011.10.009

Alcaide, E., Gil-Sanz, C., Sanjuan, E., Esteve, D., Amaro, C., and Silveira, L. (2001). Vibrio harveyi causes disease in seahorse, hippocampus sp. *J. Fish Dis.* 24 (5), 311–313. doi: 10.1046/j.1365-2761.2001.00297.x

Amemiya, C. T., Alföldi, J., Lee, A. P., Fan, S., Philippe, H., MacCallum, I., et al. (2013). The African coelacanth genome provides insights into tetrapod evolution. *Nature* 496 (7445), 311–316. doi: 10.1038/nature12027

Anderson, P. A., Berzins, I. K., Fogarty, F., Hamlin, H. J., and Guillette, L. J.Jr. (2011). Sound, stress, and seahorses: the consequences of a noisy environment to animal health. *Aquaculture.* 311 (1-4), 129–138. doi: 10.1016/j.aquaculture.2010.11.013

Anderson, P. A., and Petty, B. D. (2013). Mixed metazoan and bacterial infection of the gas bladder of the lined seahorse–a case report. *J. Aquat Anim. Health* 25 (1), 42–52. doi: 10.1080/08997659.2012.743932

Appleby, C. (1996). Gyrodactylus syngnathi n. sp.(Monogenea: gyrodactylidae) from the pipefish syngnathus rostellatus Nilsson, 1855 (Syngnathiformes: Syngnathidae) from the Oslo fjord, Norway. *Syst. Parasitol.* 33 (2), 131–134. doi: 10.14411/fp.2008.034

Armwood, A. R., Cañete-Gibas, C. F., Dill-Okubo, J. A., Wiederhold, N. P., and Camus, A. C. (2021). Retrospective study of phaeohyphomycosis in aquarium-housed fish, with first descriptions of exophiala lecanii-corni and neodevriesia cladophorae in fish. *J. Fish Dis.* 44 (10), 1563–1577. doi: 10.1111/jfd.13477

Athanasiadis, E. I., Botthof, J. G., Andres, H., Ferreira, L., Lio, P., and Cvejic, A. (2017). Single-cell RNA-sequencing uncovers transcriptional states and fate decisions in haematopoiesis. *Nat. Commun.* 8 (1), 1–11. doi: 10.1038/s41467-017-02305-6

Bahr, A., and Wilson, A. (2011). The impact of sex-role reversal on the diversity of the major histocompatibility complex: insights from the seahorse (Hippocampus abdominalis). *BMC Evol. Biol.* 11 (1), 121. doi: 10.1186/1471-2148-11-121

Balcázar, J. L., Gallo-Bueno, A., Planas, M., and Pintado, J. (2010). Isolation of vibrio alginolyticus and vibrio splendidus from captive-bred seahorses with disease symptoms. *Anton Leeuw Int. J. G* 97 (2), 207–210. doi: 10.1007/s10482-009-9398-4

Balcázar, J. L., Lee, N. M., Pintado, J., and Planas, M. (2010). Phylogenetic characterization and *in situ* detection of bacterial communities associated with seahorses (Hippocampus guttulatus) in captivity. *Syst. Appl. Microbiol.* 33 (2), 71–77. doi: 10.1016/j.syapm.2009.11.005

Balcázar, J. L., Planas, M., and Pintado, J. (2011). Novel mycobacterium species in seahorses with tail rot. *Emerg. Infect. Dis.* 17 (9), 1770. doi: 10.3201/eid1709.101289

Balla, K. M., Rice, M. C., Gagnon, J. A., and Elde, N. C. (2020). Linking virus discovery to immune responses visualized during zebrafish infections. *Curr. Biol.* 30 (11), 2092–2103. doi: 10.1016/j.cub.2020.04.031

Beemelmanns, A., Poirier, M., Bayer, T., Kuenzel, S., and Roth, O. (2019). Microbial embryonal colonization during pipefish male pregnancy. *Sci. Rep.* 9 (1), 1–14. doi: 10.1038/s41598-018-37026-3

Beemelmanns, A., and Roth, O. (2016a). Biparental immune priming in the pipefish syngnathus typhle. *Zoology.* 119 (4), 262–272. doi: 10.1016/j.zool.2016.06.002

Beemelmanns, A., and Roth, O. (2016b). Bacteria-type-specific biparental immune priming in the pipefish syngnathus typhle. *Ecol. Evol.* 6 (18), 6735–6757. doi: 10.1002/ece3.2391

Beemelmanns, A., and Roth, O. (2017). Grandparental immune priming in the pipefish syngnathus typhle. *BMC Evol. Biol.* 17 (1), 1–15. doi: 10.1186/s12862-017-0885-3

Belkaid, Y., and Hand, T. W. (2014). Role of the microbiota in immunity and inflammation. *Cell.* 157 (1), 121–141. doi: 10.1016/j.cell.2014.03.011

Bellomy, M. (1969). *Encyclopaedia of seahorses* (Reigate, Surrey, England: Tropical Fish Hobbyist Publications).

Binh, D. T., Quyen, V., Sang, T. Q., and Oanh, T. T. (2016). Vibriosis in cultured seahorse (Hippocampus spp.) in khanh hoa province, Vietnam. *Int. J. Innov. Stud. Aquat Biol. Fish* 2, 43–50. doi: 10.20431/2455-7670.0202005

Birrer, S. C., Reusch, T. B., and Roth, O. (2012). Salinity change impairs pipefish immune defence. *Fish Shellfish Immunol.* 33 (6), 1238–1248. doi: 10.1016/j.fsi.2012.08.028

Blasiola, G. (1979). Glugea heraldi n. sp.(Microsporida, glugeidae) from the seahorse hippocampus erectus perry. *J. Fish Dis.* 2 (6), 493–500. doi: 10.1111/j.1365-2761.1979.tb00410.x

Blasiola, G. (1983). A review of brooklynella parasitization in marine fishes: diagnosis, pathology and chemotherapy. *Proc. Int. Assoc. Aquat. Med.* 12, 1.

Blazer, V. S., and Wolke, R. E. (1979). An exophiala-like fungus as the cause of a systemic mycosis of marine fish. *J. Fish Dis.* 2 (2), 145–152. doi: 10.1111/j.1365-2761.1979.tb00151.x

Boisseau, J. (1967). Recherche sur le controle hormonal de l'incubation chez l'hippocampe. *Rev. Européen d'Endocrinologie* 4 (3), 197–234.

Bombardini, C., Florio, D., Fichtel, L., and Fioravanti, M. (2006). The main disease of syngnathidae in captivity. *Ittiopatologia.* 3 (3), 205–211.

Bonar, C., Garner, M., Weber, I. I. I. E., Keller, C., Murray, M., Adams, L., et al. (2013). Pathologic findings in weedy (Phyllopteryx taeniolatus) and leafy (Phycodurus eques) seadragons. *Vet. Pathol.* 50 (3), 368–376. doi: 10.1177/0300985813482337

Bonilla, F. A., and Oettgen, H. C. (2010). Adaptive immunity. *J. Allergy Clin. Immunol.* 125 (2), S33–S40. doi: 10.1016/j.jaci.2009.09.017

Boylan, S., Camus, A., Waltzek, T., Yarbrough, L., Miller, S., and Howard, S. (2015). Liquid nitrogen cryotherapy for fibromas in tarpon, megalops atlanticus, valenciennes 1847, and neoplasia in lined sea horse, hippocampus erectus, perry 1810. *J. Fish Dis.* 38 (7), 681–685. doi: 10.1111/jfd.12276

Braicovich, P. E., González, R. A., and Tanzola, R. D. (2005). First record of corynosoma australe (Acanthocephala, polymorphidae) parasitizing seahorse, hippocampus sp.(Pisces, syngnathidae) in Patagonia (Argentina). *Acta Parasitologica* 50 (2), 145–149.

Brown, T., Millar, Z., Evans, D., Pham, P. H., LePage, V., and Lumsden, J. S. (2020). Fusarium solani haplotype 12-b and aortic and branchial arteritis in hippocampus erectus perry. *J. Fish Dis.* 43 (2), 301–304. doi: 10.1111/jfd.13099

Brubaker, S. W., Bonham, K. S., Zanoni, I., and Kagan, J. C. (2015). Innate immune pattern recognition: a cell biological perspective. *Annu. Rev. Immunol.* 33, 257. doi: 10.1146/annurev-immunol-032414-112240

Buddle, A. L., Otway, N. M., Van Dyke, J. U., Thompson, M. B., Murphy, C. R., Dowland, S. N., et al. (2020). Structural changes to the uterus of the dwarf ornate wobbegong shark (Orectolobus ornatus) during pregnancy. *J. Morphol* 281 (4-5), 428–437. doi: 10.1002/jmor.21109

Burger, J. (1967). *Problems in the electrolyte economy of the spiny dogfish, squalus acanthias. Sharks Skates Rays.* (Baltimore: Johns Hopkins Press), 177–185.

Carcupino, M., Baldacci, A., Mazzini, M., and Franzoi, P. (1997). Morphological organization of the male brood pouch epithelium of syngnathus abaster risso (Teleostea, syngnathidae) before, during, and after egg incubation. *Tissue Cell.* 29 (1), 21–30. doi: 10.1016/S0040-8166(97)80068-7

Carcupino, M., Baldacci, A., Mazzini, M., and Franzoi, P. (2002). Functional significance of the male brood pouch in the reproductive strategies of pipefishes and seahorses: a morphological and ultrastructural comparative study on three anatomically different pouches. *J. Fish Biol.* 61 (6), 1465–1480. doi: 10.1111/j.1095-8649.2002.tb02490.x

Carmona, S. J., Teichmann, S. A., Ferreira, L., Macaulay, I. C., Stubbington, M. J., Cvejic, A., et al. (2017). Single-cell transcriptome analysis of fish immune cells provides insight into the evolution of vertebrate immune cell types. *Genome Res.* 27 (3), 451–461. doi: 10.1101/gr.207704.116

Chavan, A. R., Griffith, O. W., and Wagner, G. P. (2017). The inflammation paradox in the evolution of mammalian pregnancy: turning a foe into a friend. *Curr. Opin. Genet. Dev.* 47, 24–32. doi: 10.1016/j.gde.2017.08.004

Chen, G., Ning, B., and Shi, T. (2019). Single-cell RNA-seq technologies and related computational data analysis. *Front. Genet.* 10, 317. doi: 10.3389/fgene.2019.00317

Chen, X., Qi, J., He, L., Luo, H., Lin, J., Qiu, F., et al. (2022). Isolation and identification of a new strain of nervous necrosis virus from the big-belly seahorse hippocampus abdominalis. *Virol. J.* 19 (1), 1–9. doi: 10.1186/s12985-022-01837-8

Cheung, P., Nigrelli, R., and Ruggieri, G. (1980). Studies on the morphology of uronema marinum dujardin (Ciliatea: Uronematidae) with a description of the histopathology of the infection in marine fishes. *J. Fish Dis.* 3 (4), 295–303. doi: 10.1111/j.1365-2761.1980.tb00400.x

Clarke, W. C., and Bern, H. A. (2012). Comparative endocrinology of prolactin. *Hormonal Proteins Peptides* 8, 105–197. doi: 10.1016/B978-0-12-447208-2.50010-1

Cone, D. K., Appy, R., Baggett, L., King, S., Gilmore, S., and Abbott, C. (2013). A new gyrodactylid (Monogenea) parasitizing bay pipefish (Syngnathus leptorhynchus) from the pacific coast of north America. *J. Parasitol.* 99 (2), 183–188. doi: 10.1645/GE-3224.1

Cooper, M. D., and Alder, M. N. (2006). The evolution of adaptive immune systems. *Cell.* 124 (4), 815–822. doi: 10.1016/j.cell.2006.02.001

Dawson, C. E. (1986). "Syngnathidae," in *Fishes of the north-eastern Atlantic and Mediterranean II.* Eds. P. Whitehead, M. L. Bauchot, J. C. Hureau and J. E. T. Nielsen (Paris: UNESCO), 628–639.

Declercq, A., Chiers, K., Van Den Broeck, W., Rekecki, A., Teerlinck, S., Adriaens, D., et al. (2014). White necrotic tail tips in estuary seahorses, hippocampus kuda, bleeker. *J. Fish Dis.* 37 (5), 501–504. doi: 10.1111/jfd.12138

Dee, C. T., Nagaraju, R. T., Athanasiadis, E. I., Gray, C., Del Ama, L. F., Johnston, S. A., et al. (2016). CD4-transgenic zebrafish reveal tissue-resident Th2-and regulatory T cell–like populations and diverse mononuclear phagocytes. *J. Immunol.* 197 (9), 3520–3530. doi: 10.4049/jimmunol.1600959

Dekel, N., Gnainsky, Y., Granot, I., and Mor, G. (2010). Inflammation and implantation. *Am. J. Reprod. Immunol.* 63 (1), 17–21. doi: 10.1111/j.1600-0897.2009.00792.x

Di Cicco, E., Paradis, E., Stephen, C., Turba, M. E., and Rossi, G. (2013). Scuticociliatid ciliate outbreak in australian pot-bellied seahorse, hippocampus abdominalis (Lesson, 1827): clinical signs, histopathologic findings, and treatment with metronidazole. *J. Zoo Wildl Med.* 44 (2), 435–440. doi: 10.1638/2012-127R1.1

Dijkstra, J. M., Grimholt, U., Leong, J., Koop, B. F., and Hashimoto, K. (2013). Comprehensive analysis of MHC class II genes in teleost fish genomes reveals dispensability of the peptide-loading DM system in a large part of vertebrates. *BMC Evol. Biol.* 13 (1), 1–14. doi: 10.1186/1471-2148-13-260

Dill, J., Sanchez, S., McDermott, A., and Camus, A. (2017). Disseminated nocardiosis associated with the isolation of nocardia nova in a longsnout seahorse hippocampus reidi (Ginsburg). *J. Fish Dis.* 40 (9), 1235–1239. doi: 10.1111/jfd.12589

Dubin, A., Jørgensen, T. E., Moum, T., Johansen, S. D., and Jakt, L. M. (2019). Complete loss of the MHC II pathway in an anglerfish, lophius piscatorius. *Biol. Lett.* 15 (10), 20190594. doi: 10.1098/rsbl.2019.0594

Dudley, J., Hannaford, P., Dowland, S., Lindsay, L., Thompson, M., Murphy, C., et al. (2021). Structural changes to the brood pouch of male pregnant seahorses (Hippocampus abdominalis) facilitate exchange between father and embryos. *Placenta* 114, 115–123. doi: 10.1016/j.placenta.2021.09.002

Dyková, I., and Lom, J. (2007). *Histopathology of protistan and myxozoan infections in fishes: An atlas.* (Praha: Academia), 219.

Edwards, S. V., and Hedrick, P. W. (1998). Evolution and ecology of MHC molecules: from genomics to sexual selection. *Trends Ecol. Evol.* 13 (8), 305–311. doi: 10.1016/S0169-5347(98)01416-5

Ellis, M. T., and Otway, N. M. (2011). Uterine fluid composition of the dwarf ornate wobbegong shark (Orectolobus ornatus) during gestation. *Mar. Freshw. Res.* 62 (6), 576–582. doi: 10.1071/MF10138

Evans, D. H., Oikari, A., Kormanik, G. A., and Mansberger, L. (1982). Osmoregulation by the prenatal spiny dogfish, squalus acanthias. *J. Exp. Biol.* 101 (1), 295–305. doi: 10.1242/jeb.101.1.295

Falagas, M. E., Mourtzoukou, E. G., and Vardakas, K. Z. (2007). Sex differences in the incidence and severity of respiratory tract infections. *Respir. Med.* 101 (9), 1845–1863. doi: 10.1016/j.rmed.2007.04.011

Falk-Petersen, I. (2005). Comparative organ differentiation during early life stages of marine fish. *Fish Shellfish Immunol.* 19 (5), 397–412. doi: 10.1016/j.fsi.2005.03.006

Ferrero, G., Gomez, E., Lyer, S., Rovira, M., Miserocchi, M., Langenau, D. M., et al. (2020). The macrophage-expressed gene (mpeg) 1 identifies a subpopulation of b cells in the adult zebrafish. *J. Leukoc. Biol.* 107 (3), 431–443. doi: 10.1002/JLB.1A1119-223R

Fiedler, K. (1954). Vergleichende verhaltensstudien an seenadeln, schlangen nadeln und seepferdchen (Syngnathidae) 1. *Z Tierpsychol* 11 (3), 358–416. doi: 10.1111/j.1439-0310.1954.tb02165.x

Flajnik, M. F. (2018). A cold-blooded view of adaptive immunity. *Nat. Rev. Immunol.* 18 (7), 438–453. doi: 10.1038/s41577-018-0003-9

Flajnik, M. F., and Kasahara, M. (2010). Origin and evolution of the adaptive immune system: genetic events and selective pressures. *Nat. Rev. Genet.* 11 (1), 47. doi: 10.1038/nrg2703

Florio, D., Marcer, F., and Fichtel, L. (2004). Isolamento di tsukamurellapaurometabola e vibrio alginolyticus da hippocampus barbouri (Barbour's seahorse) stabulati inacquario. *Atti XI Convegno Nazionale S.I.P.I.* 27.

Fogelson, S. B., Camus, A. C., Lorenz, W., Phillips, A., Bartlett, P., and Sanchez, S. (2018). Mycobacterium syngnathidarum sp. nov., a rapidly growing mycobacterium identified in syngnathid fish. *Int. J. Syst. Evol. Microbiol.* 68 (12), 3696–3700. doi: 10.1099/ijsem.0.002978

Fogelson, S., Fast, M., Leary, J., and Camus, A. (2017). Pathologic features of mycobacteriosis in naturally infected syngnathidae and novel transcriptome assembly in association with disease. *J. Fish Dis.* 40 (11), 1681–1694. doi: 10.1111/jfd.12634

Garner, M., Atkinson, S., Hallett, S., Bartholomew, J., Nordhausen, R., Reed, H., et al. (2008). Renal myxozoanosis in weedy sea dragons, phyllopteryx taeniolatus (Lacepede), caused by sinuolinea phyllopteryxa n. sp. *J. Fish Dis.* 31 (1), 27–35. doi: 10.1111/j.1365-2761.2007.00862.x

Goehlich, H., Sartoris, L., Wagner, K.-S., Wendling, C. C., and Roth, O. (2021). Pipefish locally adapted to low salinity in the Baltic Sea retain phenotypic plasticity to cope with ancestral salinity levels. *Front. Ecol. Evol.* 9, 93. doi: 10.3389/fevo.2021.626442

Gómez, G. D., and Balcázar, J. L. (2008). A review on the interactions between gut microbiota and innate immunity of fish. *FEMS Immunol. Med. Microbiol.* 52 (2), 145–154. doi: 10.1111/j.1574-695X.2007.00343.x

Granot, I., Gnainsky, Y., and Dekel, N. (2012). Endometrial inflammation and effect on implantation improvement and pregnancy outcome. *Reproduction.* 144 (6), 661–668. doi: 10.1530/REP-12-0217

Grimholt, U., Tsukamoto, K., Azuma, T., Leong, J., Koop, B. F., and Dijkstra, J. M. (2015). A comprehensive analysis of teleost MHC class I sequences. *BMC Evol. Biol.* 15 (1), 1–17. doi: 10.1186/s12862-015-0309-1

Grimholt, U. (2016). MHC and evolution in teleosts. *Biology* 5 (1), 6. doi: 10.3390/biology5010006

Grindstaff, J. L., Brodie Iii, E. D., and Ketterson, E. D. (2003). Immune function across generations: Integrating mechanism and evolutionary process in maternal antibody transmission. *Proc. R Soc. Lond B Biol. Sci.* 270 (1531), 2309–2319. doi: 10.1098/rspb.2003.2485

Guslund, N., Krabberød, A., Nørstebø, S., Solbakken, M., Jakobsen, K., Johansen, F.-E., et al. (2022). Lymphocyte subsets in Atlantic cod (Gadus morhua) interrogated by single-cell sequencing. *Commun. Biol.* 5, 689. doi: 10.21203/rs.3.rs-1342445/v1

Guslund, N. C., Solbakken, M. H., Brieuc, M. S., Jentoft, S., Jakobsen, K. S., and Qiao, S.-W. (2020). Single-cell transcriptome profiling of immune cell repertoire of the Atlantic cod which naturally lacks the major histocompatibility class II system. *Front. Immunol.* 11, 2602. doi: 10.3389/fimmu.2020.559555

Haase, D., Roth, O., Kalbe, M., Schmiedeskamp, G., Scharsack, J. P., Rosenstiel, P., et al. (2013). Absence of major histocompatibility complex class II mediated immunity in pipefish, syngnathus typhle: evidence from deep transcriptome sequencing. *Biol. Lett.* 9 (2), 20130044. doi: 10.1098/rsbl.2013.0044

Hacquard, S., Garrido-Oter, R., González, A., Spaepen, S., Ackermann, G., Lebeis, S., et al. (2015). Microbiota and host nutrition across plant and animal kingdoms. *Cell Host Microbe* 17 (5), 603–616. doi: 10.1016/j.chom.2015.04.009

Haley, P. J. (2017). The lymphoid system: a review of species differences. *J. Toxicol. Pathol.* 30 (2), 111–123. doi: 10.1293/tox.2016-0075

Hamilton, J. (1948). "The role of testicular secretions as indicated by the effects of castration in man and by studies of pathological conditions and the short lifespan associated with maleness," in *Recent progress in hormone research: The proceedings of the laurentian hormone conference. 3* (New York: Academic Press), 257–322.

Harlin-Cognato, A., Hoffman, E. A., and Jones, A. G. (2006). Gene cooption without duplication during the evolution of a male-pregnancy gene in pipefish. *PNAS.* 103 (51), 19407–19412. doi: 10.1073/pnas.0603000103

Hedlund, M., Stenqvist, A.-C., Nagaeva, O., Kjellberg, L., Wulff, M., Baranov, V., et al. (2009). Human placenta expresses and secretes NKG2D ligands via exosomes that down-modulate the cognate receptor expression: evidence for immunosuppressive function. *J. Immunol.* 183 (1), 340–351. doi: 10.4049/jimmunol.0803477

He, L., Long, X., Qi, J., Wang, Z., Huang, Z., Wu, S., et al. (2021). Genome and gene evolution of seahorse species revealed by the chromosome-level genome of hippocampus abdominalis. *Mol. Ecol. Resour* 22 (4), 1465–1477. doi: 10.1111/1755-0998.13541

Herald, E. S. (1959). From pipefish to seahorse — a study of phylogenetic relationships. *Proc. Calif Acad. Sci.* 29, 465–473.

Hernández, P. P., Strzelecka, P. M., Athanasiadis, E. I., Hall, D., Robalo, A. F., Collins, C. M., et al. (2018). Single-cell transcriptional analysis reveals ILC-like cells in zebrafish. *Sci. Immunol.* 3 (29), 29. doi: 10.1126/sciimmunol.aau5265

Hilgers, L., Roth, O., Nolte, A. W., Schüller, A., Spanke, T., Flury, J. M., et al. (2022). Inflammation and convergent placenta gene co-option contributed to a novel reproductive tissue. *Curr. Biol.* 32 (3), 715–24. e4. doi: 10.1016/j.cub.2021.12.004

Holliman, R. B. (1963). Gyrodactylus shorti, a new species of monogenetic trematode from the brood pouch of the southern pipefish, Syngnathus scovelli (Evermann and Kendall). *Tulane Stud. Zool.* 10, 83–86. doi: 10.5962/bhl.part.4639

Hooper, L. V., Littman, D. R., and Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. *Science.* 336 (6086), 1268–1273. doi: 10.1126/science.1223490

Islam, S., Zeisel, A., Joost, S., La Manno, G., Zajac, P., Kasper, M., et al. (2014). Quantitative single-cell RNA-seq with unique molecular identifiers. *Nat. Methods* 11 (2), 163–166. doi: 10.1038/nmeth.2772

Jiang, H., Li, C., Zhang, B., Wu, Y., and Lin, Q. (2022). Roles of interleukins in antibacterial immune defense of the brood pouch in the lined seahorse hippocampus erectus. *J. Oceanol Limnol* 40 (1), 235–244. doi: 10.1007/s00343-021-0310-z

Jiang, H., Wang, K., Fang, Y., Chen, J., Li, Y., Xia, G., et al. (2019). Sex-biased regulation of respiratory burst, phagocytic activity and plasma immune factors in lined seahorse (Hippocampus erectus) after subchronic benzo [a] pyrene exposure. *Fish Shellfish Immunol.* 86, 1162–1168. doi: 10.1016/j.fsi.2018.12.068

Jiang, F., Yang, N., Huang, H., Feng, H., Li, Y., and Han, B. (2020). Recovery of vibrio vulnificus from head ulceration in seahorse (Hippocampus kuda). *Aquac Int.* 28 (2), 653–660. doi: 10.1007/s10499-019-00486-z

Kang, G., Choi, K.-M., Joo, M.-S., Woo, W.-S., and Park, C.-I. (2022). A case report of secondary infection by vibrio splendidus associated with gas bubble disease in syngnathid fishes (Syngnathus schlegeli and hippocampus haema). *Fish Aquat Sci.* 25 (1), 40–48. doi: 10.47853/FAS.2022.e5

Kasahara, M. (2000). *Major histocompatibility complex: evolution, structure, and function* (Japan: Springer).

Keller, I. S., and Roth, O. (2020). Parental investment and immune dynamics in sex-role reversed pipefishes. *PLos One* 15 (9), e0228974. doi: 10.1371/journal.pone.0228974

Koldewey, H. (2005). *Syngnathid husbandry in public aquariums 2005 manual* (London, UK: Project Seahorse).

Koldewey, H. J., and Martin-Smith, K. M. (2010). A global review of seahorse aquaculture. *Aquaculture.* 302 (3-4), 131–152. doi: 10.1016/j.aquaculture.2009.11.010

Kurtz, J., Wiesner, A., Götz, P., and Sauer, K. P. (2000). Gender differences and individual variation in the immune system of the scorpionfly panorpa vulgaris (Insecta: Mecoptera). *Dev. Comp. Immunol.* 24 (1), 1–12. doi: 10.1016/S0145-305X(99)00057-9

Landis, S. H., Sundin, J., Rosenqvist, G., and Roth, O. (2012). Behavioral adjustments of a pipefish to bacterial vibrio challenge. *Behav. Ecol. Sociobiol* 66 (10), 1399–1405. doi: 10.1007/s00265-012-1395-3

Langdon, J., Elliott, K., and Mackay, B. (1991). Epitheliocystis in the leafy sea-dragon. *Aust. Vet. J.* 68, 244. doi: 10.1111/j.1751-0813.1991.tb03219.x

Langevin, C., Aleksejeva, E., Houel, A., Briolat, V., Torhy, C., Lunazzi, A., et al. (2017). FTR83, a member of the large fish-specific finTRIM family, triggers IFN pathway and counters viral infection. *Front. Immunol.* 8, 617. doi: 10.3389/fimmu.2017.00617

Larange, A., and Cheroutre, H. (2016). Retinoic acid and retinoic acid receptors as pleiotropic modulators of the immune system. *Annu. Rev. Immunol.* 34, 369–394. doi: 10.1146/annurev-immunol-041015-055427

La Rocca, C., Carbone, F., Longobardi, S., and Matarese, G. (2014). The immunology of pregnancy: regulatory T cells control maternal immune tolerance toward the fetus. *Immunol. Lett.* 162 (1), 41–48. doi: 10.1016/j.imlet.2014.06.013

LePage, V. (2012). *A study of syngnathid diseases and investigation of ulcerative dermatitis [Masters thesis]* (Ontario, Canada: University of Guelph).

LePage, V., Dutton, C. J., Kummrow, M., McLelland, D. J., Young, K., and Lumsden, J. S. (2012). Neoplasia of captive yellow sea horses (Hippocampus kuda) and weedy sea dragons (Phyllopteryx taeniolatus). *J. Zoo Wildl Med.* 43 (1), 50–58. doi: 10.1638/2010-0236.1

LePage, V., Young, J., Dutton, C., Crawshaw, G., Paré, J., Kummrow, M., et al. (2015). Diseases of captive yellow seahorse hippocampus kuda b leeker, pot-bellied seahorse hippocampus abdominalis l esson and weedy seadragon phyllopteryx taeniolatus (L acépède). *J. Fish Dis.* 38 (5), 439–450. doi: 10.1111/jfd.12254

Li, C., Li, Y., Qin, G., Chen, Z., Qu, M., Zhang, B., et al. (2020). Regulatory role of retinoic acid in Male pregnancy of the seahorse. *Innovation* 1 (3), 100052. doi: 10.1016/j.xinn.2020.100052

Limoges, M.-A., Cloutier, M., Nandi, M., Ilangumaran, S., and Ramanathan, S. (2021). The GIMAP family proteins: an incomplete puzzle. *Front. Immunol.* 12, 679739. doi: 10.3389/fimmu.2021.679739

Lin, Q., Fan, S., Zhang, Y., Xu, M., Zhang, H., Yang, Y., et al. (2016). The seahorse genome and the evolution of its specialized morphology. *Nature* 540 (7633), 395. doi: 10.1038/nature20595

Lin, Q., Lin, J., and Huang, L. (2010). Effects of light intensity, stocking density and temperature on the air-bubble disease, survivorship and growth of early juvenile seahorse hippocampus erectus perry, 1810. *Aquac Res.* 42 (1), 91–98. doi: 10.1111/j.1365-2109.2010.02573.x

Lin, T., Liu, X., Xiao, D., and Zhang, D. (2017). Plasma levels of immune factors and sex steroids in the male seahorse hippocampus erectus during a breeding cycle. *Fish Physiol. Biochem.* 43 (3), 889–899. doi: 10.1007/s10695-017-0343-6

Lin, T., Zhang, D., Liu, X., and Xiao, D. (2016a). Parental care improves immunity in the seahorse (Hippocampus erectus). *Fish Shellfish Immunol.* 58, 554–562. doi: 10.1016/j.fsi.2016.09.065

Lin, T., Zhang, D., Liu, X., and Xiao, D. (2016b). Variations of immune parameters in the lined seahorse hippocampus erectus after infection with enteritis pathogen of vibrio parahaemolyticus. *Fish Shellfish Immunol.* 50, 247–254. doi: 10.1016/j.fsi.2016.01.039

Li, C., Olave, M., Hou, Y., Qin, G., Schneider, R. F., Gao, Z., et al. (2021). Genome sequences reveal global dispersal routes and suggest convergent genetic adaptations in seahorse evolution. *Nat. Commun.* 12 (1), 1–11. doi: 10.1038/s41467-021-21379-x

Li, Y., Wang, Z., Zheng, F., Wang, B., Liu, H., Zhang, P., et al. (2020). Identification and characterization of the pathogen associated with skin ulcer syndrome in lined seahorse, hippocampus erectus. *Aquac Res.* 51 (3), 989–999. doi: 10.1111/are.14445

Ljunggren, H.-G., and Kärre, K. (1990). In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today* 11, 237–244. doi: 10.1016/0167-5699(90)90097-S

Loes, A. N., Hinman, M. N., Farnsworth, D. R., Miller, A. C., Guillemin, K., and Harms, M. J. (2021). Identification and characterization of zebrafish Tlr4 coreceptor md-2. *J. Immunol.* 206 (5), 1046–1057. doi: 10.4049/jimmunol.1901288

Lom, J., and Dyková, I. (2005). Microsporidian xenomas in fish seen in wider perspective. *Folia Parasitol. (Praha)* 52 (1-2), 69. doi: 10.14411/fp.2005.010

Longshaw, M., Green, M., and Feist, S. (2004). Histopathology of parasitic infections in greater pipefish, syngnathus acus l., from an estuary in the UK. *J. Fish Dis.* 27 (4), 245–248. doi: 10.1111/j.1365-2761.2004.00533.x

Lu, L., Loker, E. S., Zhang, S.-M., Buddenborg, S. K., and Bu, L. (2020). Genome-wide discovery, and computational and transcriptional characterization of an AIG gene family in the freshwater snail biomphalaria glabrata, a vector for schistosoma mansoni. *BMC Genomics* 21 (1), 1–20. doi: 10.1186/s12864-020-6534-z

Luo, W., Wang, X., Qu, H., Qin, G., Zhang, H., and Lin, Q. (2016). Genomic structure and expression pattern of MHC IIα and IIβ genes reveal an unusual immune trait in lined seahorse hippocampus erectus. *Fish Shellfish Immunol.* 58, 521–529. doi: 10.1016/j.fsi.2016.09.057

Møller, A., Sorci, G., and Erritzøe, J. (1998). Sexual dimorphism in immune defense. *Am. Nat.* 152 (4), 605–619. doi: 10.1086/286193

Malmstrøm, M., Matschiner, M., Tørresen, O. K., Star, B., Snipen, L. G., Hansen, T. F., et al. (2016). Evolution of the immune system influences speciation rates in teleost fishes. *Nat. Genet.* 48 (10), 1204–1210. doi: 10.1038/ng.3645

F. Marcer, D. Florio, R. Galuppi, A. Gustinelli, L. Fichtel and M. Fioravantil (2005). Pathological findings in Syngnathids kept in an Italian public aquarium. *Abstract book EAFP 12th International Conference*; (Copenhagen, Denmark), pp. 172.

Martins, M., Mouriño, J., Fezer, G., Buglione Neto, C., Garcia, P., Silva, B., et al. (2010). Isolation and experimental infection with vibrio alginolyticus in the sea horse, hippocampus reidi ginsburg, 1933 (Osteichthyes: Syngnathidae) in Brazil. *Braz. J. Biol.* 70, 205–209. doi: 10.1590/S1519-69842010000100028

Matsunaga, T., and Rahman, A. (1998). What brought the adaptive immune system to vertebrates?-the jaw hypothesis and the seahorse. *Immunol. Rev.* 166 (1), 177–186. doi: 10.1111/j.1600-065X.1998.tb01262.x

Mayer, I., Rosenqvist, G., Borg, B., Ahnesjö, I., Berglund, A., and Schulz, R. W. (1993). Plasma levels of sex steroids in three species of pipefish (Syngnathidae). *Can. J. Zool.* 71 (9), 1903–1907. doi: 10.1139/z93-272

Medzhitov, R., and Janeway, C. (2000). Innate immunity. *N Engl. J. Med.* 343 (5), 338–344. doi: 10.1056/NEJM200008033430506

Meijer, A., Roholl, P. J., Ossewaarde, J. M., Jones, B., and Nowak, B. F. (2006). Molecular evidence for association of chlamydiales bacteria with epitheliocystis in leafy seadragon (Phycodurus eques), silver perch (Bidyanus bidyanus), and barramundi (Lates calcarifer). *Appl. Environ. Microbiol.* 72 (1), 284–290. doi: 10.1128/AEM.72.1.284-290.2006

Melamed, P., Xue, Y., Poon, J. F. D., Wu, Q., Xie, H., Yeo, J., et al. (2005). The male seahorse synthesizes and secretes a novel c-type lectin into the brood pouch during early pregnancy. *FEBS J.* 272 (5), 1221–1235. doi: 10.1111/j.1742-4658.2005.04556.x

Meng, Q., and Yu, K. (1985). A new species of ciliata, licnophora hippocampi sp. nov., from the seahorse hippocampus trimaculatus leach, with considerations of its control in the host. *Acta Zoologica Sinica* 31 (1), 65–69.

Milan, M., Dalla Rovere, G., Smits, M., Ferraresso, S., Pastore, P., Marin, M., et al. (2018). Ecotoxicological effects of the herbicide glyphosate in non-target aquatic species: Transcriptional responses in the mussel mytilus galloprovincialis. *Environ. pollut.* 237, 442–451. doi: 10.1016/j.envpol.2018.02.049

Moffett-King, A. (2002). Natural killer cells and pregnancy. *Nat. Rev. Immunol.* 2 (9), 656–663. doi: 10.1038/nri886

Moffett, A., and Loke, C. (2006). Immunology of placentation in eutherian mammals. *Nat. Rev. Immunol.* 6 (8), 584–594. doi: 10.1038/nri1897

Mor, G., and Abrahams, V. M. (2002). Immunology of implantation. *Immunol. Allergy Clin. North Am.* 22 (3), 545–565. doi: 10.1016/S0889-8561(02)00009-7

Neefjes, J., Jongsma, M. L., Paul, P., and Bakke, O. (2011). Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat. Rev. Immunol.* 11 (12), 823–836. doi: 10.1038/nri3084

Niu, J., Huang, Y., Liu, X., Zhang, Z., Tang, J., Wang, B., et al. (2020). Single-cell RNA-seq reveals different subsets of non-specific cytotoxic cells in teleost. *Genomics* 112 (6), 5170–5179. doi: 10.1016/j.ygeno.2020.09.031

Novelli, B., Socorro, J., Caballero, M., Otero-Ferrer, F., Segade-Botella, A., and Molina Domínguez, L. (2015). Development of seahorse (Hippocampus reidi, ginsburg 1933): histological and histochemical study. *Fish Physiol. Biochem.* 41 (5), 1233–1251. doi: 10.1007/s10695-015-0082-5

Nyaoke, A., Weber, E. S., Innis, C., Stremme, D., Dowd, C., Hinckley, L., et al. (2009). Disseminated phaeohyphomycosis in weedy seadragons (Phyllopteryx taeniolatus) and leafy seadragons (Phycodurus eques) caused by species of exophiala, including a novel species. *J. Vet. Diagn. Invest.* 21 (1), 69–79. doi: 10.1177/104063870902100111

Ofelio, C., Blanco, A., Roura, Á., Pintado, J., Pascual, S., and Planas, M. (2014). Isolation and molecular identification of the scuticociliate p orpostoma notata moebius, 1888 from moribund reared h ippocampus hippocampus (L.) seahorses, by amplification of the SSU rRNA gene sequences. *J. Fish Dis.* 37 (12), 1061–1065. doi: 10.1111/jfd.12207

Ofelio, C., Díaz, A. O., Radaelli, G., and Planas, M. (2018). Histological development of the long-snouted seahorse hippocampus guttulatus during ontogeny. *J. Fish Biol.* 93 (1), 72–87. doi: 10.1111/jfb.13668

Osborn, A., Stamper, M., Reimschuessel, R., Greenwell, M., Zwick, L., and Kinsel, M. (1999). Coccidiosis in the weedy seadragon (Phyllopteryx taeniolatus). *Proc. Int. Assoc. Aquat Med.* 30, 77–80.

Ozato, K., Shin, D.-M., Chang, T.-H., and Morse, H. C. (2008). TRIM family proteins and their emerging roles in innate immunity. *Nat. Rev. Immunol.* 8 (11), 849–860. doi: 10.1038/nri2413

Paladini, G., Fioravanti, M. L., Cable, J., and Shinn, A. (2010). The description of gyrodactylus corleonis sp. n. and g. neretum sp. n.(Platyhelminthes: Monogenea) with comments on other gyrodactylids parasitizing pipefish (Pisces: Syngnathidae). *Folia Parasitol. (Praha)* 57 (1), 17–30. doi: 10.14411/fp.2010.004

Páll, M., Liljander, M., and Borg, B. (2004). Prolactin diminishes courtship behaviour and stimulates fanning in nesting male three-spined sticklebacks, gasterosteus aculeatus. *Behaviour.* 141 (11-12), 1511–1519. doi: 10.1163/1568539042948088

Parker, J., Dubin, A., Schneider, R., Wagner, K., Jentoft, S., Böhne, A., et al. (2022). Immunological tolerance in the evolution of male pregnancy. *Mol. Ecol.* 00, 1–22. doi: 10.1111/mec.16333

Parker, J., Guslund N, S. J., and Roth, O. (2022). Characterization of pipefish immune cell populations through single-cell transcriptomics. *Front. Immunol.* 13, 820152. doi: 10.3389/fimmu.2022.820152

Parker, J., and Roth, O. (2022). Comparative assessment of immunological tolerance in fish with natural immunodeficiency. *Dev. Comp. Immunol.* 132, 104393. doi: 10.1016/j.dci.2022.104393

Patron, J. J., Herrera, A. A., and Oconer, E. P. (2008). Prolactin and growth hormone levels in the pouch fluid of gravid male seahorse, hipocampus barbouri Jordan and Richardson 1908. *Asia Life Sci.* 17 (2), 261–269.

Paul, J. W., Kemsley, J. O., Butler, T. A., Tolosa, J. M., Thompson, M. B., Smith, R., et al. (2020). A comparison of uterine contractile responsiveness to arginine vasopressin in oviparous and viviparous lizards. *J. Comp. Physiol B* 190 (1), 49–62. doi: 10.1007/s00360-019-01254-4

Perdiguero, P., Morel, E., and Tafalla, C. (2021). Diversity of rainbow trout blood b cells revealed by single cell RNA sequencing. *Biology.* 10 (6), 511. doi: 10.3390/biology10060511

Peuß, R., Box, A. C., Chen, S., Wang, Y., Tsuchiya, D., Persons, J. L., et al. (2020). Adaptation to low parasite abundance affects immune investment and immunopathological responses of cavefish. *Nat. Ecol. Evol.* 4 (10), 1416–1430. doi: 10.1038/s41559-020-1234-2

Poiani, A. (2006). Complexity of seminal fluid: a review. *Behav. Ecol. Sociobiol* 60 (3), 289–310. doi: 10.1007/s00265-006-0178-0

Prosser, C. M., Unger, M. A., and Vogelbein, W. K. (2011). Multistressor interactions in the zebrafish (Danio rerio): Concurrent phenanthrene exposure and mycobacterium marinum infection. *Aquat Toxicol.* 102 (3-4), 177–185. doi: 10.1016/j.aquatox.2011.01.011

Qin, G., Wang, X., Tan, S., and Lin, Q. (2017). A bacterial infection by vibrio harveyi causing heavy reduction of cultured lined seahorse hippocampus erectus. *J. Fish Dis.* 40 (4), 601–605. doi: 10.1111/jfd.12533

Qu, M., Liu, Y., Zhang, Y., Wan, S., Ravi, V., Qin, G., et al. (2021). Seadragon genome analysis provides insights into its phenotype and sex determination locus. *Sci. Adv.* 7 (34), eabg5196. doi: 10.1126/sciadv.abg5196

Raj, S. T., Lipton, A., and Chauhan, G. (2010). Characterization and infectivity evaluation of vibrio harveyi causing white patch disease among captive reared seahorses, hippocampus kuda. *Indian J. Mar. Sci.* 39 (1), 151–156.

Richards, J. G., Farrell, A. P., and Brauner, C. J. (2009). *Fish physiology: Hypoxia* (New York: Academic Press).

Ripley, J. L., and Foran, C. M. (2010). Quantification of whole brain arginine vasotocin for two syngnathus pipefishes: elevated concentrations correlated with paternal brooding. *Fish Physiol. Biochem.* 36 (4), 867–874. doi: 10.1007/s10695-009-9361-3

Ripley, J., Williams, P., and Foran, C. (2010). Morphological and quantitative changes in paternal brood-pouch vasculature during embryonic development in two syngnathus pipefishes. *J. Fish Biol.* 77 (1), 67–79. doi: 10.1111/j.1095-8649.2010.02659.x

Robertson, S. A. (2005). Seminal plasma and male factor signalling in the female reproductive tract. *Cell Tissue Res.* 322 (1), 43–52. doi: 10.1007/s00441-005-1127-3

Robertson, S. A., Care, A. S., and Moldenhauer, L. M. (2018). Regulatory T cells in embryo implantation and the immune response to pregnancy. *J. Clin. Invest* 128 (10), 4224–4235. doi: 10.1172/JCI122182

Robertson, S. A., Chin, P. Y., Glynn, D. J., and Thompson, J. G. (2011). Peri-conceptual cytokines–setting the trajectory for embryo implantation, pregnancy and beyond. *Am. J. Reprod. Immunol.* 66, 2–10. doi: 10.1111/j.1600-0897.2011.01039.x

Robertson, S. A., Prins, J. R., Sharkey, D. J., and Moldenhauer, L. M. (2013). Seminal fluid and the generation of regulatory T cells for embryo implantation. *Am. J. Reprod. Immunol.* 69 (4), 315–330. doi: 10.1111/aji.12107

Robinson, C. J., Bohannan, B. J., and Young, V. B. (2010). From structure to function: the ecology of host-associated microbial communities. *Microbiol. Mol. Biol. Rev.* 74 (3), 453–476. doi: 10.1128/MMBR.00014-10

Robinson, D. P., and Klein, S. L. (2012). Pregnancy and pregnancy-associated hormones alter immune responses and disease pathogenesis. *Horm. Behav.* 62 (3), 263–271. doi: 10.1016/j.yhbeh.2012.02.023

Rolff, J. (2002). Bateman's principle and immunity. *Proc. R Soc. Lond B Biol. Sci.* 269 (1493), 867–872. doi: 10.1098/rspb.2002.1959

Rose, E., Flanagan, S. P., and Jones, A. G. (2015). The effects of synthetic estrogen exposure on the sexually dimorphic liver transcriptome of the sex-role-reversed gulf pipefish. *PLosOne* 10 (10), e0139401. doi: 10.1371/journal.pone.0139401

Rossteuscher, S., Wenker, C., Jermann, T., Wahli, T., Oldenberg, E., and Schmidt-Posthaus, H. (2008). Severe scuticociliate (Philasterides dicentrarchi) infection in a population of sea dragons (Phycodurus eques and phyllopteryx taeniolatus). *Vet. Pathol.* 45 (4), 546–550. doi: 10.1354/vp.45-4-546

Roth, O., Beemelmanns, A., Barribeau, S. M., and Sadd, B. M. (2018). Recent advances in vertebrate and invertebrate transgenerational immunity in the light of ecology and evolution. *Heredity.* 121 (3), 225–238. doi: 10.1038/s41437-018-0101-2

Roth, O., Ebert, D., Vizoso, D. B., Bieger, A., and Lass, S. (2008). Male-Biased sex-ratio distortion caused by octosporea bayeri, a vertically and horizontally-transmitted parasite of daphnia magna. *Int. J. Parasitol.* 38 (8-9), 969–979. doi: 10.1016/j.ijpara.2007.11.009

Roth, O., Keller, I., Landis, S. H., Salzburger, W., and Reusch, T. B. (2012). Hosts are ahead in a marine host–parasite coevolutionary arms race: innate immune system adaptation in pipefish syngnathus typhle against vibrio phylotypes. *Evolution.* 66 (8), 2528–2539. doi: 10.1111/j.1558-5646.2012.01614.x

Roth, O., Klein, V., Beemelmanns, A., Scharsack, J. P., and Reusch, T. B. (2012). Male Pregnancy and biparental immune priming. *Am. Nat.* 180 (6), 802–814. doi: 10.1086/668081

Roth, O., and Landis, S. H. (2017). Trans-generational plasticity in response to immune challenge is constrained by heat stress. *Evol. Appl.* 10 (5), 514–528. doi: 10.1111/eva.12473

Roth, O., Scharsack, J., Keller, I., and Reusch, T. B. (2011). Bateman's principle and immunity in a sex-role reversed pipefish. *J. Evol. Biol.* 24 (7), 1410–1420. doi: 10.1111/j.1420-9101.2011.02273.x

Roth, O., Solbakken, M. H., Tørresen, O. K., Bayer, T., Matschiner, M., Baalsrud, H. T., et al. (2020). Evolution of male pregnancy associated with remodeling of canonical vertebrate immunity in seahorses and pipefishes. *PNAS* 117 (17), 9431–9439. doi: 10.1073/pnas.1916251117

Salinas, I. (2015). The mucosal immune system of teleost fish. *Biology* 4 (3), 525–539. doi: 10.3390/biology4030525

Salter, C. E., Donnell, K. O., Sutton, D. A., Marancik, D. P., Knowles, S., Clauss, T. M., et al. (2012). Dermatitis and systemic mycosis in lined seahorses hippocampus erectus associated with a marine-adapted fusarium solani species complex pathogen. *Dis. Aquat Organ* 101 (1), 23–31. doi: 10.3354/dao02506

Sanaye, S., Pawar, H., Murugan, A., Sreepada, R., Singh, T., and Ansari, Z. (2013). Diseases and parasites in cultured yellow seahorse, hippocampus kuda (Bleeker, 1852). *Fish Chimes* 32, 65–67.

Sang, P. S., Jee, E. H., Dennis, K. G., Ji, H. K., Casiano, H. C. J., Jin, W. J., et al. (2011). Identification of scuticociliate philasterides dicentrarchi from indo-pacific seahorses hippocampus kuda. *Afr J. Microbiol. Res.* 5 (7), 738–741. doi: 10.5897/AJMR10.294

Scobell, S., and MacKenzie, D. (2011). Reproductive endocrinology of syngnathidae. *J. Fish Biol.* 78 (6), 1662–1680. doi: 10.1111/j.1095-8649.2011.02994.x

Sears, B., Anderson, P., and Greiner, E. (2011). A new species of myxosporean (Sphaeromyxidae), a parasite of lined seahorses, hippocampus erectus, from the gulf of Mexico. *J. Parasitol.* 97 (4), 713–716. doi: 10.1645/GE-2565.1

Secombes, C. J., Wang, T., and Bird, S. (2011). The interleukins of fish. *Dev. Comp. Immunol.* 35 (12), 1336–1345. doi: 10.1016/j.dci.2011.05.001

Shao, P., Yong, P., Wang, X., Xie, S., Fan, Y., Zang, L., et al. (2019). Isolation, identification, and histopathological analysis of vibrio tubiashii from lined seahorse hippocampus erectus. *Dis. Aquat Organ* 133 (3), 195–205. doi: 10.3354/dao03350

Small, C. M., Bassham, S., Catchen, J., Amores, A., Fuiten, A. M., Brown, R. S., et al. (2016). The genome of the gulf pipefish enables understanding of evolutionary innovations. *Genome Biol.* 17 (1), 258. doi: 10.1186/s13059-016-1126-6

Small, C. M., Harlin-Cognato, A. D., and Jones, A. G. (2013). Functional similarity and molecular divergence of a novel reproductive transcriptome in two male-pregnant syngnathus pipefish species. *Ecol. Evol.* 3 (12), 4092–4108. doi: 10.1002/ece3.763

Small, C. M., Healey, H. M., Currey, M. C., Beck, E. A., Catchen, J., Lin, A. S., et al. (2022). Leafy and weedy seadragon genomes connect genic and repetitive DNA features to the extravagant biology of syngnathid fishes. *PNAS* 119 (26), e2119602119. doi: 10.1073/pnas.2119602119

Smith, N. C., Umasuthan, N., Kumar, S., Woldemariam, N. T., Andreassen, R., Christian, S. L., et al. (2021). Transcriptome profiling of Atlantic salmon adherent head kidney leukocytes reveals that macrophages are selectively enriched during culture. *Front. Immunol.* 12. doi: 10.3389/fimmu.2021.709910

Solbakken, M. H., Tørresen, O. K., Nederbragt, A. J., Seppola, M., Gregers, T. F., Jakobsen, K. S., et al. (2016). Evolutionary redesign of the Atlantic cod (Gadus morhua l.) toll-like receptor repertoire by gene losses and expansions. *Sci. Rep.* 6 (1), 1–14. doi: 10.1038/srep25211

Star, B., and Jentoft, S. (2012). Why does the immune system of Atlantic cod lack MHC II? *Bioessays.* 34 (8), 648–651. doi: 10.1002/bies.201200005

Star, B., Nederbragt, A. J., Jentoft, S., Grimholt, U., Malmstrøm, M., Gregers, T. F., et al. (2011). The genome sequence of Atlantic cod reveals a unique immune system. *Nature* 477 (7363), 207. doi: 10.1038/nature10342

Stölting, K. N., and Wilson, A. B. (2007). Male Pregnancy in seahorses and pipefish: beyond the mammalian model. *Bioessays.* 29 (9), 884–896. doi: 10.1002/bies.20626

Stoskopf, M. K. (1993). *Fish medicine* (Philadelphia, USA: W.B. Saunders Company).

Sunyem, P., and Vooren, C. (1997). On cloacal gestation in angel sharks from southern Brazil. *J. Fish Biol.* 50 (1), 86–94. doi: 10.1111/j.1095-8649.1997.tb01341.x

Swann, J. B., Holland, S. J., Petersen, M., Pietsch, T. W., and Boehm, T. (2020). The immunogenetics of sexual parasitism. *Science.* 369 (6511), 1608–1615. doi: 10.1126/science.aaz9445

Tang, Q., Iyer, S., Lobbardi, R., Moore, J. C., Chen, H., Lareau, C., et al. (2017). Dissecting hematopoietic and renal cell heterogeneity in adult zebrafish at single-cell resolution using RNA sequencing. *J. Exp. Med.* 214 (10), 2875–2887. doi: 10.1084/jem.20170976

Tendencia, E. A. (2004). The first report of vibrio harveyi infection in the sea horse hippocampus kuda bleekers 1852 in the Philippines. *Aquac Res.* 35 (13), 1292–1294. doi: 10.1111/j.1365-2109.2004.01109.x

Tharuka, M. N., Priyathilaka, T. T., Yang, H., Pavithiran, A., and Lee, J. (2019). Molecular and transcriptional insights into viperin protein from big-belly seahorse (Hippocampus abdominalis), and its potential antiviral role. *Fish Shellfish Immunol.* 86, 599–607. doi: 10.1016/j.fsi.2018.12.006

Thompson, F. L., Iida, T., and Swings, J. (2004). Biodiversity of vibrios. *Microbiol. Mol. Biol. Rev.* 68 (3), 403–431. doi: 10.1128/MMBR.68.3.403-431.2004

Thompson, J., and Moewus, L. (1964). Miamiensis avidus ng, n. sp., a marine facultative parasite in the ciliate order hymenostomatida. *J. Protozool* 11 (3), 378–381. doi: 10.1111/j.1550-7408.1964.tb01766.x

Tomita, T., Cotton, C. F., and Toda, M. (2016). Ultrasound and physical models shed light on the respiratory system of embryonic dogfishes. *Zoology.* 119 (1), 36–41. doi: 10.1016/j.zool.2015.09.002

Tomita, T., Nozu, R., Nakamura, M., Matsuzaki, S., Miyamoto, K., and Sato, K. (2017). Live-bearing without placenta: Physical estimation indicates the high oxygen-supplying ability of white shark uterus to the embryo. *Sci. Rep.* 7 (1), 1–7. doi: 10.1038/s41598-017-11973-9

Tort, L., Balasch, J., and Mackenzie, S. (2003). Fish immune system. a crossroads between innate and adaptive responses. *Inmunología.* 22 (3), 277–286.

Tremellen, K. P., and Robertson, S. A. (1999). "Seminal 'priming' for successful mammalian pregnancy." in *Reproductive immunology*. Eds. S. K. Gupta (Dordrecht: Springer) 88–98. doi: 10.1007/978-94-011-4197-0_9

Umehara, A., Kosuga, Y., and Hirose, H. (2003). Scuticociliata infection in the weedy sea dragon phyllopteryx taeniolatus. *Parasitol. Int.* 52 (2), 165–168. doi: 10.1016/S1383-5769(02)00080-6

Upton, S. J., Stamper, M. A., Osborn, A. L., Mumford, S. L., Zwick, L., Kinsel, M. J., et al. (2000). A new species of eimeria (Apicomplexa, eimeriidae) from the weedy sea dragon phyllopteryx taeniolatus (Osteichthyes: Syngnathidae). *Dis. Aquat Organ* 43 (1), 55–59. doi: 10.3354/dao043055

Van Der Aa, L. M., Levraud, J.-P., Yahmi, M., Lauret, E., Briolat, V., Herbomel, P., et al. (2009). A large new subset of TRIM genes highly diversified by duplication and positive selection in teleost fish. *BMC Biol.* 7 (1), 1–23. doi: 10.1186/1741-7007-7-7

Vasta, G. R., Nita-Lazar, M., Giomarelli, B., Ahmed, H., Du, S., Cammarata, M., et al. (2011). Structural and functional diversity of the lectin repertoire in teleost fish: relevance to innate and adaptive immunity. *Dev. Comp. Immunol.* 35 (12), 1388–1399. doi: 10.1016/j.dci.2011.08.011

Vaughan, D., Christison, K., Hansen, H., and Shinn, A. (2010). Gyrodactylus eyipayipi sp. n.(Monogenea: gyrodactylidae) from syngnathus acus (Syngnathidae) from south Africa. *Folia Parasitol. (Praha)* 57 (1), 11–15. doi: 10.14411/fp.2010.002

Venkatesh, B., Lee, A. P., Ravi, V., Maurya, A. K., Lian, M. M., Swann, J. B., et al. (2014). Elephant shark genome provides unique insights into gnathostome evolution. *Nature* 505 (7482), 174–179. doi: 10.1038/nature12826

Vincent, A. C. (1996). *The international trade in seahorses.* Cambridge, UK: TRAFFIC International

Vincent, A. C., and Clifton-Hadley, R. S. (1989). Parasitic infection of the seahorse (Hippocampus erectus)–a case report. *J. Wildl Dis.* 25 (3), 404–406. doi: 10.7589/0090-3558-25.3.404

Wagner, K.-S. (2019). *Cultivation and characterization of sex-specific microbiota in the broadnosed pipefish syngnathus typhle [Masters thesis]* (Kiel, Germany: Christian-Albrechts-Universität Kiel).

Wang, Y., Ying, N., Huang, Y., Zou, X., Liu, X., Li, L., et al. (2022). Nucleospora hippocampi n. sp., an intranuclear microsporidian infecting the seahorse hippocampus erectus from China. *Front. Cell Infect.* 12, 882843. doi: 10.3389/fcimb.2022.882843

Wang, X., Zhang, Y., Qin, G., Luo, W., and Lin, Q. (2016). A novel pathogenic bacteria (Vibrio fortis) causing enteritis in cultured seahorses, hippocampus erectus perry, 1810. *J. Fish Dis.* 39 (6), 765–769. doi: 10.1111/jfd.12411

Watanabe, S. (1999). *The role of male brood pouch in the reproduction of the seaweed pipefish, syngnathus schlegeli* (Tokyo: University of Tokyo).

Weiss, Y., Forêt, S., Hayward, D. C., Ainsworth, T., King, R., Ball, E. E., et al. (2013). The acute transcriptional response of the coral acropora millepora to immune challenge: expression of GiMAP/IAN genes links the innate immune responses of corals with those of mammals and plants. *BMC Genomics* 14 (1), 1–13. doi: 10.1186/1471-2164-14-400

Whittington, C. M., and Friesen, C. R. (2020). The evolution and physiology of male pregnancy in syngnathid fishes. *Biol. Rev.* 95 (5), 1252–1272. doi: 10.1111/brv.12607

Whittington, C. M., Griffith, O. W., Qi, W., Thompson, M. B., and Wilson, A. B. (2015). Seahorse brood pouch transcriptome reveals common genes associated with vertebrate pregnancy. *Mol. Biol. Evol.* 32 (12), 3114–3131. doi: 10.1093/molbev/msv177

Whittington, C. M., and Wilson, A. B. (2013). The role of prolactin in fish reproduction. *Gen. Comp. Endocrinol.* 191, 123–136. doi: 10.1016/j.ygcen.2013.05.027

Wijerathna, H., Nadarajapillai, K., Udayantha, H., Kasthuriarachchi, T., Shanaka, K., Kwon, H., et al. (2022). Molecular delineation, expression profiling, immune response, and anti-apoptotic function of a novel clusterin homolog from big-belly seahorse (Hippocampus abdominalis). *Fish Shellfish Immunol.* 124, 289–299. doi: 10.1016/j.fsi.2022.04.015

Willens, S., Dunn, J. L., and Frasca, S. (2004). Fibrosarcoma of the brood pouch in an aquarium-reared lined seahorse (Hippocampus erectus). *J. Zoo Wildl Med.* 35 (1), 107–109. doi: 10.1638/02-085

Williams, S. R., Kritsky, D. C., Dunnigan, B., Lash, R., and Klein, P. (2008). Gyrodactylus pisculentus sp. n.(Monogenoidea: Gyrodactylidae) associated with mortality of the northern pipefish, syngnathus fuscus (Syngnathiformes: Syngnathidae) at the woods hole science aquarium. *Folia Parasitol. (Praha)* 55 (4), 265. doi: 10.14411/fp.2008.034

Wilson, A. B., Vincent, A., Ahnesjö, I., and Meyer, A. (2001). Male Pregnancy in seahorses and pipefishes (family syngnathidae): rapid diversification of paternal brood pouch morphology inferred from a molecular phylogeny. *J. Hered.* 92 (2), 159–166. doi: 10.1093/jhered/92.2.159

Woo, P. T., and Buchmann, K. (2012). *Fish parasites: pathobiology and protection* (Wallingford, UK: CAB International).

Woods, C. M. (2000). Improving initial survival in cultured seahorses, hippocampus abdominalis leeson, 1827 (Teleostei: Syngnathidae). *Aquaculture* 190 (3-4), 377–388. doi: 10.1016/S0044-8486(00)00408-7

Wu, L., Gao, A., Li, L., Chen, J., Li, J., and Ye, J. (2021). A single-cell transcriptome profiling of anterior kidney leukocytes from Nile tilapia (Oreochromis niloticus). *Front. Immunol.* 12. doi: 10.3389/fimmu.2021.783196

Wu, Y., Liu, Y., Zhang, H., Wang, X., and Lin, Q. (2021). Expression patterns of alpha-2 macroglobulin reveal potential immune functions in brood pouch of the lined seahorse hippocampus erectus. *Aquaculture.* 533, 736064. doi: 10.1016/j.aquaculture.2020.736064

Wu, Y., Zhang, H., Zhang, B., Lin, Q., and Liu, Y. (2021). Molecular characterization of TLR22 and its role in immunological modification of the brood pouch of the lined seahorse, hippocampus erectus. *Aquaculture.* 539, 736628. doi: 10.1016/j.aquaculture.2021.736628

Xiao, W., Chen, Z., Zhang, Y., Wu, Y., Jiang, H., Zhang, H., et al. (2022). Hepcidin gene Co-option balancing paternal immune protection and Male pregnancy. *Front. Immunol.* 13, 884417. doi: 10.3389/fimmu.2022.884417

Xiao, W., Qin, G., Zhang, Y., Liu, Y., Zhang, H., Chen, Z., et al. (2022). The characterization of secretory phospholipase A2 group IB and its functional profiles during male pregnancy of lined seahorse hippocampus erectus. *Aquaculture* 553, 738065. doi: 10.1016/j.aquaculture.2022.738065

Xie, J., Bu, L., Jin, S., Wang, X., Zhao, Q., Zhou, S., et al. (2020). Outbreak of vibriosis caused by vibrio harveyi and vibrio alginolyticus in farmed seahorse hippocampus kuda in China. *Aquaculture* 523, 735168. doi: 10.1016/j.aquaculture.2020.735168

Yakimoff, W., and Gousseff, W. (1936). Eimeria syngnathi n. sp., a new coccidium from the great pipe fish (Syngnathus nigrolineatus). *J. R Microsc Soc.* 56 (4), 376.

Yang, Q., Zheng, L., Huang, Z., Lin, Q., Lu, Z., and Zhou, C. (2017). Identification and characterization of pathogen vibrio rotiferianus, a pathogen isolated from hippocampus erectus with tail-rot disease. *JFSC.* 24 (05), 1131–1140. doi: 10.3724/SP.J.1118.2017.16310

Zenclussen, A. C., Gerlof, K., Zenclussen, M. L., Ritschel, S., Zambon Bertoja, A., Fest, S., et al. (2006). Regulatory T cells induce a privileged tolerant microenvironment at the fetal-maternal interface. *Eur. J. Immunol.* 36 (1), 82–94. doi: 10.1002/eji.200535428

Zhang, Y., Qin, G., Lin, J., and Lin, Q. (2015). Growth, survivorship, air-bubble disease, and attachment of feeble juvenile seahorses, hippocampus kuda (Bleeker, 1852). *J. World Aquac Soc* 46 (3), 292–300. doi: 10.1111/jwas.12193

Zhang, Y.-H., Ravi, V., Qin, G., Dai, H., Zhang, H.-X., Han, F.-M., et al. (2020). Comparative genomics reveal shared genomic changes in syngnathid fishes and signatures of genetic convergence with placental mammals. *Natl. Sci. Rev.* 7 (6), 964–977. doi: 10.1093/nsr/nwaa002

Zhang, B., Zhang, H., Qin, G., Liu, Y., Han, X., Yin, J., et al. (2019). TLR2 gene in seahorse brood pouch plays key functional roles in LPS-induced antibacterial responses. *J. Fish Dis.* 42 (7), 1085–1089. doi: 10.1111/jfd.13006

Zhang, S., Zhang, S., Xiaohui, B., Zhang, L., Jiabing, L., and Ke, K. (2022). Pathogenic photobacterium sp. induce mortality in the lined seahorse (Hippocampus erectus): First case report from China. *Isr. J. Aquac - Bamidgeh* 74, 1–10. doi: 10.46989/001c.33603

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Predicting the impacts of climate change on the distribution of European syngnathids over the next century

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At the latitudinal interval that encompasses Europe, due to the gradual seawater warming imposed by climate change, there is now continuing evidence for a widespread pattern of poleward range shifts, often translated by expansions in the northern leading edges of distribution and contraction episodes at the southern boundaries. As the distribution of biodiversity is far from static, it seems imperative that we can predict changes in species' geographic ranges to potentially circumvent severe biodiversity losses in the near future. Here, we focus on syngnathids, flagship species and umbrella taxa for coastal marine ecosystems, and attempt to identify areas of environmental suitability in Europe over the next century, according to distinct greenhouse gas concentration trajectories. Our results seamlessly match the general and region-specific impacts of climate change projections for European seas. Especially within the Atlantic Coast, species are expected to continue poleward range shifts, with the hotspot of syngnathid diversity remaining around the Celtic-Biscay Shelf, albeit with slightly distinct species composition from that of present day. The most dramatic changes are expected within semi-enclosed areas such as the Baltic, the Mediterranean and the Black Sea, where present-day endemic species diversity is likely to plunge, especially under a 'business-as-usual' scenario. When looking at the potential protective role of 'no-take' marine protected areas (MPAs) on European syngnathids, a clear latitudinal asymmetry emerged. From the Atlantic diversity hotspot downwards, and especially in the Mediterranean and Black Sea, the current 'no-take' MPA layout, if it remains static, will not be especially effective in either safeguarding syngnathid diversity or delaying the expected gradual species disappearance. Europe needs to be prepared to implement, at a continental scale, coordinated conservation measures aimed at preventing (or delaying, at the very least) the loss of its marine endemic species. As umbrella species, conservation efforts aimed at syngnathids will surely have a positive impact on coastal ecosystems.

KEYWORDS

***Syngnathidae*, climate change, distribution, conservation, marine protected area (MPA)**

1 Introduction

Climate change is impacting all studied ecosystems, terrestrial and aquatic, with animals and plants alike forced to experience environments swiftly diverging from the physiological tolerance limits of the species they harbour (Parmesan, 2006; Doney et al., 2012; Pecl et al., 2017). As environmental change progresses faster than species can adapt, a wave of range shifts and extinction episodes ensues (Loarie et al., 2009; Burrows et al., 2011). Especially noticeable at the latitudinal interval that encompasses Europe, range shifts are being continuously documented in a broad variety of taxa, from algae to mammals (Lenoir and Svenning, 2015). At these latitudes, there is now recurrent evidence for a widespread pattern of poleward range shifts, often translated by expansions in the leading edges of distribution and extinction episodes at the trailing edges (e.g., Parmesan et al., 1999; Hickling et al., 2006).

Given that - now probably more than ever - the distribution of biodiversity is far from static, it seems imperative that we can predict changes in species' geographic ranges to minimize severe biodiversity losses in the near future (Moritz and Agudo, 2013). It is important to be mindful of two caveats when projecting species' geographic ranges: (i) forecasts tend to predict severe declines, a pattern that is often inconsistent with palaeoecological records that show resilience to past climatic warming (e.g., Warren et al., 2013), and ii) forecasts largely ignore how adaptation to new hypothetically suitable environments can be impacted by the eco-evolutionary feedbacks mediated by selective forces which are difficult to integrate in our models (e.g., sexual selection; García-Roa et al., 2020). Even so, there is still considerable value in predicting scenarios for species distribution in the future. High on the list is the opportunity to assess and adapt ongoing conservation strategies - for instance, by simultaneously evaluating the efficacy of existing protected areas (where species are) and deciding if new areas are required (where species will be).

Detailed information on current and future species distribution is vital in Europe, as elsewhere, to prepare robust conservation action for a range of possible climatic scenarios. To uphold the health of its seas, European Union (EU) initially committed to protect 10% of their coastal and marine areas by 2020 (Aichi Target 11, Convention on Biological Diversity), while recognizing that a larger area was required (especially in the Macaronesia region and the Mediterranean Sea). Now, the EU Biodiversity Strategy has set the goal of protecting 30% of its sea by 2030 (European Commission, Directorate-General for Environment, 2021). However, even considering this notable increase in coverage, there is concern that climate change may weaken the protective value of marine protected areas (MPAs), especially given the static boundaries of most (Cashion et al., 2020). Moreover, there is growing concern that conservation objectives for certain hotspots of climate change (e.g., semi-enclosed marine regions, especially in the eastern Mediterranean Sea) are unrealistic given the current trend of populations decline (Hermoso et al., 2022).

Known to act as umbrella taxa for coastal marine ecosystems (Shokri et al., 2009), seahorses and their close relatives recently evolved into powerful ambassadors for marine conservation (Vincent et al., 2011). Syngnathids (i.e., seahorses, pipefish, pipehorses and seadragons) belong to a large family of teleost fish (*Syngnathidae*), with 57 genera and approximately 300 species (Hamilton et al., 2017), that primarily inhabit shallow marine, brackish and freshwater habitats. Their extraordinary life histories (Ahnesjö and Craig, 2011), and particularly their truly unique mode of reproduction (male pregnancy), have long made syngnathids model organisms to address a diverse array of questions related to sexual selection (Berglund and Rosenqvist, 2003; Monteiro et al., 2017) and the evolution of reproductive complexity (Roth et al., 2020; Parker et al., 2023).

Even though syngnathids are iconic flagship species, they face numerous threats, including tremendous fishing pressure (Lawson et al., 2017) and notable habitat degradation and loss (e.g., Tiralongo and Baldacconi, 2014), not least from climate change (Pollom et al., 2021). Such global problems are certainly bearing on Europe's 19 syngnathid species: 15 pipefish (*Cosmocampus retropinnis*, *Entelurus aequoreus*, *Minyichthys sentus*, *Nerophis lumbriciformis*, *Nerophis maculatus*, *Nerophis ophidion*, *Syngnathus abaster*, *Syngnathus acus*, *Syngnathus phlegon*, *Syngnathus rostellatus*, *Syngnathus schmidti*, *Syngnathus taenionotus*, *Syngnathus tenuirostris*, *Syngnathus typhle* and *Syngnathus variegatus*) and 4 seahorse species (*Hippocampus algiricus*, *Hippocampus erectus*, *Hippocampus guttulatus* and *Hippocampus hippocampus*). From these, the International Union for Conservation of Nature (IUCN) lists 53% of the European syngnathids as Data Deficient, with current population status is unknown for 79% of the species (IUCN, 2022). These numbers showcase the knowledge gap on the health of European coastal ecosystems which severely restricts our ability to detect, reverse or avoid hypothetical perturbations, namely those imposed by climate change.

Evidence shows that European syngnathids are responding to the gradual warming of seawater imposed by climate change. The worm pipefish, *Nerophis lumbriciformis*, can now be found mid Norway (Monteiro et al., 2017), several kilometres north of the geographical limit described by Dawson (1986). The West African seahorse, *Hippocampus algiricus*, is also expanding its distribution northwards, and can now be found in the Canary Islands (Otero-Ferrer et al., 2015). Having recently crossed the Atlantic, the lined seahorse, *Hippocampus erectus*, has currently reached the Azores (Woodall et al., 2009). The black-striped pipefish, *Syngnathus abaster*, is gradually moving north, either through the Eastern Atlantic Coast or by colonising freshwater ecosystems in the east of the Mediterranean (Monteiro and Vieira, 2017). Although expansions have been more frequently reported, contractions in European syngnathid distributions are also occurring. For instance, current warming scenarios are raising concern for at least one population of the worm pipefish in the coldest part of the Mediterranean (Iglesias et al., 2021).

Here, using species distribution modelling, we attempt to identify areas of environmental suitability for European syngnathids over the next century, according to four scenarios representing distinct Representative Concentration Pathways (RCPs - greenhouse gas concentration trajectories adopted by the Intergovernmental Panel on Climate Change). Attempting precise estimates of the area of future syngnathid distributions would require detailed information that we lack, on habitat suitability, species interactions, limits for phenotypic plasticity or potential for adaptation. Instead, our primary aim was to uncover indications of possible contractions, expansions, and distributional shifts. To this extent, we based our work on the invaluable aggregated expert knowledge amassed by IUCN which provides curated expert range maps (ERMs; hand-drawn polygons that illustrate the geographic limits of species) and biological information on syngnathids (IUCN, 2022). Having projected future distributions, we then consider the possible conservation value of current European IUCN category I and II MPAs (Strict Nature Reserves, Wilderness Areas, and National Parks; all no-take areas) for syngnathids, now and in the future.

2 Materials and methods

2.1 Ocean data

In this study, to forecast the impact of climate change on European syngnathid distributions, we examined changes in SST (sea surface temperature), SSS (sea surface salinity) and SSV (sea surface current velocity) over the next century. From the available environmental data layers of Bio-Oracle (Tyberghein et al., 2012; Assis et al., 2018), we selected eight variables that were simultaneously available for the "present" (2000-2014) and projected for future climate scenarios (2040-2050 and 2090-2100) according to four distinct representative concentration pathways (RCP: 2.6, 4.5, 6.0 and 8.5) (Moss et al., 2010). Specifically, we selected SST (mean, minimum, maximum, range), SSS (minimum, maximum), and SSV (minimum, maximum). These variables, single or in combination, are well known to impact syngnathid reproduction (e.g., Monteiro et al., 2005; Silva et al., 2007; Monteiro et al., 2017), development (e.g., Hilomen-Garcia et al., 2003; Martinez-Cardenas et al., 2014), feeding and swimming (e.g., Qin et al., 2014), or even immunity (Birrer et al., 2012), and should be incorporated when attempting to understand current or future syngnathid distributions. Data consisted of rasters with a spatial resolution of 5 arcmin (i.e., approximately 0.08° or 9.2 km at the Equator).

2.2 Species range maps

Instead of trying to, once again, delimit the range of each European syngnathid, we opted to follow the information contained on the curated expert range maps provided by IUCN (IUCN, 2022). ERMs for syngnathids were downloaded as ESRI shapefiles. Where there was more than one record, we merged them into a single entry (e.g., *Hippocampus erectus* has two entries, the first depicting its distribution in the Atlantic American coast and the second considering solely the Azores). The exceptions were those species represented by records portraying distributions in different ecosystems. Since Bio-Oracle data are limited to the oceans, we did not consider freshwater and estuarine syngnathid distributions in our analysis. Thus, we discarded records exclusively depicting rivers, lakes, or estuaries. For example, *Syngnathus abaster* has three entries, each displaying a distinct type of distribution (marine, freshwater, or estuarine); we retained only marine. Simultaneously, also from the IUCN Red List of Threatened Species website (www.iucnredlist.org), we recorded information on species upper and lower depth limits. In two instances where depth distribution data was missing from the IUCN database (*Syngnathus phlegon* and *Syngnathus variegatus*), we used information from Fishbase (Froese and Pauly, 2021) and Akimova (2009). Given our current knowledge on syngnathid distributions, three ERMs were slightly modified: 1) *Syngnathus acus* range was trimmed to eliminate southern Africa distribution (where the local species is recognised as *Syngnathus temminckii*); 2) *Nerophis lumbriciformis* range was trimmed to eliminate Canary Islands (while the species was once present up to Western Sahara, the southern limit of distribution is currently continental Portugal); and 3) *Nerophis maculatus* range was trimmed to remove Azores and the continental Portuguese coast (the species presence has previously been questioned; see ICN, 1993).

2.3 Modelling procedures

For each syngnathid species, we refined the IUCN ERM, which exaggerate depth range, to create trimmed IUCN ERMs (tERMs). We constricted depth range by using ocean bathymetric information extracted from ETOPO1 (Amante and Eakins, 2009) with the R package "marmap" (Pante and Simon-Bouhet, 2013). We then profiled species' environments by defining a climate envelope constrained by the minimum and maximum values of all selected environmental variables within the tERM. Based on IUCN ERMs, we also calculated the maximum distance from the coast using a global dataset of distances from the nearest coastline (OBPG and Stumpf, 2022). This value was used when forecasting potential distributions (i.e., all projected distributions are below the maximum distance to the coast reported in IUCN ERMs).

To forecast distributions into the next century, we had to assume that syngnathids will persist primarily within present-day ranges of temperature, salinity, and water currents (i.e., similar climate niche or envelope). Using R (R Development Core Team, 2022) and the package "raster" (Hijmans and van Etten, 2012), we used i) the depth-corrected distribution, ii) maximum distance to the coast, and iii) present-day environmental variable ranges (i.e., between the maximum and minimum values recorded for each variable in present-day) to crop each of the selected environmental variables projected for each of the four RCP scenarios in the two time intervals (eight combinations). For each combination, the resulting eight environmental variable rasters were stacked, and a consensus area (i.e., the projected range) was extracted embracing only locations where all environmental conditions simultaneously fell between present-day limits (homoclime areas). As a way of validating the performance of our method, we also projected suitable areas in the current conditions (2000-2014), analysing how well the obtained distribution reflected that currently reported by IUCN, after correcting to tERM.

Given the low dispersal potential of most syngnathids (Mendes et al., 2020), we opted to use a partial-dispersal scenario (Bateman et al., 2013) by discarding seemingly suitable areas if they were either very distant or separated by known geographical barriers from current distributions. For instance, *Hippocampus erectus* is currently found on the Atlantic coast of America but not in the Pacific coast (despite the existence of similar temperature, salinity, and sea current areas right across the Panama Isthmus), so we omitted (i.e., masked) the Pacific for this specific seahorse. Nevertheless, as some syngnathid species have proven able in the past, we allowed for possible 'migrations' between the Mediterranean and Atlantic, or the Atlantic and the Baltic. We also indicated hypothetical future presence in Iceland even though today's currents would hardly allow for the colonisation of the Icelandic Coast (as we are especially interested in describing macro-geographical patterns, the inclusion/exclusion of the Icelandic Coast will not greatly impact our results).

We calculated the extension of the present distributions (IUCN ERMs and tERMs), as well as those of the hypothetically suitable distribution areas (in km^2). It is important to stress that our analysis, much like IUCN ERMs, does not take habitat into account (e.g., some species require the existence of seagrass meadows, which are only sparsely available within the distributional area) or species interactions (e.g., some species depend on the occurrence of specific prey or absence of certain predators). Even though range maps aim at defining edges of distribution, they have limited ability to detect gaps within the projected areas (Marsh et al., 2022). Thus, our distribution maps are designed to highlight areas where, if appropriate abiotic conditions are available within its borders, the species can potentially be found with greater probability (i.e., they are not a guarantee of presence). From this perspective, our area estimates, although likely more precise than those suggested by IUCN ERMs (which do not account for species depth distribution and distance to the coast), should still be interpreted with care, as they will necessarily overestimate the distribution.

Once range maps were available for the marine distribution of European syngnathid species (present and future scenarios), we were able to produce spatial descriptors of the European species diversity. With these data, we calculated alpha (i.e., species richness) and beta diversity (Sorenson dissimilarity index, ranging from 0, most similar, to 1, most dissimilar). The beta diversity was calculated per latitude comparing current predictions to the different time frames of the projections and RCPs. European MPAs shapefiles (containing individual polygons for each MPA, with information on its geographical coordinates, area and IUCN protection category) were obtained from UNEP-WCMC-IUCN (2022). After choosing IUCN category Ia, Ib and II MPAs ('no-take MPAs'), we calculated the number of syngnathid species per selected MPA.

Statistical analysis were conducted in R (R Development Core Team, 2022). Wilcoxon signed rank tests were conducted to evaluate differences in i) the area of IUCN ERMs and tERMS and ii) the % of species present in selected MPAs in the present versus future projections for all RCPs. After assessing data linearity, checking for normality of residuals (Kolmogorov-Smirnov test), homoscedasticity (Breusch-Pagan test) and autocorrelation (Durbin Watson Test), linear regressions were used to examine the relationship between i) tERMS and that our projections for the present areas, ii) Common and merged areas (of tERMS and our projections for the present), iii) species range minimum SST and area contraction for the four projected 2090-2100 RCP scenarios.

3 Results

By simultaneously considering species depth distribution and maximum distance to the coast, tERMs significantly contracted (-83.28 ± 13.70%; average ± standard deviation), when compared with the IUCN ERMS, due to the exclusion of large theoretically unsuitable areas (Wilcoxon signed rank test: V=190, P<0.001). With these newly redefined ranges (Supplementary Figures S1-S19), it was possible to observe with increased clarity that, as for the majority of syngnathids (Hamilton et al., 2017), European seahorses and pipefishes predominantly exist in shallow waters (N=19 species; mean minimum depth -0.79 ± 2.51 m; mean maximum depth: -52.52 ± 44.86 m; range: -170 to 0 m) and, consequently, essentially have coastal distributions (Figure 1).

Two analyses revealed that our climate envelope approach (i.e., projecting theoretically suitable areas based on sea surface temperature, salinity, and current velocity) closely mapped onto tERMs, thus validating the former. First, a linear regression revealed that our distributional area projections explained nearly 98% of the variation in tERMs ($Y = 6742 + 1.179\ X$, $R^2 = 0.978$, $F_{(1,17)} = 766$, $P < 0.001$), with our projections never producing smaller areas than those in tERMS (median = 15% larger). Second, another linear regression showed that the overlapping area of our projections and tERMs also explained approximately 98% of the merged area between both range maps ($Y = -3002 + 0.823\ X$, $R^2 = 0.978$, $F_{(1,17)} = 788.4$, $P < 0.001$).

In projecting hypothetically suitable areas across Europe, we found that a progressive range contraction is to be expected for most European syngnathids under all four selected RCP scenarios (2.6, 4.5, 6.0 and 8.5) in each of the two future periods (2040-2050 and 2090-2100) (Table 1). Across all 19 species, nearly a century from now, the forecasted ranges will have shrunk to 70.99 ± 29.27% of their current area in the mildest RCP 2.6. Mean contraction is expected to be even more severe (reduced to 53.78 ± 43.13% of the current area) in the most hostile RCP 8.5 scenario.

As climate change progresses, more northerly species will likely have an increased opportunity to expand their ranges while relatively more southerly species will tend to experience range contractions (Table 1). The most southerly species *Hippocampus algiricus*, *Syngnathus schmidti and Syngnathus variegatus* are expected to withstand the most drastic range reductions, especially under the 'business-as-usual' scenario (Table 1).

We found noticeable exceptions to the general pattern of range contraction, for five species that are all located at higher latitudes, where water temperature is cooler. *Nerophis ophidion* and *Syngnathus typhle* might experience no real range change; they will apparently expand north but may simultaneously lose areas in the enclosed Mediterranean and Black Sea (where northward

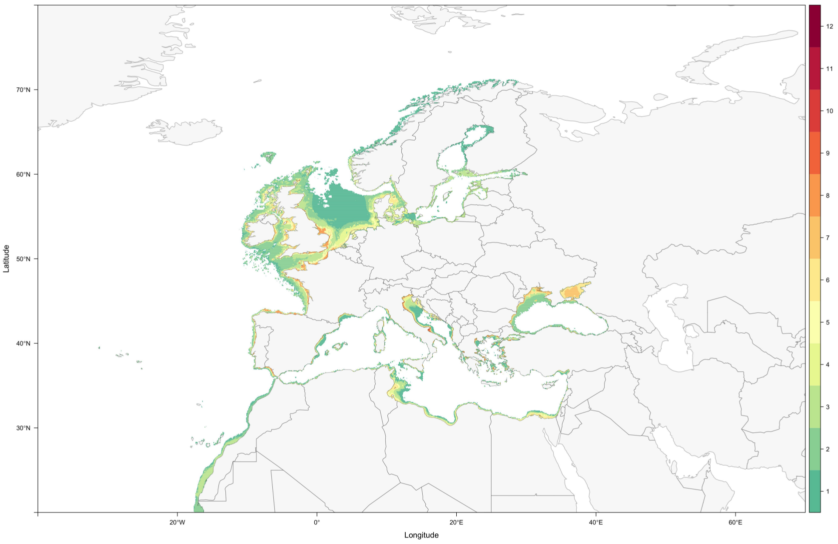

FIGURE 1
Present-day syngnathid diversity along the European coast, resulting from the sum of all nineteen syngnathid ranges (individual ranges derive from IUCN ERMs that were individually corrected for each species' depth distribution and maximum distance to the coast; colour scale highlights the number of sympatric species).

TABLE 1 Change in potentially suitable areas for European syngnathids (as % of retained present range), in two time periods (2040-2050, 2090-2100) and according to the four selected RCPs (2.6, 4.5, 6.0, 8.5).

Species	2050 2.6	2050 4.5	2050 6.0	2050 8.5	2100 2.6	2100 4.5	2100 6.0	2100 8.5
Cosmocampus retropinnis	38.5	51.1	62.4	54.9	60.7	23.8	18.2	47.4
Entelurus aequoreus	93.8	96.7	92.4	97.4	95.7	98.9	107.0	122.0
Hippocampus algiricus	10.3	5.4	15.3	1.9	33.2	2.0	2.7	2.9
Hippocampus erectus	91.0	88.0	93.6	82.9	97.4	81.6	60.6	48.8
Hippocampus guttulatus	87.3	82.6	84.8	86.1	87.2	82.8	80.7	68.8
Hippocampus hippocampus	87.7	82.2	89.1	80.8	87.3	78.4	75.1	71.6
Minyichthys sentus	125.0	173.7	109.2	131.4	111.5	55.6	114.5	19.5
Nerophis lumbriciformis	100.8	103.4	100.7	102.6	101.9	102.0	103.5	111.4
Nerophis maculatus	48.3	42.9	54.3	38.2	46.4	34.0	21.9	16.3
Nerophis ophidion	88.4	86.6	88.1	91.0	91.5	86.7	87.6	93.2
Syngnathus abaster	63.2	54.5	64.4	60.2	59.6	50.7	50.8	69.6
Syngnathus acus	93.0	89.3	93.7	88.0	91.0	85.3	83.1	82.9
Syngnathus phlegon	67.2	54.5	67.4	52.4	58.8	46.9	39.9	20.1
Syngnathus rostellatus	102.1	104.9	99.8	104.8	104.9	108.7	118.5	124.0
Syngnathus schmidti	31.1	2.5	39.5	4.4	31.4	0.2	0.0	0.0
Syngnathus taenionotus	41.8	21.1	47.3	20.5	32.6	17.5	10.5	15.6
Syngnathus tenuirostris	37.8	15.1	50.1	21.7	37.9	13.8	10.9	10.4
Syngnathus typhle	91.8	88.9	89.6	92.6	93.2	89.8	91.8	97.4
Syngnathus variegatus	23.3	0.0	37.1	5.8	26.7	0.0	0.0	0.0

Data are based on the full geographical distribution of each *species* as the range of some syngnathids occurs essentially outside Europe (*Hippocampus algiricus* and *Hippocampus erectus*).

migration is not possible). Meanwhile, the northerly species found only in the Atlantic - *Entelurus aequoreus*, *Nerophis lumbriciformis* and *Syngnathus rostellatus* – should experience range increases as they expand north into newly suitable areas without losing area in the south. Present day ranges and hypothetical future distributions (i.e., forecasted suitable areas for all combinations of the two periods and four RCP scenarios), for all nineteen European syngnathid species, can be viewed in Supplementary Figures S20-S190.

In general, linear regressions show that the present-day species experience with water temperatures (here, we used minimum SST) appropriately explain future range area reductions in the 2090-2100 interval (RCP 8.5: $Y = 16.99 - 0.09\ X$, $R^2 = 0.72$, $F_{(1,17)} = 44.06$, $P < 0.001$; RCP 6.0: $Y = 16.43 - 0.07\ X$, $R^2 = 0.49$, $F_{(1,17)} = 16.12$. $P < 0.001$; RCP 4.5: $Y = 17.65 - 0.10\ X$. $R^2 = 0.67$, $F_{(1,17)} = 34.37$, $P < 0.001$; RCP 2.6: $Y = 19.26 - 0.10\ X$, $R^2 = 0.42$, $F_{(1,17)} = 12.20$. $P < 0.005$).

Assuming no invasions by non-indigenous syngnathids, loss of species diversity will be especially apparent in the Mediterranean and Black Sea (Figure 2). Climate change-induced alteration of biotic conditions (e.g., warmer waters) linked to the lack of cooler areas that could allow substantial range shifts, might leave only a handful of refugia for the remaining Mediterranean syngnathids (Supplementary Figures S191-S198). Curiously, one of those refugia (Southern France), among the coolest sections of the Mediterranean, is the same currently occupied by what seems to be the last Mediterranean population of the worm pipefish, *Nerophis lumbriciformis*.

The forecasted progressive poleward expansions, together with the projected range contractions in the south can be summarised by looking at the latitudinal variation in both alpha diversity (species richness) and beta diversity (Sorensen dissimilarity index). While species richness will remain relatively constant between approximately 50 and 60°N (green bands in Figure 3), progressive alteration of abiotic conditions, according to emission scenarios, will affect species composition at both European latitudinal extremes (Figure 3). Due to range contractions (and possible extinctions) and poleward range shifts, we can predict an increase in the number of species at higher latitudes that contrasts with a decrease in species richness in the South. This generates areas that are predicted to have highly dissimilar composition in relation to the present as a result of increasing species richness in the north and decreasing richness in the south (orange to red bands, Figure 3).

For no-take MPAs in Europe between -40° to 70° longitude and 20° to 80° latitude, we found a strong latitudinal asymmetry, with most concentrated in northern Europe (Figure 4, top panels and bottom left panel). With rare exceptions, these MPAs are generally small (N= 849, median = 0.83 Km^2, range: 0.002- 86,971.92 Km^2; Figure 4, centre panel). In the north, the number and spatial distribution of MPAs covers most syngnathid species richness (and will probably continue to do so in the future, even under the most undesirable RCP scenario). In the south, however, the very few MPAs do not currently cover syngnathid diversity (Figure 4, top panels). Areas with high diversity (i.e., more cooccurring species) are not currently captured by any MPA and probably will continue that way in the future unless new MPAs are designated.

The skewed latitudinal distribution of MPAs, together with their generally small individual area, means that most European syngnathid species, especially those with a more southerly distribution, will really not benefit from its protection. For instance, eleven species are currently present in fewer than 1% of the 'no-take' MPAs (three of which are absent from all; Figure 4, right panel), and only four species are found in more than 10% of 'no-take' MPAs (maximum = 27.79%). The relatively small coverage of syngnathid ranges by existing 'no-take' MPAs will not significantly change in the future, irrespectively of the RCP scenario (Wilcoxon signed rank test on the % presence in MPAs in the

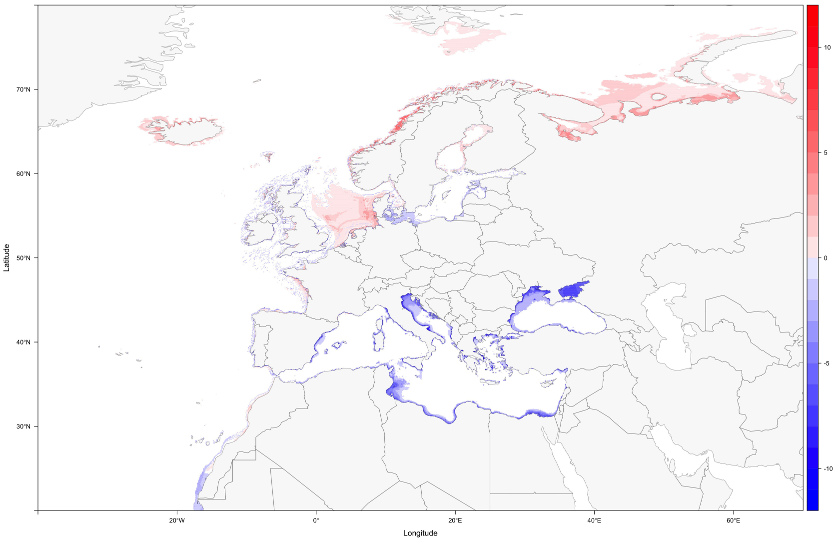

FIGURE 2
Difference in species richness from the present to the 2090-2100 time period, under RCP 8.5 (blue shows areas with loss of species, and red shows areas of increased diversity).

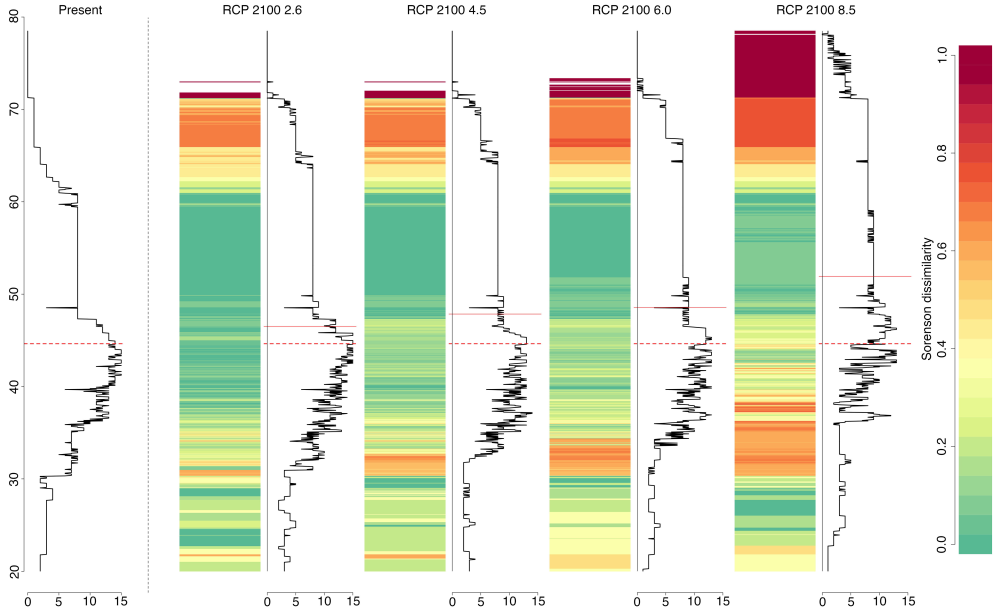

FIGURE 3
Latitudinal variation in alpha (species richness; solid black line graphs) and beta diversity (Sorensen dissimilarity index; coloured scale), for the present and the 2090-2100 time period, according to the four selected RCP scenarios. Dashed red lines indicate present latitudinal means weighted by species richness and solid red lines considers the same but for each RCP scenario.

present versus future RCP scenarios; 2050 RCP2.6: $V = 50$, $P = 0.59$; 2050 RCP4.5: $V = 61$, $P = 0.98$; 2050 RCP6.0: $V = 45$, $P = 0.41$; 2050 RCP8.5: $V = 73$, $P = 0.89$; 2100 RCP2.6: $V = 52$, $P = 0.67$; 2100 RCP4.5: $V = 68$, $P = 1$; 2100 RCP6.0: $V = 73$, $P = 0.89$; 2100 RCP8.5: $V = 66.5$, $P = 0.65$).

4 Discussion

This first dual assessment of current and forecasted distribution, under different climate change scenarios, for the full marine diversity of European syngnathids, reveals results that closely

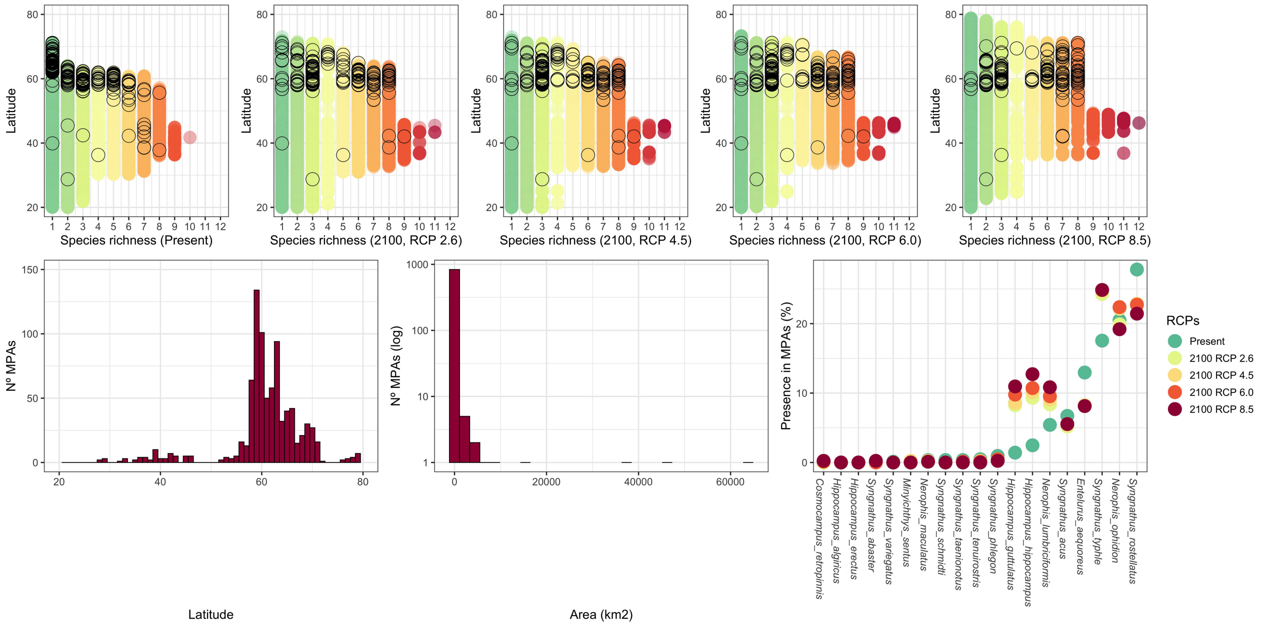

FIGURE 4
Latitudinal distribution of 'no-take' MPAs (black open circles), highlighting the number of sympatric syngnathid species, at the present and in all four considered RCP scenarios for the 2090-2100 time period (top row graphs). Latitudinal distribution of all 'no-take' MPAs (bottom left), their area distribution (histogram, log *Y* axis, bottom centre), and the percentage of 'no-take' MPAs where each of the nineteen European syngnathids is currently found or could potentially be found in the 2090-2100 tine-period, according to the four selected RCP scenarios (bottom right).

match general and region-specific impacts of climate change projections in Europe (Philippart et al., 2011). Following their climate niche, syngnathids will move north where they can, especially on the Atlantic coast, with some pipefishes, and perhaps even seahorses, extending their ranges beyond Norway, into the Barents Sea. However, in semi-enclosed areas, without real room for manoeuvre, syngnathid populations will most likely decline. Worryingly, the present-day distribution of 'no-take' MPAs throughout Europe will provide little support, especially for southern syngnathid species.

Our work has significance for regional understanding and assessment. While the Atlantic hotspot of syngnathid diversity is expected to remain around the Celtic-Biscay Shelf, its exact composition will probably slightly vary from that of present day due to the poleward movement of cold-water species and the progressive substitution by subtropical and Mediterranean species. Semi-enclosed systems such as the Baltic (especially in its southern section, together with the adjacent Kattegat), are expected to experience a considerable diversity loss. Specifically in the Baltic, in addition to the forecasted progressive drop in salinity and increased water temperatures, eutrophication (Skogen et al., 2014) could pose additional challenges to syngnathids, whose reproductive patterns are known to be impacted by turbidity (Sundin et al., 2010). But probably the most dramatic changes are expected within the Mediterranean and the Black Sea, where present-day endemic species diversity is likely to plunge. For instance, the diversity hotspot that is currently observed in the Adriatic Sea, is estimated to stop gathering suitable conditions for most of its syngnathids by 2100, under the 'business-as-usual' scenario. Together, the Black Sea and Sea of Azov are expected to experience a similar fate to that of the Mediterranean (i.e., significant loss of species).

Our work can also be significant at a continental level, highlighting the need for concerted action in Europe. The worldwide active legislation for syngnathids was recently reviewed, revealing that most European countries do not have syngnathid-specific regulations (Stanton et al., 2021). Even if currently poorly safeguarded by legislation, European syngnathids still experience some degree of protection, for instance by the action of MPAs whose number is expected to increase in the near future. Nevertheless, even though the EU Biodiversity Strategy has set the goal of significantly increasing the protection of its seas, there is concern that climate change may weaken the protective value of MPAs. As some European syngnathids are shifting their ranges (e.g., Monteiro et al., 2017), and will predictably continue to do so, adaptive management approaches will be increasingly essential (Wilson et al., 2020). Here, when focusing on 'no-take' MPAs, areas with stricter controls on fisheries and where conservation of biodiversity should be more extensive (Costello and Ballantine, 2015), the first emerging pattern retained is that there is a clear latitudinal asymmetry in Europe. Although the higher density of 'no-take' MPAs in the north (especially above 60° N) will help support connectivity, the panorama becomes quite different towards the south of Europe. From the Atlantic diversity hotspot downwards, and especially in the Mediterranean and Black Sea, 'no-take' MPAs are rare and far apart. As a result, syngnathids from southern Europe, those experiencing higher risk of population collapse, are currently much less protected by these MPAs than those from higher latitudes, and the situation will not change in the future, at least according to our distributional projections.

Our analysis on MPAs serves the specific purpose of highlighting the fact that, especially under the dynamic conditions imposed by climate change, the management for biodiversity conservation must be proactive. Especially in southern Europe, the current 'no-take' MPA layout, if it remains static, will not be especially effective in either safeguarding syngnathid diversity or delaying the expected gradual species disappearance. As MPAs are warming at roughly the same rate as unprotected areas (Bruno et al., 2018), one could naively conceive that there would be little value in repositioning MPAs or creating new protected areas. However, the establishment of effective corridors that could assist range shifts or the purposeful conservation of climatic refugia could support species persistence. Responses to paleoclimatic change show how refugia can be key landscapes worth protecting (Ashcroft, 2010). Thus, forecasting species distributions under distinct climate scenarios can be a valuable tool for conservation (of syngnathids and other taxa) by informing which MPAs will hold most diversity under climate change scenarios, where MPAs can act as climate refugia, and how MPAs can jointly serve as potential corridors that assist range shifts.

Especially if considered as a group, European syngnathids satisfy the most relevant criteria proposed for selection as umbrella species (Branton and Richardson, 2011). Firstly, they are reasonably common and well distributed around Europe (i.e., most European countries with access to the sea have one or more syngnathid species as part of their national fauna listing), thus covering wide areas that, if adequately preserved, would translate into effective conservation of cooccurring species. Secondly, syngnathids have considerable spatial range overlap with other species of conservation concern. Seagrass meadows, as an example, are hotspots of diversity that are critical for syngnathids and vulnerable early life stages of many marine species. Severely threatened in several European regions (Turschwell et al., 2021), and classified as Vulnerable by the EU, seagrass meadows and all their inhabitants would gain from effective syngnathid protection. Thirdly, we can now see a tight correspondence between the theoretical future scenarios for European coastal ecosystems and those of pipefishes and seahorses. Thus, the implementation of adequate conservation strategies for syngnathids, aimed at mitigating the effects of climate change, would certainly find echo on numerous coastal habitats (Pollom et al., 2021).

Besides the potential to act as umbrella species, syngnathids (especially seahorses) have also the ability to serve as flagship species. These fish are taxonomically distinctive, charismatic, and have successfully proven to be powerful ambassadors for marine conservation and promoters of ocean literacy. Since syngnathids are so easily recognized, and seahorses are historically embedded into European mythology (e.g., as offspring of Poseidon's steeds or protectors of dead sailors' souls transitioning into the underworld), their use as flagship species seems like a unique opportunity to better engage with the general European public, thinning potential conflicts with several socioeconomic activities and easing the implementation of conservation measures directed to coastal ecosystems.

5 Conclusion

Predicting the effects of climate change on ecosystems is now, probably more than ever, a fundamental challenge and an unavoidable requirement (Moritz and Agudo, 2013). Even considering that our selection of modelling technique (climate envelope) has limitations (see Hijmans and Graham, 2006), the resulting overall pattern of syngnathid potential distributions throughout the next century is very plausible, unsurprisingly reflecting, at both general and regional levels, the expected climate change impacts on European marine ecosystems (Philippart et al., 2011). At this point in time, a steep decline in European marine endemic species richness, especially in southern Europe, is no longer a question of "if" but rather of "how soon". Knowing that changes began many decades ago, the results of our species distribution modelling on European syngnathids suggests that the swiftness of the foreseeable diversity decline is inversely linked to our ability to curb emissions. As such, Europe needs to be prepared to implement, at a continental scale, coordinated conservation measures aimed at preventing (or delaying, at the very least) the loss of its marine endemic species. Acting as umbrella species, conservation efforts aimed at syngnathids will surely have a positive impact on coastal ecosystems. Moreover, given syngnathid potential to act as flagship species, these charismatic fish could help decisively enrol the general public into marine ecosystem preservation, leading a much-needed movement aimed at opposing climate change impacts.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

NM and AV contributed to the conception and design of the study. NM, SP, and SM contributed to data gathering. NM and PT performed the statistical analysis. NM and AV wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1138657/full#supplementary-material

References

Ahnesjö, I., and Craig, J. F. (2011). The biology of syngnathidae: pipefishes, seadragons and seahorses. *J. Fish Biol.* 78 (6), 1597–1602. doi: 10.1111/j.1095-8649.2011.03008.x

Akimova, I. A. (2009). *Red data book of Ukraine* (Kyiv, Ukraine: Animal World., Globalconsulting).

Amante, C., and Eakins, B. W. (2009). *Etopo1 1 arc-minute global relief model: Procedures, data sources and analysis* (NOAA technical memorandum).

Ashcroft, M. B. (2010). Identifying refugia from climate change. *J. Biogeogr.* 37 (8), 1407–1413. doi: 10.1111/j.1365-2699.2010.02300.x

Assis, J., Tyberghein, L., Bosch, S., Verbruggen, H., Serrão, E. A., and De Clerck, O. (2018). Bio-ORACLE v2.0: Extending marine data layers for bioclimatic modelling. *Global Ecol. Biogeogr.* 27 (3), 277–284. doi: 10.1111/geb.12693

Bateman, B. L., Murphy, H. T., Reside, A. E., Mokany, K., and VanDerWal, J. (2013). Appropriateness of full-, partial- and no-dispersal scenarios in climate change impact modelling. *Diversity Distributions* 19 (10), 1224–1234. doi: 10.1111/ddi.12107

Berglund, A., and Rosenqvist, G. (2003). Sex role reversal in pipefish. *Adv. Stud. Behav.* 32, 131–167. doi: 10.1016/S0065-3454(03)01003-9

Birrer, S. C., Reusch, T. B. H., and Roth, O. (2012). Salinity change impairs pipefish immune defence. *Fish Shellfish Immunol.* 33 (6), 1238–1248. doi: 10.1016/j.fsi.2012.08.028

Branton, M., and Richardson, J. S. (2011). Assessing the value of the umbrella-species concept for conservation planning with meta-analysis. *Conserv. Biol.* 25 (1), 9–20. doi: 10.1111/j.1523-1739.2010.01606.x

Bruno, J. F., Bates, A. E., Cacciapaglia, C., Pike, E. P., Amstrup, S. C., van Hooidonk, R., et al. (2018). Climate change threatens the world's marine protected areas. *Nat. Climate Change* 8 (6), 499–503. doi: 10.1038/s41558-018-0149-2

Burrows, M. T., Schoeman, D. S., Buckley, L. B., Moore, P., Poloczanska, E. S., Brander, K. M., et al. (2011). The pace of shifting climate in marine and terrestrial ecosystems. *Science* 334 (6056), 652–655. doi: 10.1126/science.1210288

Cashion, T., Nguyen, T., Ten Brink, T., Mook, A., Palacios-Abrantes, J., and Roberts, S. M. (2020). Shifting seas, shifting boundaries: Dynamic marine protected area designs for a changing climate. *PloS One* 15 (11), e0241771. doi: 10.1371/journal.pone.0241771

Costello, M. J., and Ballantine, B. (2015). Biodiversity conservation should focus on no-take marine reserves: 94% of marine protected areas allow fishing. *Trends Ecol. Evol.* 30 (9), 507–509. doi: 10.1016/j.tree.2015.06.011

Dawson, C. E. (1986). "Syngnathidae," in *Fishes of the north-eastern Atlantic and the Mediterranean*, vol. 2 . Eds. Whitehead,, J. C. Hureau, J. Nielson and E. Tortonese (Paris: United Nations Educational, Scientific and Cultural Organization), 628–639. M. L. B. P J P.

Doney, S. C., Ruckelshaus, M., Duffy, J. E., Barry, J. P., Chan, F., English, C. A., et al. (2012). Climate change impacts on marine ecosystems. *Ann. Rev. Mar. Sci.* 4, 11–37. doi: 10.1146/annurev-marine-041911-111611

European Commission, Directorate-General for Environment. (2021). *EU Biodiversity strategy for 2030. bringing nature back into our lives* Vol. 380 (Brussels : Publications Office of the European Union) Available at: https://data.europa.eu/doi/10.2779/677548.

Froese, R., and Pauly, D. (2021) *FishBase*. Available at: www.fishbase.org.

García-Roa, R., Garcia-Gonzalez, F., Noble, D. W. A., and Carazo, P. (2020). Temperature as a modulator of sexual selection. *Biol. Rev.* 95 (6), 1607–1629. doi: 10.1111/brv.12632

Hamilton, H., Saarman, N., Short, G., Sellas, A. B., Moore, B., Hoang, T., et al. (2017). Molecular phylogeny and patterns of diversification in syngnathid fishes. *Mol. Phylogenet Evol.* 107, 388–403. doi: 10.1016/j.ympev.2016.10.003

Hermoso, V., Carvalho, S. B., Giakoumi, S., Goldsborough, D., Katsanevakis, S., Leontiou, S., et al. (2022). The EU biodiversity strategy for 2030: Opportunities and challenges on the path towards biodiversity recovery. *Environ. Sci. Policy* 127, 263–271. doi: 10.1016/j.envsci.2021.10.028

Hickling, R., Roy, D. B., Hill, J. K., Fox, R. J., and Thomas, C. D. (2006). The distributions of a wide range of taxonomic groups are expanding polewards. *Global Change Biol.* 12 (3), 450–455. doi: 10.1111/j.1365-2486.2006.01116.x

Hijmans, R. J., and Graham, C. H. (2006). The ability of climate envelope models to predict the effect of climate change on species distributions. *Global Change Biol.* 12 (12), 2272–2281. doi: 10.1111/j.1365-2486.2006.01256.x

Hijmans, R. J., and van Etten, J. (2012). *Raster: Geographic analysis and modeling with raster data*. (R package version 2.0-12)

Hilomen-Garcia, G. V., Delos Reyes, R., and Garcia, C. M. H. (2003). Tolerance of seahorse hippocampus kuda (Bleeker) juveniles to various salinities. *J. Appl. Ichthyol.* 19 (2), 94–98. doi: 10.1046/j.1439-0426.2003.00357.x

ICN (1993). *Livro vermelho dos vertebrados de Portugal* Vol. III (Lisboa, Portugal: Peixes Marinhos e Estuarinos). Lisboa, ICN.

Iglesias, S. P., Bariche, M., Beau, F., Berenger, L., Beucher, R., Chabrolle, A., et al. (2021). French Ichthyological records for 2019. *Cybium: Rev. Internationale d'Ichtyologie* 45 (3), 169–188. doi: 10.26028/cybium/2021-453-001

IUCN (2022). *The IUCN red list of threatened species*, Version 2021-2. Available at: https://www.iucnredlist.org. Accessed on 04/08/2022.

Lawson, J. M., Foster, S. J., and Vincent, A. C. J. (2017). Low bycatch rates add up to big numbers for a genus of small fishes. *Fisheries* 42 (1), 19–33. doi: 10.1080/03632415.2017.1259944

Lenoir, J., and Svenning, J. C. (2015). Climate-related range shifts – a global multidimensional synthesis and new research directions. *Ecography* 38 (1), 15–28. doi: 10.1111/ecog.00967

Loarie, S. R., Duffy, P. B., Hamilton, H., Asner, G. P., Field, C. B., and Ackerly, D. D. (2009). The velocity of climate change. *Nature* 462 (7276), 1052–1055. doi: 10.1038/nature08649

Marsh, C. J., Sica, Y. V., Burgin, C. J., Dorman, W. A., Anderson, R. C., Del Toro Mijares, I., et al. (2022). Expert range maps of global mammal distributions harmonised to three taxonomic authorities. *J. Biogeogr.* 49 (5), 979–992. doi: 10.1111/jbi.14330

Martinez-Cardenas, L., Valdez-Hernández, E. F., González-Díaz, A. A., Soria-Barreto, M., Castañeda-Chavez, M. R., Lango-Reynoso, F., et al. (2014). Effect of salinity on growth and survival in juvenile opossum pipefish, microphis brachyurus, in culture conditions. *J. World Aquaculture Soc.* 45 (5), 577–585. doi: 10.1111/jwas.12142

Mendes, T., Gomes, C., Monteiro, N., and Antunes, A. (2020). Strong sexual selection does not induce population differentiation in a fish species with high dispersal potential: The curious case of the worm pipefish nerophis lumbriciformis (Teleostei: Syngnathidae). *J. Heredity* 111 (7), 585–592. doi: 10.1093/jhered/esaa052

Monteiro, N., Almada, V., and Vieira, M. (2005). Temporal patterns of breeding and recruitment in nerophis lumbriciformis (Pisces; syngnathidae) related to seawater temperatures. *J. Fish Biol.* 67 (5), 1475–1480. doi: 10.1111/j.0022-1112.2005.00818.x

Monteiro, N., Cunha, M., Ferreira, L., Vieira, N., Antunes, A., Lyons, D., et al. (2017). Parabolic variation in sexual selection intensity across the range of a cold-water pipefish: implications for susceptibility to climate change. *Global Change Biol.* 23 (9), 3600–3609. doi: 10.1111/gcb.13630

Monteiro, N., and Vieira, M. (2017). Rendez-vous at the Baltic? the ongoing dispersion of the black-striped pipefish, syngnathus abaster. *Oceanogr. Ocean fish Open Access J.* 3 (2), 555608. doi: 10.19080/OFOAJ.2017.03.555608

Moritz, C., and Agudo, R. (2013). The future of species under climate change: resilience or decline? *Science* 341 (6145), 504–508. doi: 10.1126/science.1237190

Moss, R. H., Edmonds, J. A., Hibbard, K. A., Manning, M. R., Rose, S. K., van Vuuren, D. P., et al. (2010). The next generation of scenarios for climate change research and assessment. *Nature* 463 (7282), 747–756. doi: 10.1038/nature08823

OBPG, Stumpf, R. P. (2022) *Distance to nearest coastline: 0.01-degree grid.* 2022 Available at: http://pacioos.org/metadata/dist2coast_1deg.html.

Otero-Ferrer, F., Herrera, R., López, A., Socorro, J., Molina, L., and Bouza, C. (2015). First records of hippocampus algiricus in the canary islands (north-east Atlantic ocean) with an observation of hybridization with hippocampus hippocampus. *J. Fish Biol.* 87 (4), 1080–1089. doi: 10.1111/jfb.12760

Pante, E., and Simon-Bouhet, B. (2013). Marmap: A package for importing, plotting and analyzing bathymetric and topographic data in r. *PloS One* 8 (9), e73051. doi: 10.1371/journal.pone.0073051

Parker, J., Dubin, A., Schneider, R., Wagner, K. S., Jentoft, S., Bõhne, A., et al. (2022). Immunological tolerance in the evolution of male pregnancy. *Mol. Ecol.* 32, 819–840. doi: 10.1111/mec.16333

Parmesan, C. (2006). Ecological and evolutionary responses to recent climate change. *Annu. Rev. Of Ecol. Evol. And Systematics* 37, 637–669. doi: 10.1146/annurev.ecolsys.37.091305.110100

Parmesan, C., Ryrholm, N., Stefanescu, C., Hill, J. K., Thomas, C. D., Descimon, H., et al. (1999). Poleward shifts in geographical ranges of butterfly species associated with regional warming. *Nature* 399 (6736), 579–583. doi: 10.1038/21181

Pecl, G. T., Araujo, M. B., Bell, J. D., Blanchard, J., Bonebrake, T. C., Chen, I. C., et al. (2017). Biodiversity redistribution under climate change: Impacts on ecosystems and human well-being. *Science* 355 (6332):eaai9214. doi: 10.1126/science.aai9214

Philippart, C. J. M., Anadón, R., Danovaro, R., Dippner, J. W., Drinkwater, K. F., Hawkins, S. J., et al. (2011). Impacts of climate change on European marine ecosystems: Observations, expectations and indicators. *J. Exp. Mar. Biol. Ecol.* 400 (1), 52–69. doi: 10.1016/j.jembe.2011.02.023

Pollom, R. A., Ralph, G. M., Pollock, C. M., and Vincent, A. C. J. (2021). Global extinction risk for seahorses, pipefishes and their near relatives (Syngnathiformes). *Oryx* 55 (4), 497–506. doi: 10.1017/S0030605320000782

Qin, G., Zhang, Y., Huang, L., and Lin, Q. (2014). Effects of water current on swimming performance, ventilation frequency, and feeding behavior of young seahorses (Hippocampus erectus). *J. Exp. Mar. Biol. Ecol.* 461, 337–343. doi: 10.1016/j.jembe.2014.09.001

R Development Core Team (2022). *R: A language and environment for statistical computing* (Vienna, Austria: R Foundation for Statistical Computing).

Roth, O., Solbakken, M. H., Torresen, O. K., Bayer, T., Matschiner, M., Baalsrud, H. T., et al. (2020). Evolution of male pregnancy associated with remodeling of canonical vertebrate immunity in seahorses and pipefishes. *Proc. Natl. Acad. Sci. U.S.A.* 117 (17), 9431–9439. doi: 10.1073/pnas.1916251117

Shokri, M. R., Gladstone, W., and Jelbart, J. (2009). The effectiveness of seahorses and pipefish (Pisces: Syngnathidae) as a flagship group to evaluate the conservation value of estuarine seagrass beds. *Aquat. Conservation: Mar. Freshw. Ecosyst.* 19 (5), 588–595. doi: 10.1002/aqc.1009

Silva, K., Vieira, M. N., Almada, V. C., and Monteiro, N. M. (2007). The effect of temperature on mate preferences and female–female interactions in syngnathus abaster. *Anim. Behav.* 74 (5), 1525–1533. doi: 10.1016/j.anbehav.2007.03.008

Skogen, M. D., Eilola, K., Hansen, J. L. S., Meier, H. E. M., Molchanov, M. S., and Ryabchenko, V. A. (2014). Eutrophication status of the north Sea, skagerrak, kattegat and the Baltic Sea in present and future climates: A model study. *J. Mar. Syst.* 132, 174–184. doi: 10.1016/j.jmarsys.2014.02.004

Stanton, L. M., Foster, S., and Vincent, A. C. J. (2021). Identifying national conservation status, legislation and priorities for syngnathid fishes globally. *Fish. Cent. Res. Rep.* 29(2):43. doi: 10.14288/1.0401950

Sundin, J., Berglund, A., and Rosenqvist, G. (2010). Turbidity hampers mate choice in a pipefish. *Ethology* 116 (8), 713–721. doi: 10.14288/1.0401950

Tiralongo, F., and Baldacconi, R. (2014). A conspicuous population of the long-snouted seahorse, hippocampus guttulatus (Actinopterygii: Syngnathiformes: Syngnathidae), in a highly polluted Mediterranean coastal lagoon. *Acta Ichthyologica Piscatoria* 44 (2):99–104. doi: 10.3750/AIP2014.44.2.02

Turschwell, M. P., Connolly, R. M., Dunic, J. C., Sievers, M., Buelow, C. A., Pearson, R. M., et al. (2021). Anthropogenic pressures and life history predict trajectories of seagrass meadow extent at a global scale. *Proc. Natl. Acad. Sci.* 118 (45), e2110802118. doi: 10.1073/pnas.2110802118

Tyberghein, L., Verbruggen, H., Pauly, K., Troupin, C., Mineur, F., and De Clerck, O. (2012). Bio-ORACLE: a global environmental dataset for marine species distribution modelling. *Global Ecol. Biogeogr.* 21 (2), 272–281. doi: 10.1111/j.1466-8238.2011.00656.x

UNEP-WCMC-IUCN (2022). *Protected planet: The world database on protected areas (WDPA) and world database on other effective area-based conservation measures (WD-OECM)* (Cambridge, UK: UNEP-WCMC and IUCN).

Vincent, A. C. J., Foster, S. J., and Koldewey, H. J. (2011). Conservation and management of seahorses and other syngnathidae. *J. Fish Biol.* 78 (6), 1681–1724. doi: 10.1111/j.1095-8649.2011.03003.x

Warren, R., VanDerWal, J., Price, J., Welbergen, J. A., Atkinson, I., Ramirez-Villegas, J., et al. (2013). Quantifying the benefit of early climate change mitigation in avoiding biodiversity loss. *Nat. Climate Change* 3 (7), 678–682. doi: 10.1038/nclimate1887

Wilson, K.L., Tittensor, D.P., , Worm, B., Lotze, H.K., et al. (2020). Incorporating climate change adaptation into marine protected area planning. *Glob. Change Biol.* 26, 3251–3267. https://doi.org/10.1111/gcb.15094

Woodall, L. C., Koldewey, H. J., Santos, S. V., and Shaw, P. W. (2009). First occurrence of the lined seahorse hippocampus erectus in the eastern Atlantic ocean. *J. Fish Biol.* 75 (6), 1505–1512. doi: 10.1111/j.1095-8649.2009.02371.x

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Hippocampus guttulatus diet based on DNA metabarcoding

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Seahorses are small sedentary fish considered flagship species of the conservation efforts. They are particularly vulnerable to human pressures because inhabiting threatened coastal ecosystems. Indeed, the worldwide decline of local populations in the last decades led to the inclusion of all seahorse species on the IUCN Red List, where most species, including *Hippocampus guttulatus*, were classified as "Data Deficient" on a global level due to the lack of relative data on several biological and ecological traits. Because of such sensitive conservation status, improvement of the current knowledge on the diet composition of wild animals and its differences among habitats could be of great importance as it could help understanding the way the environment is exploited. In the present study, we used a non-invasive DNA metabarcoding technique to further elucidate long-snouted seahorse diet and expand our understanding of prey choice among different habitats. We identified 24 families, 22 genera and 26 species, and according to the results, most of the seahorse samples contained taxa such as Amphipoda, Decapoda, Isopoda, and Mysida. Several non-native species were discovered in the diet, suggesting their dietary incorporation that could mirror high anthropogenic impacts and habitat modifications. We found significant differences in the diet composition among investigated habitats, thus indicating trophic flexibility of *H. guttulatus* among diverse habitats, a characteristic that may be essential for the resilience of this iconic yet sensitive species.

KEYWORDS
seahorses, *Hippocampus guttulatus*, diet, syngnathidae, Mar Piccolo of Taranto, DNA metabarcoding

1 Introduction

Effective biodiversity conservation depends on a good understanding of the relationships between species or populations and their environment (Bremner, 2008; Díaz-Abad et al., 2022). Identifying dietary items is necessary to acknowledge prey preference and availability in a given habitat but also to account for trophic interactions when designing species protection (Hamann et al., 2010; Díaz-Abad et al., 2022). Indeed, food is considered the most fundamental resource for the species' survival (Wang et al., 2022) as it directly influences critical traits such as body condition (Rothman et al., 2012) and reproduction (Cameron, 1996). Distribution and abundance of prey items, however, can strongly depend on environmental conditions, so predator species may be forced to modify their diets in case of eventual disturbances and/or other external pressures (Gainsbury and Meiri, 2017; Gül and Griffen, 2020). In species conservation management, understanding dietary preferences is crucial for both identifying resource requirements and predator population dynamics (Ward et al., 2012; Shao et al., 2021).

Worldwide, pressures on marine ecosystems have caused extensive modifications and/or degradation of coastal habitats (Hughes et al., 2017; Thibaut et al., 2017). Seahorses (*Hippocampus* spp.) mostly occur in threatened coastal ecosystems (Hernandez-Urcera et al., 2021; Pierri et al., 2022) and are, therefore, particularly vulnerable (IUCN, 2022). They are sedentary species whose low mobility make them sensitive to fluctuations of feeding resources at local scale as they are not able to swim over long distances in search of food (Foster and Vincent, 2004). Many studies indicated that seahorse populations declined in the past decades, and although exact causes remain unknown, habitat modifications/degradation and illegal fisheries seem main responsible (Correia et al., 2015; Pierri et al., 2021). Therefore, all seahorse species were included on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (IUCN, 2022) where many species, including the long-snouted seahorse *Hippocampus guttulatus*, were classified as "Data Deficient" on a global level (Pollom, 2017) due to the lack of information on distribution (Pierri et al., 2022), population trends (IUCN, 2022) and biodiversity-related data (Heard et al., 2019).

Seahorse distribution seems directly influenced by prey availability (Curtis and Vincent, 2006; Gristina et al., 2015; Ape et al., 2019). These fish are ambush predators that usually rely on their vision to capture prey (Kuiter, 2000). In uncovered areas, they practice a "sit-and-wait" predation strategy (Tipton and Bell, 1988; James and Heck, 1994) and rarely swim in search of food (Kendrick and Hyndes, 2005; Felício et al., 2006). Diet and foraging strategies are well documented in many scientific studies showing that seahorses principally consume epibenthic invertebrates (e.g., harpacticoid copepods, amphipods, nematodes, polychaetes), thus reflecting not only their sedentary behavior but also habitat-specific biodiversity (Kendrick and Hyndes, 2005; Castro et al., 2008; Kitsos et al., 2008; Yip et al., 2015; Ape et al., 2019). Up to date, most studies on seahorse diet employed morphological examination of gut or stomach contents, through stomach flushing or by biochemical means (e.g., Kitsos et al., 2008; Gurkan et al., 2011; Valladares et al., 2017; Ape et al., 2019; Valladares and Planas, 2021). The next-generation sequencing (NGS) technologies, combined with the expansion of DNA barcode databases (Hebert et al., 2003), has recently allowed to perform diet analysis in many fish species (e.g., Taguchi et al., 2014), including seahorses (Lazic et al., 2021; Kim et al., 2022). The analysis of the DNA barcode sequences of consumed items from fecal material indicated that DNA metabarcoding is an effective tool for studying seahorse diets (Lazic et al., 2021). Indeed, these powerful and non-invasive methods can provide accurate dietary profiles for many individuals and result in extended knowledge of foraging ecology.

In this study, we analyzed fecal samples of *H. guttulatus* from four preferential habitats in Mar Piccolo of Taranto (for this area; see Gristina et al., 2015; Gristina et al., 2017) where seahorses have recently faced a severe population decline of approximately 90% (Gristina et al., 2015; Pierri et al., 2021). We aimed at widening the knowledge of the diet of wild long-snouted seahorses by using broad-coverage metazoan DNA metabarcoding primers and investigating whether diet composition changes among different habitats. The applied protocol has been recently developed in laboratory conditions (Lazic et al., 2021) and here has been applied to wild populations. In this context, the study of the dietary composition across different habitats while using non-invasive technique could offer an important tool to help plan conservation actions that aim at reversing negative population trends.

2 Materials and methods

2.1 Study area

Mar Piccolo of Taranto is located at the northern end of the Gulf of Taranto, with a total surface area of approximately 20.63 km^2 (Figure 1). Surrounded by urban development, heavy industries and civil and military shipyards, the water body of Mar Piccolo is affected by heavy metals, hydrocarbons, pesticides, and organic waste (Cardellicchio et al., 2007; Petronio et al., 2012). Across the entire basin, natural rocky substrates are reduced, while the seafloor is dominated by soft sediments that vary from mud to mixed sands. They are sparsely covered by patches of *Cymodocea nodosa*, scattered tufts of brown algae (*Cystoseira* spp.) and large algal beds of *Cladophora prolifera* (Cecere and Petrocelli, 2009).

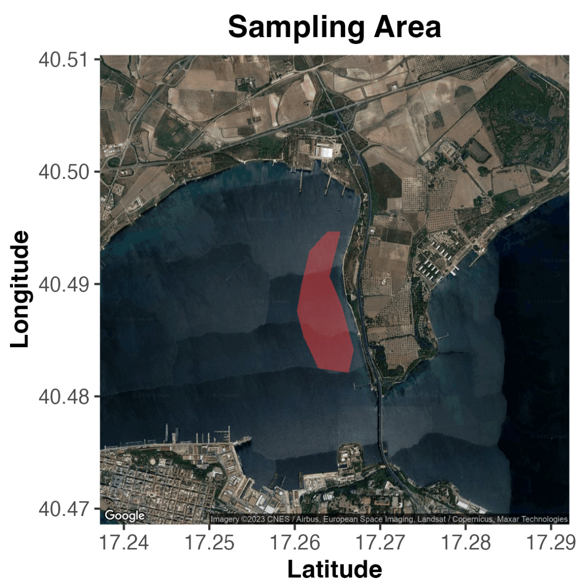

FIGURE 1
Map of the sampling area in the Mar Piccolo of Taranto.

Hard substrates, mainly of anthropogenic origin (wood poles of mussel farms, stone walls along the coastline, rocks, ropes, and discarded material), support an algal turf principally constituted of perennial *Cystoseira* spp. and other frondose algae (i.e., *Corallina elongata*, *Dictyopteris* spp.) (for further description see Gristina et al., 2015; Gristina et al., 2017).

2.2 Sample collection

Forty non-reproductive adult long-snouted seahorses were captured by diving at Mar Piccolo of Taranto. Although at least six different habitats were described at this site (Gristina et al., 2017), this study focused on the four most extensive habitats: (1) *Cladophora prolifera* (onwards *C. prolifera*) at 6 m of depth (n=10), (2) vertical stone wall (onwards stone wall) with abundant algae (*C. elongata*, *Cystoseira* sp. and *Dictyota dichotoma*) and filter feeders (sabellids, ascidians, demosponges) at 0.3 – 0.6 m of depth (n=10), (3) unvegetated sandy bottom (onwards sandy bottom) mixed with organogenous concretions (bivalve and gastropod shells) and small stones at 1.6 – 2.2 m of depth (n=10), and (4) poles of mussel farms (onwards poles) colonized by rich sessile filter-feeder fauna (bivalves, demosponges, sabellids, bryozoans and ascidians) at 3.3 – 4.2 m of depth (n=10) (for further description of habitats see Gristina et al., 2015; Gristina et al., 2017). Captured seahorses were individually placed in small aerated 2L tanks filled with surrounding seawater. Each tank was then filtered through 0.2 µm pore-size polycarbonate filters. Produced fecal samples (total n=33; *C. prolifera*=9, stone wall=9, sandy bottom=9, poles=6) were collected by syphoning, preserved in 96% ethanol, and stored at −20°C for further examination. All animals were immediately released to the original capture site in perfect health conditions. To exclude host contamination, *H. guttulatus* Cox1 amplicon sequence, produced during the previous research (Lazic et al., 2021) and available on GenBank (OQ291591), was used in subsequent analysis.

2.3 DNA extraction

For each sample, 50 mg of feces were selected and then used for the total genomic DNA extraction by using FastDNA SPIN kit for soil (BIO 101, Carlsbad, Canada) following the manufacturer's instructions. Cell lysis was achieved by bead beating in FastPrep Instrument (BIO 101) at speed 6 for 40 s. Qualitative and quantitative DNA assessment was carried out using PicoGreen®dsDNA quantitation assay (Invitrogen, Carlsbad, California) and agarose gel (1%) electrophoresis. DNA extraction blanks (sterile distilled water) were prepared and processed together with the samples to exclude contamination related to the extraction reagents and procedure. DNA extracts were stored at −20°C before amplification by PCR.

2.4 Cox1 library preparation and sequencing

Amplicon libraries were prepared from 0.5 ng of extracted DNA. The adopted strategy is described in detail by Manzari et al. (2015). The primer pair used for amplification of the mitochondrial Cytochrome oxidase subunit 1 (Cox1) gene was mlCOIintF_NextFor and dgHCO2198_NextRev (Next_For: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′, and Next_Rev: 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′) (Leray et al., 2013), designed to contain (from 5′ to 3′ ends) transposon Nextera sequences (Nextera DNA sample preparation guide, Illumina). RNase/Dnase-free Molecular Biology Grade water (Ambion) was used as a negative control of PCR amplification. Equimolar quantities of the purified amplicons were pooled and subjected to 2×250 bp paired-end sequencing on the Illumina MiSeq platform. To increase the genetic diversity of the sequenced samples, as required by the MiSeq platform, a phage PhiX genomic DNA library was added to the mix and co-sequenced (Kozich et al., 2013).

2.5 Taxonomic characterization of seahorses' preys

The quality of raw Cox1 sequence data was checked using FastQC (available at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and multiQC (Ewels et al., 2016). Illumina adapters and PCR primers were removed from raw reads using cutadapt (Martin, 2011). Retained paired-end (PE) reads were denoised into ASVs (Amplicon Sequence Variants) (Callahan et al., 2017) by applying DADA2 (version 1.18) (Callahan et al., 2016). ASVs were taxonomically annotated using a modified version of BioMaS (Bioinformatic analysis of Metagenomic amplicons) (Fosso et al., 2015) working on MetaCOXI (Balech et al., 2022) and MIDORI (Leray et al., 2018) as reference databases. Dynamic sequence similarity threshold was adopted to improve the ASVs classification accuracy (species at 97%, genus at 95%, family at 93%, class at 91%, order at 88%, and phylum at 78%) as in Lotus pipeline (Hildebrand et al., 2014). Unassigned ASVs were additionally aligned against *nt* and *nr* collections using the blastn (Mount, 2007) and diamond blastx (Buchfink et al., 2015) tools, respectively. All ASV sequences partially mapped on different reference sequences were labelled as chimeric and removed from subsequent analysis. Retained sequences were taxonomically annotated using TANGO (Alonso-Alemany et al., 2014; Fosso et al., 2018) at the same similarity percentage thresholds described above. Contaminant ASVs were identified using *decontam* (Davis et al., 2018); alignment against the human genome (Pruesse et al., 2007) allowed the removal of additional noise sources.

R packages *phyloseq* (1.26.1) (McMurdie and Holmes, 2013) and *vegan* (2.5.6) (Oksanen, 2022) were used to measure alpha and beta diversity. For this purpose, ASVs counts were normalized by rarefaction (depth values settled to 45,000). Shannon and inversed Simpson indexes were used as measures of alpha diversity (i.e., intra-sample diversity), while Bray–Curtis dissimilarity matrix was used to measure beta diversity (i.e., inter-sample diversity). The Nonmetric Multidimensional Scaling (NMDS) was applied to beta-diversity matrix to obtain graphical representations of the observed dissimilarities. Statistical differences in alpha diversity indexes were measured by Wilcoxon (W) test. PERMANOVA (Permutational Multivariate analysis of variance) was performed to infer the contribution of explanatory variables in beta diversity data partitioning by applying 999 permutations. The contribution of individual species to the overall Bray–Curtis dissimilarity was achieved by SIMPER analysis with 999 permutations. The functions *metaNDS*, *adonis* and *simper* of the Vegan package (Oksanen, 2022) were used to perform NMDS, PERMANOVA and SIMPER analysis, respectively.

3 Results

3.1 Overall sequencing results

Libraries of dual indexed amplicons of 420 bp were successfully sequenced on the MiSeq platform using 2 × 250 bp paired end (PE) sequencing strategy. Overall, approximately 8 million PE reads (mean 242,645 ± 54,990 SD) were generated across all samples. After trimming, merging and denoising procedures, approximately 87% of the produced PE reads were retained, resulting in a total of 683 ASVs. According to the ecological metrics, data were normalized by rarefaction to 45,000 sequences. Nine samples were discarded as they contained less sequences than the imposed rarefaction depth and were dominated by *H. guttulatus* Cox1 gene sequences.

3.2 Seahorse diet

Shannon index values (Figure 2) indicated statistically significant differences among stone wall and *C. prolifera* and stone wall and sandy bottom samples (p-value ≤ 0.05). Stone wall samples were characterized by the highest alpha-diversity values compared to other sampling sites (stone wall: 2.13 ± 0.93, *C. prolifera*: 0.80 ± 0.41, sandy bottom: 0.88 ± 0.63, poles: 0.76 ± 0.78). Simpson Index (Supplementary Figure 1) indicated a similar trend and revealed statistically relevant differences (p-value ≤ 0.05) between stone wall and *C. prolifera* samples (stone wall: 0.74 ± 0.17, *C. prolifera*: 0.42 ± 0.21, sandy bottom: 0.37 ± 0.24, poles: 0.34 ± 0.34).

Beta diversity values (Figure 3), measured using Bray-Curtis dissimilarity metrics and plotted using nMDS (non-metric Multi-Dimensional Scaling), indicated no clear separation of samples among different habitats. However, PERMANOVA (Table 1) highlighted that approximately 21% of data variability is associated with the sampling site.

In accordance with alpha diversity indices, SIMPER analysis (Table 2) identified stone wall ASVs contributing the most to the differences between groups. Nonetheless, the highest amount of explained between-group diversity was observed in the comparison in which sandy bottom samples were involved. These results could be probably explained by a large intra-group variability as observed in alpha (Figure 2; Supplementary Figure 1) and beta (Figure 3) diversity plots.

In terms of taxa, we identified 24 families, 22 genera and 26 species. At level of classes, Malacostraca, Gastropoda, and Polychaeta were the most observed (Table 3), and were represented by nine orders (Amphipoda, Isopoda, Decapoda, Mysida, Trochida, Phyllodocida, Sabellida, Terebellida, and Eunicida). Although with low abundances, several other classes were also detected, including Insecta, Gymnolaemata, Demospongiae and Ascidacea. Malacostracan crustaceans were the predominant food item for all habitats and had relatively high abundances (range 1 – 99,98%). There were differences in specific taxa identified among different habitats (Table 3), but also some overlap. For instance, genus *Munna* was present in samples from stone wall, *C. prolifera* and wood poles, but was absent on sandy bottom. Furthermore, the most consumed diet item differed among habitats; in samples of wood poles, for instance, the most abundant genera were *Paramysis* (Arthropoda, 16.05% ± 39.31% in one out of six samples) and *Athanas* (Arthropoda, 11.41% ± 27.96, %, in one sample), in samples from *C. prolifera*, the most observed was *Munna* (Arthropoda, 11.56% ± 33.09%, in five samples out of nine), while on sandy bottom, the most abundant were *Caprella* (Arthropoda, 10.88% ± 32.61%, in three samples out of nine) and *Paramysis* (Arthropoda, 10.30% ± 30.85%, in four samples). The same principle was also observed at the species level, where the most consumed prey differed among habitats. Indeed, on wood poles, the samples presented high content of *Amphipoda* sp. (Arthropoda, 22.27% ± 27.96%, in three out of six samples) and *Paramysis (Mesomysis) intermedia* (16.05% ± 39.31%, in one sample); in *C. prolifera*, the most abundant was *Munna japonica* (11.56% ± 33.09%, in five out of nine samples); on stone wall, *Steromphala adansonii* (Mollusca, 4.73% ± 12.75%, in five samples) and; on sandy bottom, the most consumed was *P. (M.) intermedia* (10.30% ± 30.85%, in four samples). The abundance of unassigned sequences at the species level ranged between 0.11% and 100%.

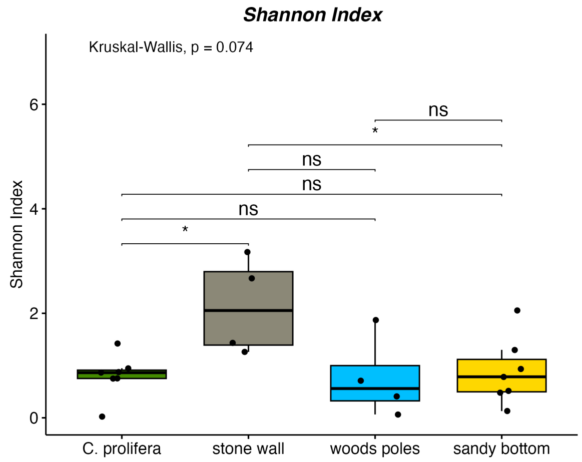

FIGURE 2
Shannon index for investigated habitats. * means "p-value ≤ 0.05" and "ns" means not significant.

4 Discussion

This study used DNA metabarcoding of feces to investigate the diet of long-snouted seahorses in several most extensive habitats at Mar Piccolo of Taranto and indicated that this technique is suitable

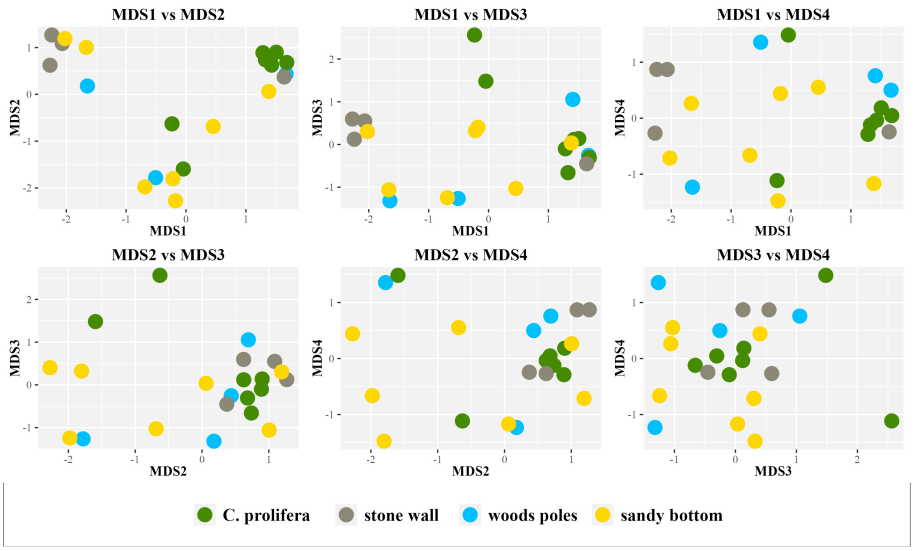

FIGURE 3
nMDS plot based on the Bray-Curtis dissimilarity matrix. Stress=0.09. nMDS was inferred using four dimensions and up to 100 iterations. All possible combinations of inferred MDS are shown.

TABLE 1 PERMANOVA results.

	Explained variability
Habitat	20.69% *
Residuals	79.31%

Residuals represent the amount of variability that a model cannot explain.
* means p-value ≤ 0.05.

for dietary assessment of wild seahorses, following previous laboratory findings (Lazic et al., 2021). Compared to other diet studies (Kitsos et al., 2008; Gurkan et al., 2011; Ape et al., 2019) using morphological analysis of undigested food remains, our study reached a more extensive taxonomic characterization, especially for small-sized and soft-bodied prey. Among all habitats, seahorses mainly preyed on crustaceans, followed by polychaetes and gastropods. Our results expand the existing knowledge on seahorse diet and elucidate differences in dietary composition among different habitats, detecting prey types previously missed by visual methods, all of which were plausible due to records from the same study site (Ape et al., 2019; Pierri pers. comment). Indeed, the analysis revealed several species that have, to our knowledge, not been identified before as seahorse preys, namely *Caprella scaura* (Amphipoda, Arthropoda), *Paramysis (Mesomysis) intermedia* (Mysida, Arthropoda) and *Steromphala adansonii* (Gastropoda, Mollusca).

Most of the samples contained crustacean taxa such as Amphipoda, Isopoda, Decapoda and Mysida; in three out of four investigated habitats, seahorses frequently fed on *Munna japonica* (Isopoda, Arthropoda). Such finding is interesting as this species seems non-native to the Mediterranean Sea (Lazic et al., 2021). Several other non-native species were also detected in the diet, including *C. scaura* and *Alpheus bellulus* (Decapoda, Arthropoda). *H. guttulatus* had ingested other invertebrates as well, including gastropods, among which species *S. adansonii* resulted frequently selected on sandy bottom and stone wall. Several insect, sponge and ascidians were also found in the stomach contents, but hardly any of the identified ones served as food. Such taxa could have been the result of secondary predation or were eaten passively with effective prey items (Kim et al., 2022), in accordance with previous studies highlighting reliability of DNA-based methods for detecting indirect predation (e.g., Sheppard et al., 2005). Seahorses usually

TABLE 2 Results of SIMPER analysis: the number of ASVs significantly (p-values ≤ 0.05) contributing to between group diversity, contribution (portion of the diversity) range and identified species.

Comparison	N° of ASV	Contribution range (total Contribution)	Identified species
wood poles *vs.* *C. prolifera*	3	0.01 – 0.3% (0.32%)	*Amphipoda* sp. DNAS-1B6-193928
wood poles *vs.* stone wall	22	0.002 – 12.3% (19.1%)	*Alpheus bellulus*
wood poles *vs.* sandy bottom	5	0.01 – 16.1% (28.2%)	*Amphipoda* sp. DNAS-1B6-193928 *Paramysis (Mesomysis) intermedia*
C. prolifera *vs.* stone wall	34	0.02 – 12.3% (21.4%)	*Alpheus bellulus*
C. prolifera *vs.* sandy bottom	9	0.0004 – 21.8% (32.2%)	*Pisidia longicornis*
stone wall *vs.* sandy bottom	36	0.002 – 12.6% (25.9%)	*Alpheus bellulus*

TABLE 3 The most abundant (relative abundance ≥ 1%) classes, genera, and species for each sampling site.

Sampling site	Rank	Taxa	Average relative abundances	Frequency of occurrence
Poles	Class	Malacostraca	91% ± 10.01%	6/6
		Insecta	1.07% ± 2.62%	1/6
	Genus	*Paramysis*	16.05% ± 39.31%	1/6
		Athanas	11.41% ± 27.96	1/6
		Liposcalis	1.07% ± 2.62%	1/6
		Munna	1.78% ± 2.75%	4/6
	Species	*Amphipoda* sp. *DNAS-1B6-193928*	22.27% ± 34.96%	3/6
		Athanas nitescens	11.41% ± 27.96%	1/6
		Paramysis (Mesomysis) intermedia	16.05% ± 39.31%	1/6
		Liposcelis entomophila	1.07% ± 2.62%	1/6
C. prolifera	Class	Malacostraca	78.92% ±	9/9
		Ascidiacea	1.69% ± 5.08%	1/9
		Demospongia	1.0% ± 3.0%	1/9
	Genus	*Munna*	11.56% ± 33.09%	5/9
		Botryllus	1.69% ± 5.08%	1/9
		Haliclona	1.0% ± 3.00%	1/9
	Species	*Botryllus schlosseri*	1.69% ± 5.08%	1/9
		Munna japonica	11.56% ± 33.09%	5/9
		Haliclona xena	1.0% ± 3.00%	1/9
Stone wall	Class	Malacostraca	18.83% ± 30.96%	9/9
		Gastropoda	4.73% ± 12.75%	5/9
		Polychaeta	3.17% ± 8.92%	3/9
		Insecta	1.06% ± 2.21%	3/9
		Gymmoleamata	1.71% ± 5.13%	1/9
	Genus	*Steromphala*	4.73% ± 12.75%	5/9
		Munna	1.98% ± 5.92%	3/9
		Pisidia	2.35% ± 7.04%	2/9
		Watersipora	1.71% ± 5.13%	1/9
	Species	*Steromphala adansonii*	4.73% ± 12.75%	5/9
		Gammaridae sp. *HKL 32*	2.08% ± 5.75%	4/9
		Munna japonica	1.98% ± 5.92%	3/9
		Pisidia longicornis	2.35% ± 7.04%	2/9
		Watersipora subovoidea	1.71% ± 5.13%	1/9
Sandy bottom	Class	Malacostraca	45.92% ± 38.91%	9/9
		Polychaeta	10.33% ± 29.26%	5/9
		Insecta	2.61% ± 7.63%	3/9
	Genus	*Paramysis*	10.30% ± 30.85%	4/9
		Caprella	10.88% ± 32.61%	3/9
	Species	*Paramysis (Mesomysis) intermedia*	10.30% ± 30.85%	4/9

(Continued)

TABLE 3 Continued

Sampling site	Rank	Taxa	Average relative abundances	Frequency of occurrence
		Hemiptera sp. *BIOUG03768-E03*	2.55% ± 7.63%	1/9
		Caprella scaura	1.88% ± 36.61%	3/9

ingest their prey *via* a strong suction action down their tubular snout (Bergert and Wainwright, 1997; Woods, 2002). Indeed, the presence of this unlikely prey items was not a common occurrence, and their ingestion could also have occurred accidentally during ingestion of effective prey since small crustaceans frequently live and hide in sessile benthic organisms such as sponges. It is not possible to reliably distinguish between primary, secondary, or accidentally ingested prey items using DNA or visual analyses of gut contents, however, inferences can be made based on the overall composition of the gut contents, which for seahorses are usually dominated by a mix of crustacean species (Woods, 2002; Castro et al., 2008; Storero and González, 2008; Gurkan et al., 2011; Valladares et al., 2017; Manning et al., 2019). The likely secondary and/or accidental prey items may be useful in providing some insights of the food webs and habitats that the primary prey species rely on. Among these unlikely prey items, we also identified non-native species, such as *Haliclystus tenuis* (Staurozoa, Cnidaria) (Holst and Laakmann, 2019), thus pointing out that the gained data could also help at evaluating ecosystem structure and health.

The general pattern that *H. guttulatus* eats different prey items among different habitats has also been found in another study at the same site which indicated stone wall habitats as the most diversified in terms of species biodiversity probably due to complexity of algal turfs (Ape et al., 2019). Seahorses are showing trophic flexibility and can modify their foraging strategy depending on the habitat complexity (James and Heck, 1994; Felício et al., 2006; Ape et al., 2019) by catching the prey that is more accessible in a specific habitat (Ape et al., 2019) and that can fit into their mouth (Wilson and Vincent, 1998). The studies of Ryer (1988) and Franzoi et al. (1993) on species of the genus *Syngnathus* indicated that the availability of prey species influences the consumption rates and contribution to the diet (Oliveira et al., 2007). In this regard, frequent consumption of several prey items may indicate their higher availability at Mar Piccolo of Taranto rather than seahorse dietary preference. In addition to crustaceans, gastropod *S. adansonii* seems to be important in the diet of seahorses. Although adults of this species are relatively large, and it is possible that seahorses preyed on its larvae, there are indications that seahorses can extract large-sized prey from its shell and suck it while integer (Ape et al., 2019). Indeed, many fish species select larger prey items as more energy can be obtained by this choice (Gerking, 1994).

Notably, the present results were more accurate and consistent in terms of identified species than observed in previous studies on the diet of the long-snouted seahorses (Kitsos et al., 2008; Gurkan et al., 2011; Ape et al., 2019; Valladares and Planas, 2021). Indeed, many prey taxa discovered in this study were identified at the species level, while previous work (Ape et al., 2019) at Taranto Mar Piccolo only provided identification at the order or family level. Although this method could be useful in detecting species that would be otherwise difficult to observe, identification of prey DNA in fecal samples, however, depend on many factors, including differential digestion of soft-bodied and hard-bodied prey, variable gut transition times for different prey components and prey types (Nielsen et al., 2018), and choice of target sequences (Weber and Lundgren, 2009). Furthermore, this approach is strongly limited by the reference databases, whose level of completeness represents one of the most critical issues in DNA-based species diversity analyses. We used several databases available, but the capacity to identify diet items could still be improved by creating representative, possibly local, databases at least at the family level to limit misidentifications or unassigned sequences (Santos and Branco, 2011). Furthermore, the diet results presented in this study provide a single snapshot of what the animals had eaten in recent feeding events and although seahorses have limited swimming abilities (Curtis and Vincent, 2006; Caldwell and Vincent, 2013; Pierri et al., 2020), it could not be excluded that some displacement among habitats however occurred. Since both biomass and species composition of local communities can vary over time, mainly because of natural or human-induced impacts, samples should be collected at different periods of time, encompassing any existing variations. This is particularly important when having in mind that several dietary detections were of non-native species that could *de facto* act as dietary replacements. Mar Piccolo of Taranto is one of the most heavily polluted and human-impacted water bodies in southern Italy (Cardellicchio et al., 2007; Petronio et al., 2012) and the dietary incorporation of non-native species could mirror high anthropogenic impacts and habitat modifications.

Using a non-invasive and accurate molecular technique, our findings further elucidate long-snouted seahorse diet and expand our understanding of prey choice. However, future studies should evaluate how diets change by season as both availability and need may differ. Management goals should probably target different habitats during different periods of year and hopefully emblematic seahorses may be able to respond positively to these management actions.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA918558.

Ethics statement

Ethical review and approval was not required for the animal study because according to the art. 2 of D.P.R. 1639/1968 the Department of Biosciences, Biotechnologies and Environment is legally accredited for permission of fishing for scientific purposes. Moreover according to art. 2 of D. Lg. 26 04/03/2016, which recipes the European regulation 2010/63/EU about the protection of animals involved in scientific researches, for "practices not likely to cause pain, suffering, distress or lasting damage equivalent to or greater than that caused by the insertion of a needle according to good veterinary practice" it is not required the authorization of an Ethical review and approval. Indeed, according to art. 2 of D. Lg. 26 04/03/2016 in this study during all activities involving seahorses, no damaging, sacrificing, or experimenting was performed on animals.

Author contributions

LT, BF, BB, MM, SM, PG, and CP conceived the ideas and designed the methodology. LT, CG, GM, and PC collected the data. BF analyzed the data. LT, BF, and CP led the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1138279/full#supplementary-material

References

Alonso-Alemany, D., Barré, A., Beretta, S., Bonizzoni, P., Nikolski, M., and Valiente, G. (2014). Further steps in TANGO: Improved taxonomic assignment in metagenomics. *Bioinformatics* 30, 17–23. doi: 10.1093/bioinformatics/btt256

Ape, F., Corriero, G., Mirto, S., Pierri, C., Lazic, T., and Gristina, M. (2019). Trophic flexibility and prey selection of the wild long-snouted seahorse hippocampus guttulatus cuvier 1829 in three coastal habitats. *Estuar. Coast. Shelf Sci.* 224, 1–10. doi: 10.1016/j.ecss.2019.04.034

Balech, B., Sandionigi, A., Marzano, M., Pesole, G., and Santamaria, M. (2022). MetaCOXI: an integrated collection of metazoan mitochondrial cytochrome oxidase subunit-I DNA sequences. *Database* 2022, baab084. doi: 10.1093/database/baab084

Bergert, B., and Wainwright, P. C. (1997). Morphology and kinematics of prey capture in the syngnathid fishes hippocampus erectus and syngnathus floridae. *Mar. Biol.* 127, 563–565. doi: 10.1007/s002270050046

Bremner, J. (2008). Species' traits and ecological functioning in marine conservation and management. *J. Exp. Mar. Biol. Ecol.* 366, 37–47. doi: 10.1016/j.jembe.2008.07.007

Buchfink, B., Xie, C., and Huson, D. H. (2015). Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* 12, 59–60. doi: 10.1038/nmeth.3176

Caldwell, I. R., and Vincent, A. C. J. (2013). A sedentary fish on the move: Effects of displacement on long-snouted seahorse (Hippocampus guttulatus cuvier) movement and habitat use. *Environ. Biol. Fish.* 96, 67–75. doi: 10.1007/s10641-012-0023-4

Callahan, B. J., McMurdie, P. J., and Holmes, S. P. (2017). Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME J.* 11, 2639–2643. doi: 10.1038/ismej.2017.119

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: High-resolution sample inference from illumina amplicon data. *Nat. Methods* 13, 581. doi: 10.1038/nmeth.3869

Cameron, J. L. (1996). Regulation of reproductive hormone secretion in primates by short-term changes in nutrition. *Rev. Reprod.* 1, 117–126. doi: 10.1530/ror.0.0010117

Cardellicchio, N., Buccolieri, A., Giandomenico, S., Lopez, L., Pizzulli, L., and Spada, L. (2007). Organic pollutants (PAHs, PCBs) in sediments from the mar piccolo in taranto (Ionian Sea, southern Italy). *Mar. pollut. Bull.* 55, 451–458. doi: 10.1016/j.marpolbul.2007.09.007

Castro, A. L. C., De Farias Diniz, A., Martins, I. Z., Vendel, A. L., De Oliveira, T. P. R., and De Lucena Rosa, I. M. (2008). Assessing diet composition of seahorses in the wild using a non-destructive method: Hippocampus reidi (Teleostei: syngnathidae) as a study-case. *Neotrop. Ichthyol.* 6, 637–644. doi: 10.1590/S1679-62252008000400012

Cecere, E., and Petrocelli, A. (2009). "The mar piccolo of taranto," in *Flora and vegetation of the Italian transitional water systems.* Eds. E. Cecere, A. Petrocelli, A. Sfriso and G. Izzo (algal beds of Cladophora prolifera:CoRiLa. Multigraf, Spinea, Venezia), 195–227.

Correia, M., Caldwell, I. R., Koldewey, H. J., Andrade, J. P., and Palma, J. (2015). Seahorse (Hippocampinae) population fluctuations in the ria Formosa lagoon, south Portugal. *J. Fish Biol.* 87, 679–690. doi: 10.1111/jfb.12748

Curtis, J. M. R., and Vincent, A. C. J. (2006). Life history of an unusual marine fish: Survival, growth and movement patterns of hippocampus guttulatus cuvier 1829. *J. Fish Biol.* 68, 707–733. doi: 10.1111/j.0022-1112.2006.00952.x

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A., and Callahan, B. J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* 6, 226. doi: 10.1186/s40168-018-0605-2

Díaz-Abad, L., Bacco-Mannina, N., Madeira, F. M., Neiva, J., Aires, T., Serrao, E. A., et al. (2022). eDNA metabarcoding for diet analyses of green sea turtles (Chelonia mydas). *Mar. Biol.* 169, 18. doi: 10.1007/s00227-021-04002-x

Ewels, P., Magnusson, M., Lundin, S., and Kaller, M. (2016). MultiQC: Summarize analysis results for multiple tools and samples in a single report. *Bioinform.* 32, 3047–3048. doi: 10.1093/bioinformatics/btw354

Felício, A. K. C., Rosa, I. L., Souto, A., and Freitas, R. H. A. (2006). Feeding behavior of the longsnout seahorse hippocampus reidi ginsburg 1933. *J. Ethol.* 24, 219–225. doi: 10.1007/s10164-005-0189-8

Fosso, B., Pesole, G., Rosselló, F., and Valiente, G. (2018). Unbiased taxonomic annotation of metagenomic samples. *J. Comput. Biol.* 25, 348–360. doi: 10.1089/cmb.2017.0144

Fosso, B., Santamaria, M., Marzano, M., Alonso-Alemany, D., Valiente, G., Donvito, G., et al. (2015). BioMaS: A modular pipeline for bioinformatic analysis of metagenomic AmpliconS. *BMC Bioinform.* 16, 203. doi: 10.1186/s12859-015-0595-z

Foster, S. J., and Vincent, A. C. J. (2004). The life history and ecology of seahorses, hippocampus spp.: implications for conservation and management. *J. Fish Biol.* 65, 1–61. doi: 10.1111/j.0022-1112.2004.00429.x

Franzoi, P., Maccagnani, R., Rossi, R., and Ceccherelli, V. U. (1993). Life cycles and feeding habits of syngnathus taenionotus and s. abaster (Pisces, syngnathidae) in a brackish bay of the po river delta (Adriatic Sea). *Mar. Ecol. Prog. Ser.* 97, 71–81. doi: 10.3354/MEPS097071

Gainsbury, S., and Meiri, S. (2017). The latitudinal diversity gradient and interspecific competition: No global relationship between lizard dietary niche breadth and species richness global ecol. *Biogeogr* 26, 563–572. doi: 10.1111/geb.12560

Gerking, S. D. (1994). *Feeding ecology of fish* (London: Academic Press).

Gristina, M., Cardone, F., Carlucci, R., Castellano, L., Passarelli, S., and Corriero, G. (2015). Abundance, distribution and habitat preference of hippocampus guttulatus and hippocampus hippocampus in a semi-enclosed central Mediterranean marine area. *Mar. Ecol.* 36, 57–66. doi: 10.1111/maec.12116

Gristina, M., Cardone, F., Desiderato, A., Mucciolo, S., Lazic, T., and Corriero, G. (2017). Habitat use in juvenile and adult life stages of the sedentary fish hippocampus guttulatus. *Hydrobiologia* 784, 9–19. doi: 10.1007/s10750-016-2818-3

Gül, M. R., and Griffen, B. D. (2020). Diet, energy storage, and reproductive condition in a bioindicator species across beaches with different levels of human disturbance. *Ecol. Indicat.* 117, 106636. doi: 10.1016/j.ecolind.2020.106636

Gurkan, S., Taskavak, E., Sever, T. M., and Akalin, S. (2011). Gut contents of two European seahorses hippocampus hippocampus and hippocampus guttulatus in the Aegean Sea, coasts of Turkey. *Pakistan J. Zool.* 43, 1197–1201.

Hamann, M., Godfrey, M. H., Seminoff, J. A., Arthur, K., Barata, P. C. R., Bjorndal, K. A., et al. (2010). Global research priorities for sea turtles: Informing management and conservation in the 21st century. *Endanger. Spec. Res.* 11, 245–269. doi: 10.3354/esr00279

Heard, J., Chen, J. P., and Wen, C. K. C. (2019). Citizen science yields first records of hippocampus japapigu and hippocampus denise (Syngnathidae) from Taiwan: A hotspot for pygmy seahorse diversity. *Zookeys* 883, 83–90. doi: 10.3897/zookeys.883.39662

Hebert, P. D. N., Cywinska, A., Ball, S. L., and De Waard, J. R. (2003). Biological identifications through DNA barcodes. *Proc. Biol. Sci.* 270, 313–321. doi: 10.1098/rspb.2002.2218

Hernandez-Urcera, J., Murillo, F. J., Regueira, M., Cabanellas-Reboredo, M., and Planas, M. (2021). Preferential habitats prediction in syngnathids using species distribution models. *Mar. Environ. Res.* 172, 105488. doi: 10.1016/j.marenvres.2021.105488

Hildebrand, F., Tadeo, R., Voigt, A. Y., Bork, P., and Raes, J. (2014). LotuS: An efficient and user-friendly OTU processing pipeline. *Microbiome* 2, 30. doi: 10.1186/2049-2618-2-30

Holst, S., and Laakmann, S. (2019). First record of the stalked jellyfish haliclystus tenuis kishinouye 1910 (Cnidaria: Staurozoa) in Atlantic waters. *Mar. Biodiv* 49, 1061–1066. doi: 10.1007/s12526-018-0888-3

Hughes, T. P., Kerry, J. T., Álvarez-Noriega, M., Álvarez-Romero, J. G., Anderson, K. D., Baird, A. H., et al. (2017). Global warming and recurrent mass bleaching of corals. *Nature* 543, 373–377. doi: 10.1038/nature21707

IUCN (2022) *The IUCN red list of threatened species*. Available at: http://www.iucnredlist.org (Accessed December 20, 2022).

James, P. L., and Heck, K. (1994). The effects of habitat complexity and light intensity on ambush predation within a simulated seagrass habitat. *J. Exp. Mar. Biol. Ecol.* 176, 187–200. doi: 10.1016/0022-0981(94)90184-8

Kendrick, A. J., and Hyndes, G. A. (2005). Variations in the dietary compositions of morphologically diverse syngnathid fishes. *Environ. Biol. Fish.* 72, 415–427. doi: 10.1007/s10641-004-2597-y

Kim, M. J., Kim, H. W., Lee, S. R., Kim, N. Y., Lee, Y. J., Joo, H. T., et al. (2022). Feeding strategy of the wild Korean seahorse (Hippocampus haema). *J. Mar. Sci. Eng.* 10, 357. doi: 10.3390/jmse10030357

Kitsos, M. S., Tzomos, T., Anagnostopoulou, L., and Koukouras, A. (2008). Diet composition of the seahorses, hippocampus guttulatus cuvier 1829 and hippocampus hippocampus (L. 1758) (teleostei, syngnathidae) in the Aegean Sea. *J. Fish Biol.* 72, 1259–1267. doi: 10.1111/j.1095-8649.2007.01789.x

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., and Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq illumina sequencing platform. *Appl. Environ. Microbiol.* 79, 5112–5120. doi: 10.1128/AEM.01043-13

Kuiter, R. H. (2000). *Seahorses, pipefishes and their relatives: A comprehensive guide to syngnathiformes* (Chorleywood, UK: TMC Publishing).

Lazic, T., Pierri, C., Corriero, G., Balech, B., Cardone, F., Deflorio, M., et al. (2021). Evaluating the efficiency of DNA metabarcoding to analyze the diet of hippocampus guttulatus (Teleostea: Syngnathidae). *Life* 11, 998. doi: 10.3390/life11100998

Leray, M., Ho, S. L., Lin, I. J., and Machida, R. J. (2018). MIDORI server: A webserver for taxonomic assignment of unknown metazoan mitochondrial-encoded sequences using a curated database. *Bioinformatics* 34, 3753–3754. doi: 10.1093/bioinformatics/bty454

Leray, M., Yang, J. Y., Meyer, C. P., Mills, S. C., Agudelo, N., Ranwez, V., et al. (2013). A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: Application for characterizing coral reef fish gut contents. *Front. Zool* 10, 34. doi: 10.1186/1742-9994-10-34

Manning, C. G., Foster, S. J., and Vincent, A. C. J. (2019). A review of the diets and feeding behaviours of a family of biologically diverse marine fishes (Family syngnathidae). *Rev. Fish Biol. Fish.* 29, 197–221. doi: 10.1007/s11160-019-09549-z

Manzari, C., Fosso, B., Marzano, M., Annese, A., Caprioli, R., D'Erchia, A. M., et al. (2015). The influence of invasive jellyfish blooms on the aquatic microbiome in a coastal lagoon (Varano, SE Italy) detected by an illumina-based deep sequencing strategy. *Biol. Invasions* 17, 923–940. doi: 10.1007/s10530-014-0810-2

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. J.* 17, 10–12. doi: 10.14806/ej.17.1.200

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: An r package for reproducible interactive analysis and graphics of microbiome census data. *PloS One* 8, e61217. doi: 10.1371/journal.pone.0061217

Mount, D. W. (2007). Using the basic local alignment search tool (BLAST). *Cold Spring Harb. Protoc.* 2007, pdb.top17. doi: 10.1101/pdb.top17

Nielsen, J. M., Clare, E. L., Hayden, B., Brett, M. T., and Kratina, P. (2018). Diet tracing in ecology: Method comparison and selection. *Methods Ecol. Evol.* 9, 278–291. doi: 10.1111/2041-210X.12869

Oksanen, J. (2022) *Vegan: An introduction to ordination*. Available at: https://CRAN.R-project.org/package=vegan (Accessed August 1 2022).

Oliveira, F., Erzini, K., and Gonçalves, J. M. S. (2007). Feeding habits of the deep-snouted pipefish syngnathus typhle in a temperate coastal lagoon. *Estuar. Coast. Shelf Sci.* 72, 337–347. doi: 10.1016/j.ecss.2006.11.003

Petronio, B., Cardellicchio, N., Calace, N., Pietroletti, M., Pietrantonio, M., and Caliandro, L. (2012). Spatial and temporal heavy metal concentration (Cu, Pb, zn, Hg, fe, Mn, Hg) in sediments of the mar piccolo in taranto (Ionian Sea, Italy). *Water Air Soil pollut.* 223, 863–875. doi: 10.1007/s11270-011-0908-4

Pierri, C., Cardone, F., Corriero, G., Lazic, T., Quattrocchi, F., Alabiso, G., et al. (2021). Density decline in a Mediterranean seahorse population: Natural fluctuations or new emerging threats? *Front. Mar. Sci.* 8. doi: 10.3389/fmars.2021.692068

Pierri, C., Lazic, T., Corriero, G., Cardone, F., Onen Tarantini, S., Desiderato, A., et al. (2020). Site fidelity of hippocampus guttulatus cuvier 1829 at mar piccolo of taranto (Southern italy; Ionian Sea). *Environ. Biol. Fish* 103, 1105–1118. doi: 10.1007/s10641-020-01008-0

Pierri, C., Lazic, T., Gristina, M., Corriero, G., and Sinopoli, M. (2022). Large-Scale distribution of the European seahorses (Hippocampus rafinesque 1810): A systematic review. *Biology* 11, 325. doi: 10.3390/biology11020325

Pollom, R. (2017)Hippocampus guttulatus. In: *The IUCN red list of threatened species*. Available at: https://www.iucnredlist.org/species/41006/67617766 (Accessed December 10, 2022).

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J., et al. (2007). SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* 35, 7188–7196. doi: 10.1093/nar/gkm864

Rothman, J. M., Chapman, C. A., and Van Soest, P. J. (2012). Methods in primate nutritional ecology: A user's guide. *Int. J. Primatol.* 33, 542–566. doi: 10.1007/s10764-011-9568-x

Ryer, C. H. (1988). Pipefish foraging: Effects of fish size, prey size and altered habitat complexity. *Mar. Ecol. Prog. Ser.* 48, 37–45. doi: 10.3354/meps048037

Santos, A. M., and Branco, M. (2011). The quality of name-based species records in databases. *Trends Ecol. Evol.* 27, 6–7. doi: 10.1016/j.tree.2011.10.004

Shao, X., Lu, Q., Xiong, M., Bu, H., Shi, X., Wang, D., et al. (2021). Prey partitioning and livestock consumption in the world's richest large carnivore assemblage. *Curr. Biol.* 31, 4887–4897. doi: 10.1016/j.cub.2021.08.067

Sheppard, S. K., Bell, J., Sunderland, K. D., Fenlon, J., Skervin, D., and Symondson, W. O. C. (2005). Detection of secondary predation by PCR analyses of the gut contents of invertebrate generalist predators. *Mol. Ecol.* 14, 4461–4468. doi: 10.1111/j.1365-294X.2005.02742.x

Storero, L. P., and González, R. A. (2008). Feeding habits of the seahorse hippocampus patagonicus in San Antonio bay (Patagonia, Argentina). *J. Mar. Biol. Assoc. U. K* 88, 1503–1508. doi: 10.1017/S0025315408002506

Taguchi, T., Miura, Y., Krueger, D., and Sugiura, S. (2014). Utilizing stomach content and faecal DNA analysis techniques to assess the feeding behaviour of largemouth bass micropterus salmoides and bluegill lepomis macrochirus. *J. Fish. Biol.* 84, 1271–1288. doi: 10.1111/jfb.12341

Thibaut, T., Blanfuné, A., Boudouresque, C. F., Personnic, S., Ruitton, S., Ballesteros, E., et al. (2017). An ecosystem-based approach to assess the status of Mediterranean algae-dominated shallow rocky reefs. *Mar. Poll. Bull.* 117, 311–329. doi: 10.1016/j.marpolbul.2017.01.029

Tipton, K., and Bell, S. S. (1988). Foraging patterns of two syngnathid fishes: Importance of harpacticoid copepods. *Mar. Ecol. Prog.* 21, 31–43. doi: 10.3354/meps047031

Valladares, S., and Planas, M. (2021). Nutrient incorporation in first feeding seahorses evidenced by stable carbon isotopes. *Animals* 11, 470. doi: 10.3390/ani11020470

Valladares, S., Soto, D. X., and Planas, M. (2017). Dietary composition of endangered seahorses determined by stable isotope analysis. *Mar. Freshw. Res.* 68, 831–839. doi: 10.1071/MF16013

Wang, B., Li, Y., Zhang, G., Yang, J., Deng, C., Hu, H., et al. (2022). Seasonal variations in the plant diet of the Chinese monal revealed by fecal DNA metabarcoding analysis. *Avian Res.* 13, 100034. doi: 10.1016/j.avrs.2022.100034

Ward, E. J., Levin, P. S., Lance, M. M., Jeffries, S. J., and Acevedo-Gutierrez, A. (2012). Integrating diet and movement data to identify hot spots of predation risk and areas of conservation concern for endangered species. *Conserv. Lett.* 5, 37–47. doi: 10.1111/j.1755-263X.2011.00210.x

Weber, D., and Lundgren, J. (2009). Detection of predation using qPCR: Effect of prey quantity, elapsed time, chaser diet, and sample preservation on detectable quantity of prey DNA. *J. Insect Sci.* 9, 12. doi: 10.1673/031.009.4101

Wilson, M. J., and Vincent, A. C. J. (1998). Preliminary success in closing the life cycle of exploited seahorse species, hippocampus spp., in captivity. *Aq. Sci. Conser.* 2, 179–196. doi: 10.1023/A:1009629130932

Woods, C. M. C. (2002). Natural diet of the seahorse hippocampus abdominalis. *New Zeal. J. Mar. Freshw. Res.* 36, 655–660. doi: 10.1080/00288330.2002.9517121

Yip, M. Y., Lim, A. C. O., Chong, V. C., Lawson, J. M., and Foster, S. J. (2015). Food and feeding habits of the seahorses hippocampus spinosissimus and hippocampus trimaculatus (Malaysia). *J. Mar. Biol. Assoc. U. K.* 95, 1033–1040. doi: 10.1017/S0025315414001660

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Performance of the long-snouted seahorse, *Hippocampus guttulatus*, under warming conditions

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Temperature is a determinant cue for several behavioral, physiological, and metabolic processes in fish, which occur within a range set to optimize species fitness. Understanding how ocean warming will impact species, at individual and population levels, is, therefore, of utmost relevance for management and conservation purposes. This knowledge assumes particular relevance when it comes to species with unique life history traits that experience multiple threats, such as seahorses. This study aimed to assess the effects of warming on growth, feed intake, and behavioral patterns of the long-snouted seahorse, *Hippocampus guttulatus*. Fish were collected, before the breeding season, in the Sado estuary, Portugal, and subsequently exposed for an 8-week period to three different temperatures: 17°C, 20°C, and 24°C. Three times a week, behavioral observations were performed. Feed intake was measured every day and individuals were weighed once a week. Results indicate differences in behavioral responses of seahorses exposed to increasingly warming conditions. Under extreme temperature conditions (24°C), fish were more active, and fed more, but this increment of energy through feeding did not translate into increased growth in weight. Altogether, these results indicate that Sado's population of *H. guttulatus* may become under thermal stress when exposed, for a long term, to warming conditions that are expected to occur by the end of the century.

KEYWORDS
activity, feed intake, climate change, syngnathidae, body condition

1 Introduction

Anthropogenic influence on the production and release of greenhouse gases into the atmosphere has rapidly increased over the last century (Rahman, 2013; Fawzy et al., 2020). Since the mid of the 20th century, the ocean absorbed more than 90% of the excess heat in the atmosphere (Jewett and Romanou, 2017) and more than 25% of the atmospheric CO_2

(Sabine et al., 2004). According to the Intergovernmental Panel on Climate Change (IPCC) models, the temperature is projected to increase 1.4 to 4.4°C (more conservative and more extreme scenarios, respectively) (IPCC, 2021), until the end of the century. Ocean warming is expected to affect all levels of the structure and function of marine ecosystems and organisms (Parmesan, 1996; McCarthy et al., 2001; Walther et al., 2002. For ectotherms, such as fish, the temperature is a determinant factor for several behavioral (Wong and Candolin, 2015), physiological (Pörtner and Farrell, 2008), and metabolic processes (Clarke and Fraser, 2004). Warming has been shown to lead to increased metabolic rates (Roessig et al., 2004), interfere with growth (Boltaña et al., 2017), reproduction (Lopes et al., 2020; Servili et al., 2020), digestion processes and feeding rates (Rangel and Johnson, 2018; Pilakouta et al., 2020). It has also the potential to induce behavioral deviations, such as changes in swimming performance (Simon et al., 2017), predator-prey interactions (Domenici et al., 2019), and social interactions (Colchen et al., 2017).

In addition to ocean warming and other climate change-related stressors (e.g. acidification, hypoxia) (Sampaio et al., 2021), marine species are also being impacted by other human-related pressures, such as overexploitation of resources (Jackson et al., 2001), poaching (Correia, 2022), habitat degradation and fragmentation (Airoldi et al., 2008) and pollution (Shahidul Islam and Tanaka, 2004). When combined, these pressures are particularly severe for species with unique life history traits, such as seahorses. Seahorses (*Hippocampus* spp.) are benthic species, with low mobility, small home ranges, low fecundity, sparse distribution, mate fidelity, and lengthy parental care (Foster and Vincent, 2004). These traits, combined with illegal trade for Traditional Chinese Medicine and unsustainable aquarium display (Foster and Vincent, 2004), and habitat loss (Harasti, 2016; Correia, 2022), render them extremely vulnerable to population declines and local extinctions (Foster and Vincent, 2004; Chong et al., 2010; Macusi et al., 2011; Vincent et al., 2011). Hence, for better control over seahorse trade, all species in the genus have been included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES Convention) (Foster and Vincent, 2005), and in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (IUCN, 2020). More recently, IUCN World Conservation Congress approved a resolution (WCC-2020-Res-095) that urges IUCN members to take action toward ensuring the status assessment of all syngnathids (seahorses, pipefish, and seadragons), and their inclusion in national/regional Red Lists, while promoting the protection and restoration of their habitats.

Experimental Studies on the effects of global warming on the behavior and physiology of seahorses are still scarce and limited to just a few species. The temperate long-snouted seahorse, *Hippocampus guttulatus*, distributes across most of Europe and North Africa, including the Atlantic Ocean, Mediterranean Sea, and Black Sea (Woodall et al., 2015; Lourie et al., 2016). Concurrent with this wide geographic range, the species can also experience a wide range of temperatures throughout the year (Planas et al., 2012), which will vary depending on the latitude. Temperatures in the South of the Iberian Peninsula can range from 10 to 28 °C (Ria Formosa lagoon, Curtis and Vincent, 2006), which clearly differs from temperatures experienced in Northern France or the British Isles, where temperatures can range from 4 to 20°C (Garrick-Maidment et al., 2014). To date, two studies looked into the effects of prolonged exposure (1 month) to increasingly warmer water conditions (18°C – 26°C – 30°C) on *H. guttulatus* (Aurélio et al., 2013; Faleiro et al., 2015). Authors report an increase in ventilation and metabolic rates of adult seahorses with warming, but fail to detect any further physiological and behavioral impairments, which led the authors to conclude that adults of the species are thermally resilient to heat stress. A similar study on a congener sub-tropical species, *H. erectus*, which occurs from the Gulf of Mexico to Nova Scotia in water temperatures ranging from 5 to 28°C (Teixeira and Musick, 2001; Lourie et al., 2016), exposed fish for 1 month to increasingly warmer water conditions (22°C – 26°C – 30°C) (Qin et al., 2018). Authors found evidence of persistently higher basal metabolic rate at 30°C, higher stress response on gene expression, and higher mortality rates. Further, a transcriptomic analysis suggests an arrest of muscle development processes, activation of heat shock proteins, and a switch to anaerobic metabolism from the first moments of exposure to high temperatures (30 or 33 °C) (del Vecchio et al., 2022).

The extensive latitudinal range these species inhabit might contribute to differences in thermal tolerance across populations (Helmuth et al., 2002; Pereira et al., 2017), with population-specific thermal limits being set by physiological limitations in aerobic performance (Eliason et al., 2011). Therefore, when assessing the potential impact of climate change it is important to use scenarios that reflect temperatures within and outside a population's natural variability, and not only scenarios that reflect the variability across the species geographical distribution.

'The present study aimed to assess the effects of chronic exposure to increasing temperature scenarios on growth, feed intake, and behavioral patterns of adults of *H. guttulatus*. Individuals were collected in the Sado estuary (Portugal), where temperature can annually range from average 15°C to 19°C, but minimum and maximum temperatures range from 13.8°C (January) and 20.5°C (September) (European Centre for Medium-Range Weather Forecasts, ECMWF, 2022) (Table S1). Fish were exposed for a 2-month period to three different temperature scenarios: ambient temperature (17°C); high temperature (20°C); and an extreme temperature scenario (24°C), which is likely to occur by the end of the century (IPCC, 2021). Furthermore, as the study took place during the species' breeding season, we provide a descriptive discussion of temperature effects on reproduction based on anecdotal observations.

2 Materials and methods

2.1 Study species

Two species of seahorses inhabit the Portuguese coastal areas, *H. guttulatus* (Pollom, 2017), and *Hippocampus hippocampus* (Pollom, 2016). Both species can be found in inshore waters from the coastal environment and are associated with habitats of different complexity (Correia et al., 2015; Correia et al., 2018; Woodall et al., 2018). National legislation has recently included syngnathids under Decree-Law n°38/2021, which approves the legal regime applicable to the protection and conservation of wild flora and fauna and natural habitats of the species listed in the Berne and Bonn Conventions (https://dre.pt/dre/detalhe/decreto-lei/38-2021-164258742). The most extensively studied population in Portugal is the Ria Formosa lagoon's population, where several studies have taken place to assess population status, identify major threats and apply mitigation measures to support its conservation (Curtis and Vincent, 2006; Caldwell and Vincent, 2012; Correia et al., 2013; Correia et al., 2015; Correia et al., 2018; Correia, 2022). However, there are newly recorded seahorse populations, such as in the Sado and Tagus estuaries which are understudied and in need of attention (unpublished data). The Sado Estuary is the second-largest estuary in Portugal and one of the most important wetlands in the national territory, integrating an important natural reserve. This region has the largest distribution of seagrass species in Portugal (Cunha and Serrão, 2011), only surpassed by the Ria Formosa lagoon, and supports species with both economic and conservation value, including seahorses.

2.2 Fish collection and experimental setup

Adult males and females (n=22 individuals; 11 males and 11 females) of the long-snouted seahorse were individually collected by scuba diving in the seagrass meadows of Tróia, Portugal (38.462311, -8.856218), on April 8th, 2022 (Figure 1). Permit to collect fish from the wild was granted by the National Institute of Nature Conservation and Forests (ICNF, permits 288 – 290/2022/CAPT). Individuals were immediately placed in large containers filled with seawater from the collection site, with aeration, and controlled temperature, and transported to ISPA fish facilities.

At arrival, seahorses were placed in six 80-L aquaria (40x40x60 cm), at a density of 2 males and 2 females per aquarium (except 1 tank, with only 1 male and 1 female). Soon after being in these conditions, males and females paired and formed couples based on body size. All aquaria were equipped with a protein skimmer (TMC Reef-Skim 200), biological and mechanical filtration (internal filter, Max 104 F), and maintained under temperature and salinity conditions matching field site (average 15°C and 35 PSU), and a photoperiod of 14L: 10D. To provide environmental enrichment and holdfast, plastic chains were added to each aquarium (Correia et al., 2013). Seahorses were left under these conditions for 15 days, to acclimate to laboratory conditions. Fish were fed once per day, *ad libitum*, with frozen wild-caught mysis (*Mesopodopsis slabberi*). Temperature and salinity were daily measured, in the morning. Ammonia, nitrites and nitrates, were monitored frequently, and kept below critical levels (0.1, 0.3, and 10.0 $mg\ L^{-1}$, respectively). Aquaria were cleaned daily, in the morning, before feeding. Subsequently, aquaria were randomly assigned to three temperature treatments: ambient temperature (17°C), matching average temperature at the beginning of the species breeding season; high temperature (20°C), matching temperatures within the species range of variability which they can experience in their natural habitat for a short period; and extreme temperature (24°C), reflecting a scenario that falls outside the natural thermal variability of this population and are likely to occur by the end of the century. Sea surface temperatures, represented as monthly averages from daily means, were extracted from remotely sensed climate models (European Centre for Medium-Range Weather Forecasts, ECMWF, 2022) from spatial points (geo-referenced occurrence data) representing the habitat where the species was captured (Table S1). Temperatures in all treatments increased gradually, using heaters, by about 1°C per day to avoid stress and heat shock associated with rapid temperature changes. Aquaria from the extreme treatment (24°C) were the first to start increasing temperature, followed by aquaria from the high treatment (20°C), 4 days later, and finally, aquaria from the ambient treatment (17°C) had their temperature increased 7 days after the first aquaria. This ensured that all treatments reached the experimental treatment temperature (considered day 0 of exposure) at the same time. At this stage, each individual was measured for height (Lourie, 2003) and weight (wet weight) (Table S2). Within sex, initial weight (W) and height (H) did not differ across treatments (W_{males}: F = 0.176, p = 0.277; H_{males}: F = 0.933, p = 0.432; $W_{females}$: F = 0.677, p = 0.535; $H_{females}$: F = 0.402, p = 0.682), with males average weight and height of 11.99 ± 1.62 g, and 13.79 ± 0.75 cm, respectively, and females average weight and height of 10.70 ± 1.40 g, and 13.71 ± 0.70 cm,

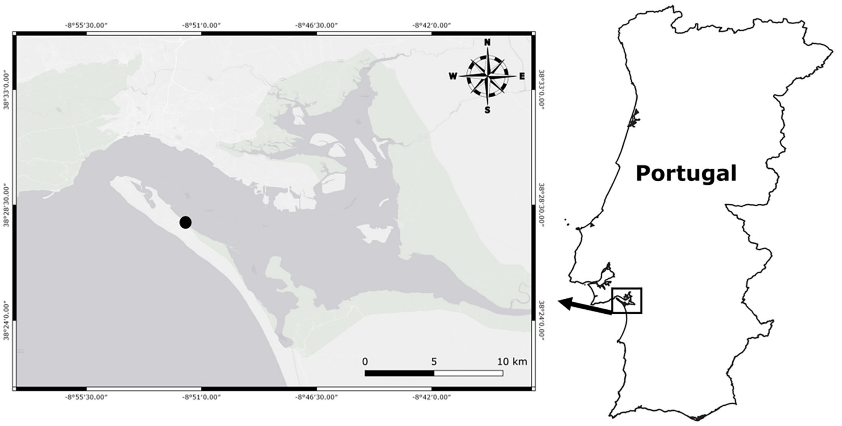

FIGURE 1
Field site location.

respectively. Each treatment had two replicate aquaria, each with 2 couples (i.e., 4 couples for treatment). Aquaria were placed side by side, divided by a white opaque plastic sheet to avoid intraspecific interactions between tanks (Papoutsoglou et al., 2000; Tamazouzt et al., 2000). Ambient temperature treatment had only 3 couples (one aquarium had only 1 couple), since it was only possible to collect 11 females and 11 males. As this is a species with national conservation status and protected by law, it was decided to keep only three couples under control conditions.

Exposure to temperature treatments lasted 2 months. At the end of the experimental period, temperatures from high and extreme treatments were gradually decreased till the matching temperature from ambient treatment, which matched the temperature at the collection site at that time of the year (*i.e.* July). Seahorses were then released back to the same area where were previously collected, as per requirement from the permit issuer.

2.3 Behavior

Behavioral observations were performed three times a week, based on an ethogram adapted from Faleiro et al. (Faleiro et al., 2008; Faleiro et al., 2015) (Table 1). Focal observations were made in the morning (between 07:00 and 10:00 hours), before feeding, to avoid post-prandial bias. Observations were made by a motionless observer standing in front of the aquarium. Each focal observation lasted 3 minutes, and sea horses were observed one at a time in each aquarium. In every observation day, the order of the seahorses observed changed, to add randomization to the process and avoid an effect of the order of observation. As individuals from each aquarium had a set of distinctive traits (body size and spot patterns), we were able to keep the ID of each individual throughout the exposure period (Correia et al., 2014) and thus avoiding invasive individual identification methods such as VIE tagging (Curtis, 2006).

2.4 Feed intake

To allow *ad libitum* conditions for every fish, fish under treatments A and B were daily fed 5% of body weight (BW) and fish from treatment C were daily fed 7% of BW (Palma et al., 2011) . To maintain the proper feed rations, the daily wet weight of each ration was altered in accordance with the average wet weight growth for each treatment (Palma et al., 2008). The values were initially adjusted based on uneaten food to avoid degradation of water quality. Food was weighed and thawed before feeding seahorses, daily. After 24h, uneaten food items were siphoned out of the tank and collected in a hand-held net with fine mesh, over a paper to dry. Then, every piece of uneaten food was sorted using a tweezer and placed in a plastic petri dish for weighing. Indistinct matter was considered as debri and part of faeces and discarded from the weighing. Feed intake (FI) was calculated using the formula:

$$FI = \frac{FS - FU}{FS}$$

where FS is the amount of food supplied (g) and FU is the amount of uneaten food (g). Feed intake was measured daily, except for weekends.

2.5 Weight

Individuals were weighed (g) on a weekly basis to determine the amount of food (5 and 7% of body weight) to provide throughout the exposure period. The procedure involved placing each individual in a shallow tray with seawater and placed on a scale, and was performed as quickly as possible. Manipulation of individuals might induce stress and alter behavioral responses. Although we did not measure stress responses to manipulation, we did not observe signs of disturbance attributable to handling stress. Fish resumed their normal rest state as soon as they were placed back in the aquaria, and were seen feeding when food was supplied. Therefore, we consider that fish recovered from handling stress in just a few hours, and this standardized procedure across treatments did not alter behavioral observations throughout the study period.

Data on the initial and final weight were used to calculate mean weight gain (Palma et al., 2011):

$$WG(\%)\frac{Wf - Wi}{Wi} \times 100$$

where Wf is the final seahorse wet weight and Wi is the initial wet weight.

2.6 Data analysis

Linear mixed-effects models (“lmerTest” package in R) were used to access the effect of temperature treatments throughout time in the different seahorse behaviours: rest, swimming, body movement, attach and unattached, and also feed intake.

TABLE 1 Ethogram of *Hippocampus guttulatus* describing activity patterns and holdfast attachment (adapted from Faleiro et al., 2008).

Behaviour	Description
Rest	The seahorse stays immobile, without any movement
Body movement	The seahorse remains in the same place, but moving either the body or the fins
Swimming	The seahorse swims actively
Attachment	The seahorse is attached or to a holdfast or to another individual
Unattachment	The seahorse is not attached to anything

Treatment and Day of Observation (Dobs) were used as fixed effects and Animal ID (Individual) and Holding Aquarium as random effects, for all behaviours, except feed intake. For feed intake only Holding Aquarium was considered as random effect. Day of Observation (Dobs) considered the first day of observation as day 0. A generalized linear model was used to determine the effect of the treatments in the weight gain of the seahorses. Assumptions of homoscedasticity and normality of the residuals were examined by visual inspection of residual fitted plots.The statistical analysis was performed with R statistics, Rstudio (Version 4.2.1). Statistical significance was considered when p<0.05.

3 Results

3.1 Temperature effects on behaviour

Long-snouted seahorses spent significantly less time resting when exposed to extreme (24°C) temperature, and spent significantly more time swimming when compared with ambient temperature (17°C), where individuals spent more time motionless (Figures 2, 3; Table 2, Table S3). Dobs had also a significant effect on rest and swimming behaviors, but no interation between Dobs and Temperature was detected (Table 2).

Under extreme temperature (24 °C) fish were also seen spending less time attached compared to fish from ambient temperature, and Dobs had also a significant effect (Figure 4, Table 2, Table S3).

Time spent stationary but moving either the body or the fins (body movement) did not differ across treatments, or Dobs (Table 2, Table S3).

3.2 Temperature effects on feed intake

Fish under warming conditions (20 and 24°C) showed significantly higher feed intake when compared to fish under ambient temperature (17°C) (Figure 5, Table 2, Table S3). Dobs had also a significant effect on feed intake, but no interation between Dobs and Temperature was detected (Table 3).

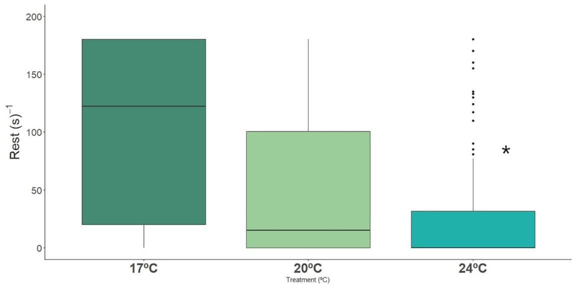

FIGURE 2
Time spent in stationary behavior (s^{-1}) by long-snouted seahorse exposed to different temperature treatments (17°C, 20°C and 24°C). Bold * represent significant differences from control treatment.

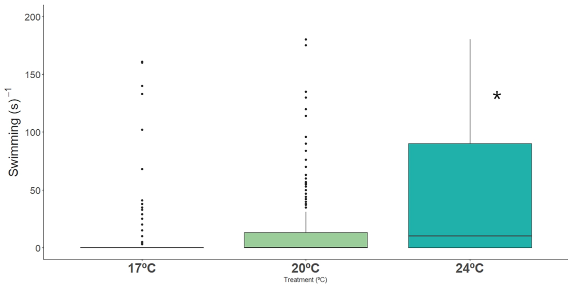

FIGURE 3
Time spent swimming (s^{-1}) by long-snouted seahorse exposed to different temperature treatments (17°C, 20°C and 24°C). Bold * represent significant differences from control treatment.

3.3 Temperature effects on weight gain

Weight gain, at the end of the exposure period, did not differ across treatments (Figure 6, Table 3, Table S2).

4 Discussion

Results from the present study reflect differences in behavioral responses in seahorses exposed to different elevated temperatures for a long-term period (2 months). Fish were significantly more active under extreme temperature conditions (24°C) (less time resting, more time swimming and unattached), and were also seen feeding more under both high and extreme temperatures (20°C and 24°C). Higher food ingestion did not translate into an increased body condition, though, as the weight gain of the fish did not differ across treatments. Temperature can impact differently fish locomotion and activity. While some studies suggest that individual activity increases with temperature (Ojanguren and Brañta, 2000; Biro et al., 2010), others conclude that swimming ability decreases under warming conditions (Johansen and Jones, 2011). The elevated temperature was shown to increase swimming activity in Atlantic cod, *Gadus morhua* (Claireaux et al., 2000), but impact negatively swimming activity in juvenile brown trout, *Salmo trutta* (Ojanguren and Brañta, 2000). In the current study, we found a significant temperature effect on swimming activity, with fish under extreme temperature treatment (24°C) being more active (more time spent swimming) and spending less time resting. Similarly, a study on the activity of adult lined seahorses showed that when they were exposed to higher temperatures close to the species' thermal limit (30°C), they increased their swimming as opposed to seahorses maintained at lower temperatures (22 and 26° C) (Qin et al., 2018). Interestingly, two other studies conducted on the long-snouted seahorse, from the same collection site, exposed to much more extreme temperature conditions (26°C, 28°C, 30°C), did not find an effect of temperature on frequency of swimming or periods of inactivity when compared to fish from control

TABLE 2 Statistical results of the Linear mixed effects models on time (s) seahorse spent in rest, body movement, swimming, attached and unattached, and feed intake of long-snouted seahorse exposed to three temperature treatments – control (17°C), high (20°C), and extreme (24°C).

Variable		Estimate	S.E.	df	t-value	pr(>|t|)
Rest	Treatment High		-38.900	21.637	41.289	-1.798
	0.080					
	Treatment Extreme		-68.143	21.637	41.289	-3.149
	0.003					
	Day of Observation	0.811	0.371	440.000	2.183	**0.029**
Treatment High x Day of Observation	-0.404	0.491	440.000	-0.822	0.412	
Treatment Extreme x Day of Observation	0.099	0.491	440.000	0.201	0.841	
Body Movement	Treatment High	29.919	18.189	63.830	1.645	0.105
	Treatment Extreme	22.937	18.189	63.830	1.261	0.212
	Day of Observation	-0.325	0.388	440.000	-0.837	0.403
	Treatment High x Day of Observation	0.489	0.513	440.000	0.953	0.341
	Treatment Extreme x Day of Observation	0.432	0.513	440.000	0.843	0.400
Swimming	Treatment High	8.978	10.245	87.650	0.876	0.383
	Treatment Extreme	45.206	10.245	87.650	4.412	**<0.001**
	Day of Observation	-0.486	0.241	440.000	-2.017	**0.044**
	Treatment High x Day of Observation	-0.085	0.319	440.000	-0.268	0.789
	Treatment Extreme x Day of Observation	-0.531	0.319	440.000	-1.666	0.096
Unattachment	Treatment High	19.380	18.297	52.448	1.059	0.294
	Treatment Extreme	44.756	18.297	52.448	2.446	**0.018**
	Day of Observation	-0.720	0.359	440.000	-2.002	**0.046**
	Treatment High x Day of Observation	-0.346	0.476	440.000	-0.729	0.467
	Treatment Extreme x Day of Observation	-0.443	0.476	440.000	-0.932	0.353
Attachment	Treatment High	-2.329	18.127	65.105	-0.128	0.898
	Treatment Extreme		-36.434	18.127	65.105	-2.010
	0.049					
	Day of Observation	1.086	0.389	440.000	2.791	**0.006**
	Treatment High x Day of Observation	-0.350	0.515	440.000	-0.680	0.497
Treatment Extreme x Day of Observation	0.105	0.515	440.000	0.204	0.839	
Feed Intake	Treatment High	0.329	0.081	15.974	4.037	**<0.001**
	Treatment Extreme	0.417	0.081	15.974	5.130	**<0.001**
	Day of Observation	0.004	0.002	120.000	2.338	**0.021**
	Treatment High x Day of Observation	-0.001	0.002	120.000	-0.368	0.714
	Treatment Extreme x Day of Observation	-0.005	0.002	120.000	-1.803	0.074

Shown are the parameter estimates, standard error (SE), degrees of freedom (df), t-statistic value, and P-value. Significant differences ($p<0.05$) are indicated in bold.

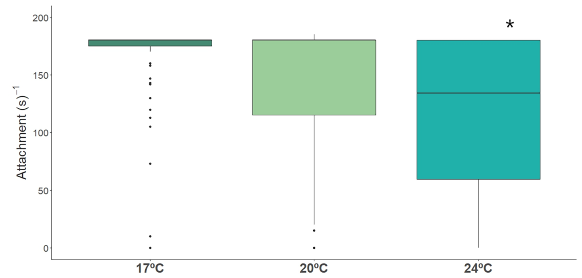

FIGURE 4
Time spent attached (s^{-1}) by long-snouted seahorse exposed to different temperature treatments (17°C, 20°C and 24°C). Bold * represent significant differences from control treatment.

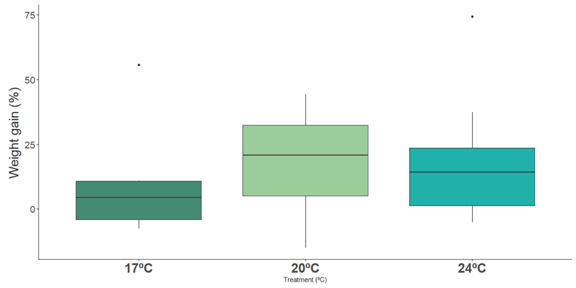

FIGURE 6
Impact of ocean warming on weight gain (%) of long-snouted seahorse exposed to different temperature treatments (17°C, 20°C and 24°C).

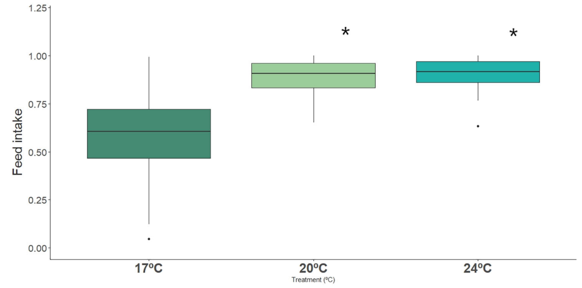

FIGURE 5
Impact of ocean warming on the feed intake of long-snouted seahorse exposed to different temperature treatments (17°C, 20°C and 24°C). Bold * represent significant differences from control treatment.

temperatures (18°C) (Aurélio et al., 2013; Faleiro et al., 2015). Some studies in other fish species have demonstrated that swimming activity decreases at low temperatures, increase to a peak and then decrease as the temperature approaches the upper thermal limit (Myrick and Cech, 2000; Ojanguren and Brañta, 2000). This could explain the results from those authors' studies – the extreme temperatures the seahorses were exposed to might already be close to their thermal limit. Another possible explanation for the observed differences might relate to methodological aspects. In the current study, we run behavioral observations three times a week, for 2 months, and observations were performed early in the morning before feeding. Aurélio et al. (2013) and Faleiro et al. (2015) had shorter periods of behavioral observations and run observations 30 min after feeding. This might have influenced the time spent swimming/inactive as fish might still be under post-prandial effects.

In response to a warmer environment, fish will increase their energetic demands and consequently will need to ingest more food (Sswat et al., 2018) , to maintain core activities. As expected, in our study, fish under the extreme temperature treatment (24°C) significantly ingested more food than fish under ambient and high-temperature treatments (17°C and 20°C). Contrasting results were reported by Aurélio et al. (2013) and Faleiro et al. (2015), with no changes in feed intake rates of *H. guttulatus* under extreme water temperatures (26°C, 28°C, 30°C). Also, Qin et al. (2018) fail to detect an influence of temperature on the feeding frequency of the *H. erectus* after 1 week at 30°C, which is close to the species' thermal limit. However, in these 3 studies, authors observed an increase in metabolic rates and ventilation rates (which we did not measure in the current study), supporting an increased metabolic activity under higher temperatures. The increase in metabolic rates but the absence of a temperature effect on feeding rates might indicate that the species was already outside their optimum temperature limit, as feed intake might decrease if the temperature surpasses the species' optimum limits (Volkoff and Rønnestad, 2020). Therefore, individuals might have adopted a saving-energy strategy by not spending energy on feeding but making digestion more efficient.

Temperature increases within an appropriate range usually promote fish growth (Green and Fisher, 2004), and therefore we were expecting to have fish under the better condition at higher temperatures. In our study, fish did not show any significant change in their body weight among the different temperatures they were exposed to. Similarly, Qin et al. (2018) did not find differences in

TABLE 3 Statistical results of the Generalized Linear on weight gain (%) of long-snouted seahorse exposed to three temperature treatments – control (17°C), high (20°C), and extreme (24°C).

Variable		Estimate	Std.Error	t-value	Pr(>|t|)
Weight gain	Treatment High	7.289	12.935	0.564	0.580
	Treatment Extreme	8.712	12.935	0.674	0.509

Shown are the parameter estimates, standard error (SE), t-statistic value, and P-value. Significant differences (p<0.05) are indicated in bold.

weight or body height of adult *H. erectus* exposed to different temperature scenarios (22, 26, and 30 °C) for a 4-week period. However, the seahorse hepatosomatic index (HSI) was lower under 30 °C, while metabolic rates were higher at this temperature. Authors argue that thermal stress inhibited hepatic energy reserves and leads to lower HSI by increasing energy consumption. In our study the lack of temperature-induces effects in fish growth was possible due to fish being more active under extreme temperatures (24°C), thus spending more energy. Nonetheless, we can also not rule out the possibility that seahorses were already under thermal stress at these extreme temperatures. Mascaró et al. (Mascaró et al., 2016, Mascaró et al., 2019), for example, report that chronic exposure (1 month) to the extreme temperature of 30°C significantly reduced juvenile *H. erectus* growth. Using transcriptome data, del Vecchio et al. (2022) provide evidence of repression of the MEF2A gene within the first 0.5 h of exposure of the *H. erectus* to target temperatures (30 and 33 °C). This gene is implicated in the regulation of growth and differentiation of muscle fibers, and its repression might justify changes in growth under temperatures close to the s'ecies' thermal limits. Despite the relevance of this study, the rate of thermal increase untill reaching the target temperatures (abrupt: < 5 min; gradual, 1-1.5 °C every 3 h) and the fact that the authors only collected data after 0.5 h of exposure prevents us from making further conclusions on the effects of prolonged warming on growth.

A word of caution is needed when interpreting results of the current study, as it took place during the breeding season of the species, and reproduction changes activity rate (which likely justifies the observed significant effect of Day of Observation) and physiological condition (weight gain) of individuals. Therefore, its influence on the response variables under measure is difficult to control. Nevertheless, courtship behaviors and daily greetings (Vincent, 1995) were regularly and evenly recorded across the three temperature treatments during the 8-week exposure period (19 courtship events under ambient temperature; 21 events under high temperature; 21 events under extreme temperatures). Moreover, all males across treatments were seen to become pregnant at a certain point of the experimental period, even though the number of successful pregnancies differed across treatments. Therefore, the observed changes at the behavioral level and feeding rate are likely to truly reflect a temperature effect, rather than just an influence of reproductive activity. A total of 7 successful breeding events were registered, being 4 events (in 3 out of the 4 males) in couples from the extreme temperature (24°C), 2 events (in 2 out of the 4 males) in couples from the high temperature (20°C), and 1 event (in 1 out of the 3 males) in ambient temperature (17°C). As reproduction is considered to be sensitive to thermal stress (Pörtner and Farrell, 2008) our results, then, might indicate that the range of tested temperatures was not enough to negatively impact reproduction, and it could even suggest that breeding is stimulated at higher temperatures. Qin et al. (2018) found no significant negative effects on gonad development or reproductive endocrine regulation genes in response to chronic thermal stress (1 month, at 30 °C) in the *H. erectus*. The authors argue that the species' reproductive behavior is adapted to higher-temperature conditions, as *H. erectus* seasonally migrate into warm waters for breeding and return to offshore areas when the water cools (Boehm et al., 2015). Nevertheless, in the present study, and despite the suggestion of increased reproductive activity under high and extreme temperature conditions (20 and 24°C), episodes of unsuccessful egg transfer from the female to the male were recorded only under these temperature conditions and were frequently observed in couples where males were not responsive to females approach and courtship. Moreover, abortion was also registered at high temperatures (20°C), with the male releasing embryos of several developmental stages. Altogether, these observations might suggest that there is a high but unsuccessful investment in reproduction under warming conditions. Consequences at the offspring level were not possible to infer, as we were unable to maintain the newborns. Future studies should take a closer look at the impacts of high temperatures on the reproductive success of these species.

Overall, and despite the limited number of individuals available to run this experimental study, our results points to a negative consequence of ocean warming on behavior and feeding rate of adult *H. guttulatus*. To truly understand if these individuals were already under thermal stress at the extreme temperature of 24°C, additional physiological and biochemical endpoints would be needed. Determination of metabolic rates, such as minimum and maximum metabolic rate, is a highly useful physiological tool that allows a better understanding of fish metabolic performance under different environmental conditions, and, as it involves minimum manipulation of the individual, there is a low risk of death. The set of physiological responses to warming is the reflection of a prompt cellular stress reaction triggered to maintain the organism's homeostasis, and it involves biochemical adjustments of metabolic pathways and the regulatory activity of the antioxidant system (Sopinka et al., 2016). These indicators provide highly relevant information for evaluating stress response levels, but the downside is that they require the euthanasia of individuals, for tissue and blood sampling. To avoid invasive and lethal practices with a species that holds a special conservation status, we decided not to evaluate these physiological responses.

Seahorses are a great model to study the effects of climate change on the fitness and behavior of sedentary and low dispersal species, acting as an indicator of ecosystem health. These species can be used as flagship species for conservation issues and can be found worldwide in some of the most vulnerable marine ecosystems in shallow waters (Vincent et al., 2011). The population of the *H. guttulatus* from the Sado estuary is subject to intense pressure from other anthropogenic activities, such as overexploitation, pollution, and habitat destruction. All these pressures reduce the likelihood of these species to acclimatize and hinder the opportunity to persist in a changing environment. If protective measures are not taken, seahorse populations might suffer a sharp decline in numbers, and in long term affect this population's fitness and survival.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study was carried out under the approval of Direção-Geral de Alimentação e Veterinária (DGAV, Portuguese Authority for Animal Health, permit 0421/000/000/2020) and according to the ISPA University's animal ethics guidelines.

Author contributions

AC: methodology, data collection, data analysis, writing- original draft preparation, writing - review and editing; MC: conceptualization, methodology, writing- original draft preparation, writing - review and editing; GS: methodology, writing - review and editing; AL: data analysis, writing - review and editing; AF: funding acquisition, project administration, conceptualization, methodology, writing- original draft preparation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1136748/full#supplementary-material

References

Airoldi, L., Balata, D., and Beck, M. W. (2008). The Gray zone: Relationships between habitat loss and marine diversity and their applications in conservation. *J. Exp. Mar. Biol. Ecol.* 366 (1–2), 8–15. doi: 10.1016/j.jembe.2008.07.034

Aurélio, M., Faleiro, F., Lopes, V. M., Pires, V., Lopes, A. R., Pimentel, M. S., et al. (2013). Physiological and behavioral responses of temperate seahorses (Hippocampus guttulatus) to environmental warming. *Mar. Biol.* 160 (10), 2663–2670. doi: 10.1007/s00227-013-2259-8

Biro, P. A., Beckmann, C., and Stamps, J. A. (2010). Small within-day increases in temperature affects boldness and alters personality in coral reef fish. *Proc. R. Soc. B: Biol. Sci.* 277 (1678), 71–77. doi: 10.1098/rspb.2009.1346

Boehm, J. T., Waldman, J., Robinson, J. D., and Hickerson, M. J. (2015). Population genomics reveals seahorses (Hippocampus erectus) of the Western mid-Atlantic coast to be residents rather than vagrants. *PloS One* 10 (1), e0116219-. doi: 10.1371/journal.pone.0116219

Boltaña, S., Sanhueza, N., Aguilar, A., Gallardo-Escarate, C., Arriagada, G., Valdes, J. A., et al. (2017). Influences of thermal environment on fish growth. *Ecol. Evol.* 7 (17), 6814–6825. doi: 10.1002/ece3.3239

Caldwell, I. R., and Vincent, A. C. J. (2012). Revisiting two sympatric European seahorse species: apparent decline in the absence of exploitation. *Aquat. Conserv.: Mar. Freshw. Ecosyst.* 22 (4), 427–435. doi: 10.1002/aqc.2238

Chong, V. C., Lee, P. K. Y., and Lau, C. M. (2010). Diversity, extinction risk and conservation of Malaysian fishes. *J. Fish Biol.* 76 (9), 2009–2066. doi: 10.1111/j.1095-8649.2010.02685.x

Claireaux, G., Webber, D. M., Lagardère, J.-P., and Kerr, S. R. (2000). Influence of water temperature and oxygenation on the aerobic metabolic scope of Atlantic cod (Gadus morhua). *J. Sea Res.* 44 (3–4), 257–265. doi: 10.1016/S1385-1101(00)00053-8

Clarke, A., and Fraser, K. P. P. (2004). Why does metabolism scale with temperature? *Functional Ecology* 18 (2), 243–251. doi: 10.1111/j.0269-8463.2004.00841.x

Colchen, T., Teletchea, F., Fontaine, P., and Pasquet, A. (2017). Temperature modifies activity, inter-individual relationships and group structure in a fish. *Curr. Zool.* 63 (2), 175–183. doi: 10.1093/cz/zow048

Correia, M. (2022). Monitoring of seahorse populations, in the ria Formosa lagoon (Portugal), reveals steep fluctuations: Potential causes and future mitigations. *Proc. Zool. Soc.* 75 (2), 190–199. doi: 10.1007/s12595-021-00394-2

Correia, M., Caldwell, I. R., Koldewey, H. J., Andrade, J. P., and Palma, J. (2015). Seahorse (Hippocampinae) population fluctuations in the ria Formosa lagoon, south Portugal. *J. Fish Biol.* 87 (3), 679–690. doi: 10.1111/jfb.12748

Correia, M., Koldewey, H. J., Andrade, J. P., Esteves, E., and Palma, J. (2018). Identifying key environmental variables of two seahorse species (Hippocampus guttulatus and hippocampus hippocampus) in the ria Formosa lagoon, south Portugal. *Environ. Biol. Fish.* 101 (9), 1357–1367. doi: 10.1007/s10641-018-0782-7

Correia, M., Palma, J., Koldewey, H., and Andrade, J. P. (2013). Can artificial holdfast units work as a habitat restoration tool for long-snouted seahorse (Hippocampus guttulatus cuvier)? *J. Exp. Mar. Biol. Ecol.* 448, 258–264. doi: 10.1016/j.jembe.2013.08.001

Correia, M., Palma, J., Koldewey, H., and Andrade, J. P. (2014). The use of a non-invasive tool for capture–recapture studies on a seahorse hippocampus guttulatus population. *J. Fish Biol.* 84 (4), 872–884. doi: 10.1111/jfb.12304

Cunha, A. H., and Serrão, E. A. (2011). Tools for seagrass conservation and management in Portugal. *Ecologia* 3, 23–36.

Curtis, J. M. R. (2006). Visible implant elastomer color determination, tag visibility, and tag loss: Potential sources of error for mark–recapture studies. *North Am. J. Fish. Manage.* 26 (2), 327–337. doi: 10.1577/M05-099.1

Curtis, J. M. R., and Vincent, A. C. J. (2006). Life history of an unusual marine fish: survival, growth and movement patterns of hippocampus guttulatus cuvier 1829. *J. Fish Biol.* 68 (3), 707–733. doi: 10.1111/j.0022-1112.2006.00952.x

del Vecchio, G., Galindo-Sánchez, C. E., Tripp-Valdez, M. A., López-Landavery, E. A., Rosas, C., and Mascaró, M. (2022). Transcriptomic response in thermally challenged seahorses hippocampus erectus: The effect of magnitude and rate of temperature change. *Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol.* 262, 110771. doi: 10.1016/j.cbpb.2022.110771

Domenici, P., Allan, B. J. M., Lefrançois, C., and McCormick, M. I. (2019). The effect of climate change on the escape kinematics and performance of fishes: implications for future predator–prey interactions. *Conserv. Physiol.* 7 (1), coz078. doi: 10.1093/conphys/coz078

ECMWF. (2022). Available at: https://www.ecmwf.int/

Eliason, E. J., Clark, T. D., Hague, M. J., Hanson, L. M., Gallagher, Z. S., Jeffries, K. M., et al. (2011). Differences in thermal tolerance among sockeye salmon populations. *Science* 332 (6025), 109–112. doi: 10.1126/science.1199158

Faleiro, F., Baptista, M., Santos, C., Aurélio, M. L., Pimentel, M., Pegado, M. R., et al. (2015). Seahorses under a changing ocean: the impact of warming and acidification on the behaviour and physiology of a poor-swimming bony-armoured fish. *Conserv. Physiol.* 3 (1), cov009. doi: 10.1093/conphys/cov009

Faleiro, F., Narciso, L., and Vicente, L. (2008). Seahorse behaviour and aquaculture: How to improve hippocampus guttulatus husbandry and reproduction? *Aquaculture* 282 (1), 33–40. doi: 10.1016/j.aquaculture.2008.05.038

Fawzy, S., Osman, A., Doran, J. W., and Rooney, D. (2020). Strategies for mitigation of climate change: A review. *Environ. Chem.Lett.* 18 (6), 2069–2094. doi: 10.1007/s10311-020-01059-w

Foster, S. J., and Vincent, A. C. J. (2004). Life history and ecology of seahorses: implications for conservation and management. *J. Fish Biol.* 65 (1), 1–61. doi: 10.1111/j.0022-1112.2004.00429.x

Foster, S. J., and Vincent, A. C. J. (2005). Enhancing sustainability of the international trade in seahorses with a single minimum size limit. *Conserv. Biol.* 19 (4), 1044–1050. doi: 10.1111/j.1523-1739.2004.00157.x-i1

Garrick-Maidment, N., Durant, E., and Newman, J. (2014). Year 5 report on the Seahorse Tagging Project at South Beach, Studland Bay in Dorset. *The Seahorse Trust.* Published online. http://www.theseahorsetrust.org.

Green, B. S., and Fisher, R. (2004). Temperature influences swimming speed, growth and larval duration in coral reef fish larvae. *J. Exp. Mar. Biol. Ecol.* 299 (1), 115–132. doi: 10.1016/j.jembe.2003.09.001

Harasti, D. (2016). Declining seahorse populations linked to loss of essential marine habitats. *Mar. Ecol. Prog. Ser.* 546, 173–181. doi: 10.3354/meps11619

Helmuth, B., Harley, C. D. G., Halpin, P. A., O'Donnell, M. P., Hofmann, G. E., and Blanchette, C. A. (2002). Climate change and latitudinal patterns of intertidal thermal stress. *Science* 298(5595), 1015–1017. doi: 10.1126/science.1076814

IPCC (2021). "Climate change 2021 the physical science basis summary for policymakers working group I contribution to the sixth assessment report of the intergovernmental panel on climate change," in *Climate change 2021: The physical science basis.*

IUCN (2020) *The IUCN red list of threatened species version 2020–1.* Available at: http://www.iucnredlist.org.

Jackson, J. B., Kirby, M. X., Berger, W. H., Bjorndal, K., Botsford, L. W., Bourque, B. J., et al. (2001). Historical overfishing and the recent collapse of coastal ecosystems. *Science* 293, 629–637. doi: 10.1126/science.1059199

Jewett, L., and Romanou, A. (2017). Ocean acidification and other ocean changes. In: *Climate Science Special Report: Fourth National Climate Assessment*, Volume I [D. J. Wuebbles, D. W. Fahey, K. A. Hibbard, D. J. Dokken, B.C. Stewart, T. K. Maycock, et al (eds.)]. U.S. Global Change Research Program, Washington, DC, USA, pp. 364–392. doi: 10.7930/J0QV3JQB

Johansen, J. L., and Jones, G. P. (2011). Increasing ocean temperature reduces the metabolic performance and swimming ability of coral reef damselfishes. *Global Change Biol.* 17 (9), 2971–2979. doi: 10.1111/j.1365-2486.2011.02436.x

Lopes, A. F., Faria, A. M., and Dupont, S. (2020). Elevated temperature, but not decreased pH, impairs reproduction in a temperate fish. *Sci. Rep.* 10 (1). doi: 10.1038/s41598-020-77906-1

Lourie, S. (2004). A Guide to the Identification of Seahorses. *Project Seahorse and TRAFFIC North America.* (Washington D.C.: University of British Columbia and World Wildlife Fund). Available at: https://projectseahorse.org/wp-content/uploads/2021/06/Seahorse_ID_Guide_2004.pdf

Lourie, S. A., Pollom, R. A., and Foster, S. J. (2016). A global revision of the seahorses hippocampus rafinesque 1810 (Actinopterygii: Syngnathiformes): taxonomy and biogeography with recommendations for further research. *Zootaxa* 4146 (1), 1–66. doi: 10.11646/zootaxa.4146.1.1

Macusi, E. D., Katikiro, R. E., Deepananda, K. A., Jimenez, L. A., Conte, A. R., and Fadli, N. (2011). Human induced degradation of coastal resources in Asia Pacific and implications on management and food security. *J. Nat. Studies* 9(10), 13–28.

Mascaró, M., Amaral-Ruiz, M., Huipe-Zamora, I., Martínez-Moreno, G., Simões, N., and Rosas, C. (2016). Thermal tolerance and phenotypic plasticity in juvenile hippocampus erectus perry 1810: Effect of acute and chronic exposure to contrasting temperatures. *J. Exp. Mar. Biol. Ecol.* 483, 112–119. doi: 10.1016/j.jembe.2016.07.005

Mascaró, M., Horta, J. L., Diaz, F., Paschke, K., Rosas, C., and Simões, N. (2019). Effect of a gradually increasing temperature on the behavioural and physiological response of juvenile hippocampus erectus: Thermal preference, tolerance, energy balance and growth. *J. Thermal Biol.* 85, 102406.

McCarthy, J. J., Canziani, O. F., Leary, N. A., Dokken, D. J., and White, K. S. (Eds.) (2001). *Climate change 2001: impacts, adaptation, and vulnerability: contribution of Working Group II to the third assessment report of the Intergovernmental Panel on Climate Change.* (Vol. 2). Cambridge: Cambridge University Press.

Myrick, C. A., and Cech, J. J. (2000). Swimming Performances of Four California Stream Fishes: Temperature Effects. *Environmental Biology of Fishes* 58(3), 289–295. doi: 10.1023/a:1007649931414

Ojanguren, A. F., and Brañta, F. (2000). Thermal dependence of swimming endurance in juvenile brown trout. *J. Fish Biol.* 56 (6), 1342–1347. doi: 10.1111/j.1095-8649.2000.tb02147.x

Palma, J., Bureau, D. P., and Andrade, J. P. (2011). Effect of different artemia enrichments and feeding protocol for rearing juvenile long snout seahorse, hippocampus guttulatus. *Aquaculture* 318 (3), 439–443. doi: 10.1016/j.aquaculture.2011.05.035

Palma, J., Stockdale, J., Correia, M., and Andrade, J. P. (2008). Growth and survival of adult long snout seahorse (Hippocampus guttulatus) using frozen diets. *Aquaculture* 278 (1), 55–59. doi: 10.1016/j.aquaculture.2008.03.019

Papoutsoglou, S. E., Mylonakis, G., Miliou, H., Karakatsouli, N. P., and Chadio, S. (2000). Effects of background color on growth performances and physiological responses of scaled carp (Cyprinus carpio l.) reared in a closed circulated system. *Aquacult. Eng.* 22 (4), 309–318. doi: 10.1016/S0144-8609(00)00056-X

Parmesan, C. (1996). Climate and species' range. *Nature* 382 (6594), 765–766. doi: 10.1038/382765a0

Pereira, R. J., Sasaki, M. C., and Burton, R. S. (2017). Adaptation to a latitudinal thermal gradient within a widespread copepod species: the contributions of genetic divergence and phenotypic plasticity. *Proc. R. Soc London Ser.B* 284, 20170236. doi: 10.1098/rspb.2017.0236

Pilakouta, N., Killen, S. S., Kristjánsson, B. K., Skúlason, S., Lindström, J., Metcalfe, N. B., et al. (2020). Multigenerational exposure to elevated temperatures leads to a reduction in standard metabolic rate in the wild. *Funct. Ecol.* 34 (6), 1205–1214. doi: 10.1111/1365-2435.13538

Planas, M., Blanco, A., Chamorro, A., Valladares, S., and Pintado, J. (2012). Temperature-induced changes of growth and survival in the early development of the seahorse hippocampus guttulatus. *J. Exp. Mar. Biol. Ecol.* 438, 154–162. doi: 10.1016/j.jembe.2012.10.003

Pollom, R. (2017). Hippocampus guttulatus. *The IUCN Red List of Threatened Species 2017: e.T41006A67617766.* doi: 10.2305/IUCN.UK.2017-3.RLTS.T41006A67617766.en

Pörtner, H. O., and Farrell, A. P. (2008). Physiology and climate change. *Science* 322 (5902), 690–692. doi: 10.1126/science.1163156

Qin, G., Johnson, C., Zhang, Y., Zhang, H., Yin, J., Miller, G., et al. (2018). Temperature-induced physiological stress and reproductive characteristics of the migratory seahorse hippocampus erectus during a thermal stress simulation. *Biol. Open* 7 (6), bio032888. doi: 10.1242/bio.032888

Rahman, M. I. (2013). Climate change: a theoretical review. *Interdiscip. Description Complex Syst.* 11 (1), 1–13. doi: 10.7906/indecs.11.1.1

Rangel, R. E., and Johnson, D. W. (2018). Metabolic responses to temperature in a sedentary reef fish, the bluebanded goby (Lythrypnus dalli, Gilbert). *J. Exp. Mar. Biol. Ecol.* 501, 83–89. doi: 10.1016/j.jembe.2018.01.011

Roessig, J. M., Woodley, C. M., Cech, J. J., and Hansen, L. J. (2004). Effects of global climate change on marine and estuarine fishes and fisheries. *Rev. Fish Biol. Fish.* 14 (2), 251–275. doi: 10.1007/s11160-004-6749-0

Sabine, C. L., Feely, R. A., Gruber, N., Key, R. M., Lee, K., Bullister, J. L., et al. (2004). The Oceanic Sink for Anthropogenic CO 2. *Science* 305 (5682), 367–371. doi: 10.1126/science.1097403

Sampaio, E., Santos, C., Rosa, I. C., Ferreira, V., Pörtner, H.-O., Duarte, C. M., et al. (2021). Impacts of hypoxic events surpass those of future ocean warming and acidification. *Nat. Ecol. Evol.* 5 (3), 311–321. doi: 10.1038/s41559-020-01370-3

Servili, A., Canario, A. V. M., Mouchel, O., and Muñoz-Cueto, J. A. (2020). Climate change impacts on fish reproduction are mediated at multiple levels of the brain-pituitary-gonad axis. *Gen. Comp. Endocrinol.* 291, 113439. doi: 10.1016/j.ygcen.2020.113439

Shahidul Islam, Md., and Tanaka, M. (2004). Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: a review and synthesis. *Mar. pollut. Bull.* 48 (7), 624–649. doi: 10.1016/j.marpolbul.2003.12.004

Simon, Y., Levavi-Sivan, B., Cahaner, A., Hulata, G., Antler, A., Rozenfeld, L., et al. (2017). A behavioural sensor for fish stress. *Aquacult. Eng.* 77, 107–111. doi: 10.1016/j.aquaeng.2017.04.001

Sopinka, N. M., Donaldson, M. R., O'Connor, C. M., Suski, C. D., and Cooke, S. J. (2016). Stress indicators in fish. In *Fish physiology* (Elsevier Inc) 35, 405–462. doi: 10.1016/B978-0-12-802728-8.00011-4

Sswat, M., Stiasny, M. H., Taucher, J., Algueró-Muñiz, M., Bach, L. T., Jutfelt, F., et al. (2018). Food web changes under ocean acidification promote herring larvae survival /631/158/2165 /631/158/2446 article. *Nat. Ecol. Evol.* 2 (5), 836–840. doi: 10.1038/s41559-018-0514-6

Tamazouzt, L., Chatain, B., and Fontaine, P. (2000). Tank wall colour and light level affect growth and survival of Eurasian perch larvae (Perca fluviatilis l.). *Aquaculture* 182 (1), 85–90. doi: 10.1016/S0044-8486(99)00244-6

Teixeira, R., and Musick, J. A. (2001). Reproduction and food habits of the lined seahorse, Hippocampus erectus (Teleostei: Syngnathidae) of Chesapeake Bay, Virginia. *Revista Brasileira De Biologia* 61 (1), 79–90. doi: 10.1590/s0034-71082001000100011

Vincent, A. C. J. (1995). A role for daily greetings in maintaining seahorse pair bonds. *Anim. Behav.* 49, 258–260. doi: 10.1016/0003-3472(95)80178-2

Vincent, A. C. J., Foster, S. J., and Koldewey, H. J. (2011). Conservation and management of seahorses and other syngnathidae. *J. Fish Biol.* 78 (6), 1681–1724. doi: 10.1111/j.1095-8649.2011.03003.x

Volkoff, H., and Rønnestad, I. (2020). Effects of temperature on feeding and digestive processes in fish. *Temperature* 7 (4), 307–320. doi: 10.1080/23328940.2020.1765950

Walther, G.-R., Post, E., Convey, P., Menzel, A., Parmesan, C., Beebee, T. J. C., et al. (2002). Ecological responses to recent climate change. *Nature* 416 (6879), 389–395. doi: 10.1038/416389a

Wong, B. B. M., and Candolin, U. (2015). Behavioral responses to changing environments. *Behavioral Ecology* 26 (3), 665–673. doi: 10.1093/beheco/aru183

Woodall, L. (2017). Hippocampus hippocampus. *The IUCN Red List of Threatened Species 2017: e.T10069A67618259*. doi: 10.2305/IUCN.UK.2017-3.RLTS.T10069A67618259.en

Woodall, L. C., Koldewey, H. J., Boehm, J. T., and Shaw, P. E. (2015). Past and present drivers of population structure in a small coastal fish, the European long snouted seahorse Hippocampus guttulatus. *Conservation Genetics* 16 (5), 1139–1153. doi: 10.1007/s10592-015-0728-y

Woodall, L. C., Otero-Ferrer, F., Correia, M., Curtis, J. M. R., Garrick-Maidment, N., Shaw, P. W., et al. (2018). A synthesis of European seahorse taxonomy, population structure, and habitat use as a basis for assessment, monitoring and conservation. *Mar. Biol.* 165 (1), 1–19. doi: 10.1007/s00227-017-3274-y

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The development of a quantification method for measuring iridescence using sexually selected traits in the Gulf pipefish (*Syngnathus scovelli*)

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Reliably quantifying the strength of visual sexual signals, such as iridescence, has been challenging across the field of evolutionary biology, but is critically important for studying biologically relevant trait variation. To address this issue, we present the Iridescence Detection and Isolation Algorithm (IDIA), which was designed to isolate the iridescent signal from photographs for quantification of ornamentation. The Gulf pipefish, *Syngnathus scovelli*, served as a model system for testing the limits of the algorithm, and was an ideal test case due to their female-specific iridescent bands on their abdomens with a large degree of among-individual variation. Specifically, we tested the repeatability of iridescence estimates in a variety of settings, including manual versus automated measurements, a gradient of lighting intensities, observational data from multiple populations, and in detecting exposure to synthetic estrogen. Using the IDIA, female iridescence was quantified in two ways with results indicating a manual measurement of each individual band may be more reliable than the automated measurement taken by drawing a polygon around all bands. However, the intensity of the lighting the photographs were taken in did not significantly affect repeatability of the measurement of iridescence no matter how it was taken. The IDIA was able to detect geographical variation in female ornamentation of *S. scovelli*, demonstrating that our automated approach can potentially replicate previously-described population-level variation. Differences in the iridescent signal were significant when comparing female pipefish from the Florida coast to females collected from the Texas coast, indicating the possibility that external factors, such as differing environmental conditions, could affect the strength of female visual signals. Lastly, the IDIA was applied in an ecotoxicology application to detect the development of iridescence in male pipefish exposed to synthetic estrogen. Exposed males began expressing banding patterns with iridescence levels within the range of females. The results from this study confirm the feasibility of using the IDIA for measuring iridescence in fish across a variety of applications.

KEYWORDS

iridescence, sexual selection, ornamentation, pipefish, secondary sexual traits, *Syngnathus scovelli*, ecotoxicology

1 Introduction

The evolution of elaborate ornamentation in sexually mature organisms that are advantageous for mating success, rather than survival (Kirkpatrick, 1982), can be explained by the theory of sexual selection (Darwin, 1871; Clutton-Brock, 2007; Clutton-Brock, 2017). Darwin distinguished primary sexual characteristics, such as the organs for reproduction, from secondary sexual characters, which include morphological differences seen between sexes that are not directly involved in reproduction (Darwin, 1871; Jones and Ratterman, 2009). Exaggerated traits can be used for either intrasexual selection, where members of the same sex evolve weaponry used in mating competition for access to the other sex, or intersexual mate choice resulting in ornamentation to attract the choosy sex (Emlen and Oring, 1977; Moore, 1990; Clutton-Brock, 2007). Secondary sexual traits, such as bold coloration or horns and tusks in males, do not seem to fit into the theory of natural selection because they are costly to produce, maintain, and in many cases, make the organisms more evident to potential predators, decreasing survivorship (West-Eberhard, 1979; Muniz and Machado, 2018; Hare and Simmons, 2019). The evolution and persistence of secondary sexually selected traits emerge from the preference of large or elaborate ornaments; therefore increasing the reproductive success of individuals bearing these traits (Fisher, 1915; Kirkpatrick et al., 1990).

The study of sexual selection has focused predominantly on male ornamentation and the corresponding female mate choice; however, these studies do not reflect the diversity of the animal kingdom and may lead to biases in research and the development of evolutionary theory (Clutton-Brock, 2009; Számadó and Penn, 2015; Hare and Simmons, 2019). The presence of female secondary sexual characteristics raises important unanswered questions about the evolutionary mechanisms influencing sexual selection acting upon female ornamentation. Female intrasexual and intersexual selection exist across a wide variety of taxa, including fishes (Dijkstra et al., 2009; Flanagan et al., 2014; Rometsch et al., 2021), insects (Rillich et al., 2009), crabs (Baldwin and Johnsen, 2012), amphibians (Pröhl and Hödl, 1999), birds (Trail, 1990; Sæther, 2002; Lipshutz, 2017), and mammals (Bebié and McElligott, 2006; Powell, 2008), often resulting in the development of elaborate female traits. The investigation of female ornamentation in sex-role reversed systems creates the opportunity to understand sexual selection pressures more fully on females, but currently repeatable tools for quantifying variation in many ornaments are lacking.

Testing the measurement of female sexual signals should ideally be conducted within a group of organisms where the mating system and sexually selected traits are well understood. One of the most apparent cases of role reversal with higher sexual selection pressures on females is seen in the family Syngnathidae. Syngnathids, including seahorses, pipefishes, pipehorses, and seadragons, provide a unique platform to examine female ornamentation and male mate choice as they exhibit male pregnancy with several species possessing a sex-role-reversed mating system (Vincent et al., 1992; Jones et al., 2001). In this family of fishes, females transfer eggs to the males' brooding structures following elaborate courtship displays, and the male then fertilizes the eggs and houses the embryos as they develop (Wilson et al., 2003; Silva et al., 2010). Although seahorses and some pipefish species display monogamous behaviors, pipefish exhibit a range of polyandrous to polygynandrous behaviors, with sexual dimorphism with more elaborate traits in females (Rosenqvist and Berglund, 2011). This role reversal results in females competing for access to mates and therefore experiencing stronger sexual selection pressures (Jones et al., 2005). Female ornamentation in pipefishes has been shown to predict mating success (Flanagan et al., 2014; Monteiro et al., 2017), reproductive success (Berglund et al., 1997), fecundity (Mobley et al., 2018), female-female competitiveness (Bernet et al., 1998), and female and offspring quality (Cunha et al., 2017).

Female signals have evolved across pipefish species, and due to the energetic costs of producing both eggs and ornamentation, are often honest indicators of a females' quality (Berglund, 2000; Sogabe and Ahnesjö, 2011; Rose et al., 2013b). Sexually selected signals in female pipefish include dimorphic bodies (longer torsos, increased body width, ventral flaps, or skin folds), displays (temporary melanistic changes, dancing vertically in the water column, body twitches, or postures), and markings on the body (stripes, spots, banding patterns, or iridescence; reviewed in Rosenqvist and Berglund, 2011) and have been quantified in a variety of ways. Developing tools to measure the various sexually selected ornaments can therefore provide information about an individual's fitness. For example, temporary female ornamentation expressed during courtship in the broadnose pipefish, *Syngnathus typhle*, has been shown to be strongly correlated with the number of eggs females have, indicating ornamentation is an honest signal of the female's fertility (Berglund et al., 1997). Mobley and colleagues (Mobley et al., 2018) measured the thickness of the stripes found on the female torso of the sex-role-reversed *Stigmatopora nigra*, the wide-bodied pipefish, and found correlations between the ornament and fecundity. Band width was also used in the sex-role-reversed species *Syngnathus abaster*, the black-striped pipefish, in addition to a measurement of luminance, to show selection for wider stripes in females and a higher overall fertility and fecundity in females with darker stripes (Cunha et al., 2017). Additionally, the number of female secondary selected bands has been shown to be a good predictor of reproductive success in the Gulf pipefish, *Syngnathus scovelli*. A component of these previously-counted bands is their iridescence. While iridescence has been quantified in several organisms (reviewed in Gruson et al., 2019), this trait has not been commonly measured in fish.

Iridescence is found across a broad distribution of taxa ranging from ctenophores to cephalopods to various insects, fish, reptiles, amphibians, birds and even the fur of some small mammals (Doucet and Meadows, 2009). Within these taxa, the iridescent ornamentation fulfills a variety of roles such as species recognition (Silberglied and Taylor, 1978), intraspecies competition (Fitzstephens and Getty, 2000; Lim and Li, 2006), or age determination (Doucet et al., 2006; Bitton and Dawson, 2008). Similar to many other types of ornamentation, iridescent signals have been found to be costly to maintain as depleted environments result in organisms with lower levels of iridescence (McGraw et al., 2002; Kemp et al., 2006; Lim and Li, 2007). Iridescence has also been found to be an important component of mate choice. In male peacocks (*Pavo cristatus*), both the brightness of the eyespots and the degree of iridescence on the train was correlated to frequency of visitation by females and male mating success (Loyau et al., 2007). Among syngnathids, multiple species possess some variation of iridescent markings, including the weedy seadragon (*Phyllopteryx taeniolatus*), the African freshwater pipefish (*Enneacampus ansorgii*), the straightnose pipefish (*Nerophis ophidion*), the worm pipefish (*Nerophis lumbriciformis*), the Gulf pipefish (*Syngnathus scovelli*), the blue-stripe pipefish (*Doryrhamphus excisus*), and the sargassum pipefish (*Syngnathus pelagicus*). Measuring this observed iridescence has varied across different researchers, with iridescence being quantified as the area of the blue sexual markings (Berglund et al., 1986; Flanagan et al., 2014), in terms of presence/absence of iridescence (Partridge et al., 2010), and as a rating on a scale (Sundin et al., 2016). To collect data that can be compared across studies, and across species, a reliable and repeatable method for measuring iridescent signals in pipefishes is needed.

Syngnathus scovelli is commonly used as a model system for the understanding of sexual selection pressures on females due to their sexual dimorphism playing an important role in polyandrous mate choice decisions (Jones et al., 2001; Rose et al., 2013a; Partridge et al., 2013; Flanagan et al., 2014; Anderson and Jones, 2019). At sexual maturity, females of this species possess an intricate array of ornamentation along their abdomens of multiple components. The permanent light-colored bands located on the middle of the female's torso are estrogen-responsive (Partridge et al., 2010; Anderson et al., 2020), and are overlaid by a bluish iridescent signal covering part or all of the band. Additionally, during courtship and other social behaviors, female pipefish develop alternating light-dark bars along the top of their abdomen and proceeding down the length of the tail. This melanistic change usually corresponds with the S-shaped display of *S. scovelli* courtship. Previous studies have shown that females are selected for by the number and area of these permanent light-colored bands, with females that possess either many intermediately sized bands, or a small number of large bands experiencing the greatest mating success (Flanagan et al., 2014), but the iridescent signal of these bands has not been investigated.

To fully understand the mechanisms involved with the evolution of secondary sexual traits, reliable measurements of elaborate ornaments are necessary to determine the relationship between a selected trait and an organism's fitness. Identifying the strength of these visual sexual signals reliably and without human bias has been challenging across the field, and little is known about the extent of natural variation in many of these signals (Rosenqvist and Berglund, 2011). In this study, the Iridescence Detection and Isolation Algorithm (IDIA) was developed and tested as a quantification method for iridescent signals. This program was used on many individual Gulf pipefish to investigate the effect of lighting intensity on the strength of the iridescent signal, detect geographical variation in the female ornamentation, and identify morphological changes in male *S. scovelli* as a result of estrogen exposure.

2 Materials and methods

2.1 Ethics statement

Syngnathus scovelli used to test the effects of lighting intensity in this study were collected in Tampa Bay, under special activities license # SAL-18-0182-E granted by the State of Florida in June 2019. All animal use protocols were approved under The University of Tampa AUP #2018-1. Photos of animals used in the geographic variation portion of this study were new analyses conducted using data from Flanagan et al. (2016). For the study investigating the role of synthetic estrogen on band development and iridescence in male pipefish, animals were collected from Redfish Bay near Aransas Pass, Texas (N 27 53 39.07, W 97 7 51.69) in July 2013 under the Texas Parks and Wildlife permit number SPR-0808-307 with protocols approved by the Institutional Animal Care and Use Committee at Texas A&M University and outlined in AUP # 2013–0020 (Reference #001898).

2.2 The development and use of the iridescence detection and isolation algorithm

2.2.1 The program-specifics of the IDIA

The Iridescence Detection and Isolation Algorithm (IDIA) was designed as a macro for MS Access Database to isolate the iridescent signal into a false-color image from photographs. The false color image then allows for automation of the process of quantifying iridescent ornamentation based on the spectral characteristics of each pixel rather than solely human identification of the signal. To generate the equation for the Iridescence Detection and Isolation Algorithm (IDIA), we first explored parameters that could report iridescence from the brightness and color patterns from images of known iridescence. Images of pipefish with iridescent signals were linked to a Microsoft Access database and were visualized within the program using package modules mIntelJPEGLibrary.bas and cDIBSection.cls (from VB Accelerator) to convert images from JPEG to Device-Independent Bitmaps (DIB file formats). DIB formats store data as a color table by mapping RGB values to individual pixels in the image, while keeping original image size and pixel scaling consistent across digital platforms. Converted images were displayed and used to develop the algorithm and were initially visually assessed for specific pixels in the image that represented iridescence, in this case, on the torso of pipefish females. The corresponding RGB values from pixels we identified as iridescent were statistically analyzed for their parameters to create the optimal values. Equations (see below) were developed to consistently identify the pixels throughout the entire images that met the combination of criteria of known iridescent pixels. After this initial empirical development step, where trial and error were used to determine the visual fit to images and the appropriate thresholds (visually analyzing pixel by pixel by eye), coding in MSAccess used the equations described below to assign red to each pixel in the entire image where pixels with the spectral characteristics of iridescence was detected. Developing the correct placement of red signal replacing iridescence for quantification was not a completely automated process because the user was able to optimize the parameters for their specific image and iridescence or colors of the trait under review. Reflections and inconsistencies in images due to variation in lighting conditions caused some non-specific shading and false iridescent signals on the fish, therefore we generated multiple photos with the false coloration over the iridescent signals with different cutoff thresholds to better fit the iridescence profile for each individual image. The overall goal of defining the parameters was to be able to replace pixels that met the correct criteria with a false signal that could be quantified, replacing the need to make decisions on a pixel by pixel bases for images.

Optimization of the parameters that could best identify pixels with iridescence was empirically derived from images of pipefish with known iridescence across a range of color parameters. Pixels were assessed in ImageJ for their red, green, and blue hexadecimal channel intensity using a scale of 0 – 255 color units, where 0 represented an absence of that color and 255 indicated a maximum brightness, a parameter which is defined as x_{ijk}, where i refers to the color (R for red, G for green, or B for blue) and j and k refer to the x and y coordinates of the pixel in the image, respectively. A minority of pixels were too dark to measure so were excluded from the analysis. A minimum value of brightness for each color (x_{c_i}) was determined using pixels with known iridescence and pixel lacking iridescence, which allowed us to identify the optimal iridescence values and optimal ratios of red to green, green to blue, and red to blue for the study species. This process of selecting pixels corresponding to blue hue iridescence in the bands on Gulf pipefish allowed us to develop the parameters for the equation supporting the IDIA program that could highlight all pixels within that range, removing human biases in the downstream process of iridescent selection for quantification. The particular criterions for these parameters listed below can be optimized for other iridescent traits on different species in the future.

Four main parameters were combined to generate the IDIA equation and get an overall iridescence score. The four parameters were chosen because they were identified as the most variable components in the iridescent pipefish bands, which are brightness and saturation of the blue hue. The parameters used were suggested by Gruson and colleagues (Gruson et al., 2019) as the best parameters for isolating these components of the blue hue. The first parameter addresses the difference between the color scores of the pixels (x_{ijk}) and a determined minimum brightness value (x_{c_i}) to detect the potential for iridescence and ensure pixels are not too dark (Equation 1). The optimal values for *S. scovelli* were set at $x_{c_R} > 80$, $x_{c_G} > 90$, and $x_{c_B} > 60$.

$$SSdark_{jk} = \sum_i \begin{cases} (x_{ijk} - x_{c_i})^2, & where\ x_{ijk} < x_{c_i} \\ 0, & where\ x_{ijk} \geq x_{c_i} \end{cases} \tag{1}$$

Ratios of the color hues were then used to identify the specific color patterns for Gulf pipefish bands compared to background color of the fish's abdomen as the second parameter (Equation 2). The values 0.9, 1.3, and 1.4 were used for the red:green, red:blue, and green:blue ratios respectively to distinguish iridescence for *S. scovelli*, as described in the previous paragraph.

$$SSratio_{jk} = 2\left[(x_{Rjk} - 0.9x_{Gjk})^2 + (x_{Gjk} - 1.4x_{Bjk})^2 + (x_{Rjk} - 1.3x_{Bjk})^2\right] \tag{2}$$

The third parameter measures the difference between green and blue channels (Equation 3), with a value of 40 being selected out of 0-255 range for the *S. scovelli* bands.

$$SSdiff_{jk} = 2(x_{Bjk} + 40 - x_{Gjk})^2 \tag{3}$$

The fourth parameter adds an additional verification of iridescence based on the total size of the image. Here, we measure the value of blue intensity (x_{Bjk}) for each pixel relative to the minimum and maximum values for the blue intensity in the surrounding pixels (Equation 4). The surrounding pixels that are incorporated into the calculation of the local range is determined by the photo size (# of pixels). For a 5 MP photo, a window of 3 pixels away in each direction was used, resulting in a 7x7 grid used to calculate the local range of blue intensity for the included pixels. The number of pixels in the image and size of this pixel window (n) is determined by calculating $7 \times \sqrt{\frac{All\ pixels}{5000000}}$ and rounding its product down to the nearest odd number. This value is then used to establish how many pixels in each direction surrounding the pixel of interest should be included in the local range (m) by calculating $m = \frac{(n-1)}{2}$. With this local range now defined, the local minimum and maximum blue values ($Bmin_{jk}$ and $Bmax_{jk}$) are used to define the contrast in iridescence between the focal pixel and its local range (Equation 4).

$$Bmax_{jk} = max\Big|_{p=j-m}^{j+m}\left(max\Big|_{q=k-m}^{k+m}(x_{Bpq})\right)$$

$$Bmin_{jk} = min\Big|_{p=j-m}^{j+m}\left(min\Big|_{q=k-m}^{k+m}(x_{Bpq})\right)$$

$$SScontrast_{jk} = -(Bmax_{jk} - Bmin_{jk})^2 + 2(Bmax_{jk} - x_{Bjk})^2 - (x_{Bjk} - Bmin_{jk})^2 \tag{4}$$

The size of this pixel window scales inversely with pixel dimension, as a higher resolution leads to a larger window. The pixel blue band value (x_{Bjk}) for Equation 4 should be as close as possible to the high end (and far from the low) of the local range of the blue band, and the local range should be as wide as possible to assure the pixel is within a cluster of iridescent and not a false signal.

The IDIA equation creates a final value for each pixel to be used for replacing iridescence with a false-color signal that can be quantified in ImageJ. To calculate the IDIA, a series of squared residual functions for each parameter (Equation 1-4) are calculated for each pixel in the selected area of images were then added together to get an iridescence score (targeting the 0-255 range; Equation 5).

$$IDIA_{jk} = \min\left(255,\ \sqrt{\max\left(0,\ SSdark_{jk} + SSratio_{jk} + SSdiff_{jk} + SScontrast_{jk} + 100^2\right)}\right) \tag{5}$$

The final residuals penalization function is adjusted up by 10000, cut off below 0, and square root is taken, to get an IDIA score in the 0-255 range. Five images are created in MSAccess by red replacing the color for any pixel with an IDIA value of 140,150,160,170, and 180, which allows the user to set the sensitivity of the program by determining which IDIA value best represents the iridescence for each particular image to account for any variation in background signals. Once the user selects the best image, the corresponding IDIA value is used for quantifying the number of pixels that meet that threshold in a given area of the photo that the user selects in ImageJ.

2.2.2 The quantification of iridescence using the IDIA

All pipefish used in the study were photographed to include the entirety of the iridescent bands located on their torsos in a single image, with a scale included in every image. Olympus cameras used for photographs that ranged in model, but the photos taken fell into two categories, 1) 3968 X 2976 pixels (1.2 to 2.3MB), and 2) 4608 X 3456 pixels (5.85 to 6.12MB).

For quantification analysis of the iridescence area on the body, pixel-scale data containing the RGB values were extracted from photographs using ImageJ 1.8.0 for the location set by the user for measurement (Schneider et al., 2012). In ImageJ, the area of the torso that includes the colored bands was selected using the polygon tool, and the x/y coordinate system was used to identify specific pixel locations to analyze their spectral characteristics (using Analyze→Tools→Set XY Coordinates). Using the scale in ImageJ, the number of pixels per millimeter were measured and entered into the IDIA MSAccess macro in addition to the IDIA threshold previously selected from the 140-180 range by the user. The 140-180 threshold was chosen because those values produced the most reliable and repeatable identification of the iridescence found in the bands by the IDIA program, rather than background reflectance that could falsely identify non-iridescence in the photos. For example, since we are imaging fish that have a reflective surface, we aimed to identify just the iridescence in the given area that is associated with the bands, which was verified by human eye (similar to the butterfly example in Gruson et al., 2019). The final calculation of iridescence is generated by MSAccess by summing the total number of pixels within the area of the torso box that meet the IDIA threshold and converting the pixels to area using the scale in each image to provide the final measurement of the iridescent area in squared millimeters. In *S. scovelli*, iridescence is predominantly distributed on the torso, beginning just behind the operculum and usually extending to the urogenital opening (Figure 1B; although in some females, it extends onto the tail, Figures 1C, 2).

2.2.3 Approaches to measurement of iridescence methodology

This study used two approaches to quantify torso iridescence, 1) measuring all iridescence from the torso of pipefish and 2) isolating the signal from the bands themselves. This distinction allows us to determine the area of iridescence from the sexually-selected bands themselves relative to the overall iridescence shown in the torso area, which might be responding to different selective pressures, such as pre- and post-copulatory sexual selection, environmental conditions, or ecological competition. After the images were processed using the IDIA, iridescence was quantified and reported in two ways, overall iridescence, and mean band iridescence (Figure 1).

Overall iridescence was measured by outlining the focal area of iridescence on the pipefish using the polygon tool in ImageJ (Figure 1E), using a series of selected landmark locations around the area of interest on the torso of the pipefish, including the banding pattern. The selected x/y coordinates and pixel RGB values from the polygon area were used in the IDIA algorithm to sum up the pixels that contain the iridescent signal within the designated polygon, converting it to area using the metric scale in the image (mm^2). The use of this tool allows for all of the iridescence to be measured on the fish without human decisions, therefore removing user bias associated with outlining the banding patterns. Overall iridescence measurements used in the analyses were not adjusted for by the number of bands.

To measure the contribution of the individual bands to the overall iridescence on the torso of the fish, the band iridescence method was calculated by replicating methods used in Flanagan et al. (2014). However, instead of measuring the physical area of the band *via* the permanent coloration located on the female torsos, the iridescent signal identified by the red from the IDIA false-color image was measured instead. The false-color images were exported to ImageJ where the scale was set, and the red coloration signified as iridescence for each individual band was outlined by hand (Figure 1D). With the outlines, area of iridescence (mm^2) was

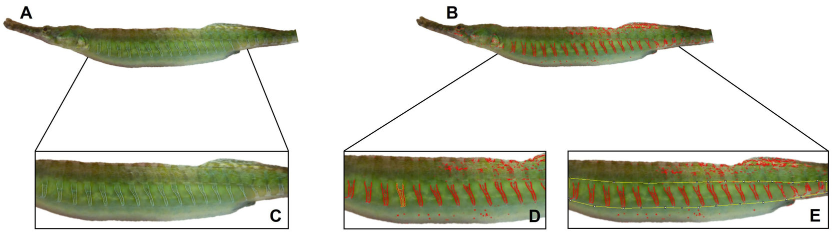

FIGURE 1
(A) Photos of the iridescent bands found on female *S. scovelli* and **(B)** the corresponding iridescent signal that is highlighted by the Iridescence Detection and Isolation Algorithm (IDIA). **(C)** represents the area of the torso that possesses the iridescent bands while **(D, E)** show the two ways of quantifying iridescence though using the IDIA and ImageJ (mean band iridescence and overall iridescence respectively).

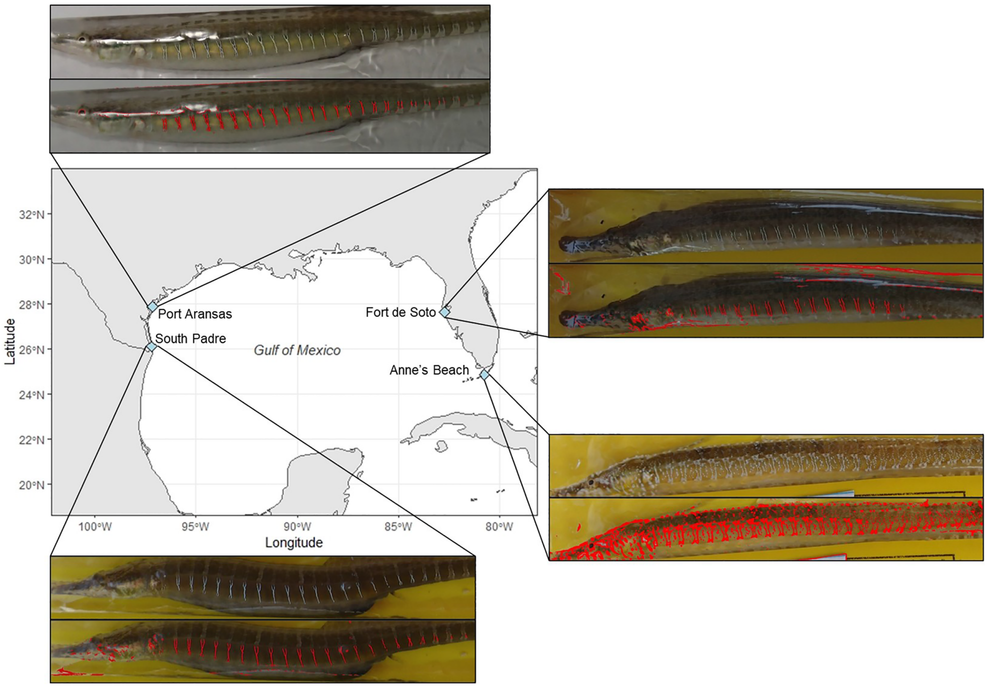

FIGURE 2
A map of the four geographic sites chosen to analyze for this study from Flanagan et al. (2016). For each location, there is a photo of a female *S. scovelli* collected at that site (top) paired with that same photograph after being processed through the Iridescence Detection and Isolation Algorithm (IDIA) (bottom). The red colouration in these photographs represents areas distinguished as iridescent by the IDIA. Due to limitations in the field all photos were taken in various lighting conditions. However, we conducted experiments under varied lighting conditions and found that iridescence does not significantly differ in response to lighting (Figure 3).

generated for every band. For each pipefish, the individual band area measurements were summed and divided by the total number of iridescent bands resulting in a mean band iridescence.

2.3 Morphological analyses

Each pipefish whose image was measured for iridescence was also measured for length and dorso-ventral body depth. The length was measured from the tip of the snout to the end of the tail, and depth was measured from the point in front of the dorsal fin down the bottom of the torso. All statistical analyses were conducted in R Studio version 1.2.5 using packages MASS (Venables and Ripley, 2002) and car (Fox and Weisberg, 2019) for the generalized linear models.

2.4 Proof of concept: Testing the reliability and repeatability of the iridescence detection and isolation algorithm

Previous studies have indicated that color perception may be affected by additional components, including the background pattern and color of the organism, color sensitivity of the receiver, or environmental factors, such as ambient light (Gomez and Théry, 2007; Loyau et al., 2007). Because of this, the purpose of this initial study was to test the reliability of the IDIA and determine the effect of varying light intensities on the strength of the iridescent signal found on *S. scovelli*. Twenty-four sexually mature female *S. scovelli* were collected in seagrass beds located in Tampa Bay using a push net in June 2019 and transported to The University of Tampa in Tampa, FL. Pipefish were photographed in 2.5-gallon tanks filled with seawater made to 28 ppt using Instant Ocean® Sea Salt (http://www.instantocean.com) across three different lighting conditions previously used in other pipefish studies (Masonjones et al., 2010; Flanagan et al., 2014; Flanagan et al., 2016), including 9, 13, and 21 lumens using an Olympus ToughTG-5 digital camera placed 8.4 cm in front of the tank. White, waterproof paper was added to the back and bottom of all tanks to keep the background coloration constant and a PAR sensor was used to indicate the intensity of the light. A white photo box was utilized for the two lower range lighting levels (9 and 13 lumens) with an LED light bar placed on the top, and all other light sources were eliminated, and the highest lighting level was set-up outside of the photo box in regular fluorescent lighting (21 lumens). At each lighting level, three photos were taken of the individual pipefish to account for any inherent variation in the photo-taking process and test the repeatability of the program, resulting in nine photographs per pipefish. The entire photographing process took less than five minutes.

For all images, both band and overall iridescence were measured. To remove any biases, a double-blind procedure was implemented when the photos were read into the IDIA. The first researcher sorted images into their own folders and created a key that linked the photo ID to the specific pipefish and lighting condition. These folders were then given to a second researcher who used a random number generator to assign each folder a new number. These new numbers were added to the original key and given back to the first researcher. The randomized photos were used when collecting iridescence measurements and at the end, the measurements were matched to the original pipefish ID and lighting condition using the key. As there was no interest in the interaction between the different lighting conditions and the two measurement methods, mean band and overall iridescence were analyzed separately. Overall and mean band iridescence expressed normality and equal variances, therefore, one-way ANOVAs between the two iridescent measurements and lighting conditions were performed.

2.5 An evolutionary application of the IDIA: Quantifying geographical variation in the iridescent signal

Iridescence was quantified for female pipefish from four geographic locations that were previously collected for population genomics analyses (Flanagan et al., 2016). Females were selected from two Texas populations (South Padre and Port Aransas) and two Florida populations (Fort De Soto and Anne's Beach) and the IDIA was used to detect potential variation in iridescence (Figure 2). In addition to the mean band and overall iridescence measurement, the iridescence on the torso that was not on the banding patterns was calculated. Torso iridescence was calculated by subtracting the mean band iridescence from the overall iridescence.

Seventeen pipefish were randomly selected from each location to create equal sample sizes for the analysis. An initial analysis into the morphometrics of the female *S. scovelli* indicated body length and depth significantly varied across the four geographic locations (One-way ANOVA: $F_{3,64}$ = 27.29, $p< 0.0001$; One-way ANOVA: $F_{3,64}$ = 48.43, $p< 0.0001$ respectively). Overall, the Florida pipefish were longer and deeper than the Texas populations. Additionally, the females from the Port Aransas TX population expressed significantly greater length and depth than the South Padre location (Figure S2A, One-Way ANOVA: $F_{3,\ 64}$ = 11.79, $p< 0.0001$). Due to the strong relationship between length and depth (Pearson's correlation: r = 0.87, df = 66, $p< 0.0001$), only one was incorporated into our models. The iridescent ornament is largely located on the torso of the females and stops before or just after the urogenital pore; therefore the depth of the female is more likely to affect the amount of iridescence present. All iridescence measurements were divided by body depth to adjust for depth variation prior to the analysis of band and overall iridescence. The depth-adjusted overall iridescence expressed normality and equal variances therefore a one-way ANOVA between overall iridescence and location was performed. Mean band and torso iridescence residuals were not normally distributed and log-transforming the data did not achieve normality. These two iridescence measurements were analyzed separately using a GLM with an underlying quasi-Poisson distribution comparing the iridescence measurements across locations.

The relationship between the permanent band area, measured by Flanagan et al. (2016), and the iridescent signal of the band, measured using the IDIA in this study, was analyzed. Additionally, we tested whether this relationship was consistent across three of the different populations of *S. scovelli*. Mean band area measurements were obtained from Flanagan et al. (2016) for the South Padre, Texas population and both Florida locations (Anne's Beach and Fort De Soto) to compare against corresponding mean band iridescence calculations. Seventeen pipefish were randomly selected for each of the three locations from the original dataset to obtain equal sample sizes for the analysis. Pearson correlation coefficients were first determined between mean band area and mean band iridescence independent of location. In addition, three subsets were created for each of the individual populations and Pearson correlation coefficients were found between mean band area and mean band iridescence for each location.

2.6 An ecological application of the IDIA: Detection of iridescence as a biological indicator

Non-pregnant male *S. scovelli* were collected from Redfish Bay near Aransas Pass, Texas (N 27 53 39.07, W 97 7 51.69) in July 2013 under the Texas Parks and Wildlife permit number SPR-0808-307. Fish were housed for 18 days and exposed to 5-10 ng/l 17α-ethinylestradiol (EE2) (n=8) using a solution of EE2 dissolved in ethanol described in Rose et al. (2015), or non-exposed in EE2-free saltwater with trace amounts of ethanol for the control (n=5). The males used in the control and experimental groups were sized matched, with no significant difference in length or depth, to ensure size was not impacting any reaction to the EE2. Water changes were conducted every other day replacing 10% of the water to maintain the EE2 levels, following protocols approved by the Institutional Animal Care and Use Committee at Texas A&M University and outlined in AUP # 2013–0020 (Reference #001898). The polygon tool approach was used to measure the overall iridescence on the first and last day of the study to detect morphological changes resulting from environmental contaminants. The overall iridescence value from Day 1 was subtracted from the final overall iridescence measurement to calculate an overall change in iridescence for males in both the estrogen-exposed and unexposed treatments. Overall iridescence measurements from the day 18 photographs of male *S. scovelli* exposed to estrogen were also compared to the overall iridescence measurements from unexposed female *S. scovelli* collected from the same location in Port Aransas, Texas. Due to a violation of normality, a Wilcoxon rank sum test was used to compare the difference in the iridescent signal expressed between the unexposed and exposed males as well as exposed males and unexposed females.

3 Results

3.1 Proof of concept: Testing the reliability and repeatability of the iridescence detection and isolation algorithm

Twenty-four pipefish were originally caught and photographed; however, three presented too dark of a background coloration on the torso for the IDIA to confidently detect any iridescence and were thus removed from the analysis. Mean band and overall iridescence did not vary between the different lighting conditions (9 lumens = 0.099 ± 0.0033, 13 lumens = 0.099 ± 0.0028, 21 lumens = 0.107 ± 0.0022; One-way ANOVA: $F_{2,60} = 2.29$, p= 0.109); however, the highest level of lighting (21 lumens) was shown to detect greater overall iridescence compared to the others, but was not statistically significant (9 lumens = 3.93 ± 0.297, 13 lumens = 4.00 ± 0.314, 21 lumens = 4.89 ± 0.312; One-way ANOVA: $F_{2,60} = 3.01$, p= 0.057) (Figure 3). Further *post hoc* analysis of the overall iridescence showed that none of the pairwise comparisons were significantly different (Tukey HSD all p> 0.05). Variation of overall iridescence was highlighted through looking at the three iridescent measurements per lighting condition for each individual pipefish (Figure S1). Within each fish there was inherent variation of the signal as the boxplots representing the different lighting conditions were not always in line, with some cases more extreme than others (Figure S1B).

3.2 An evolutionary application of the IDIA: Quantifying geographical variation in the iridescent signal

Using the IDIA, both overall iridescence (One-way ANOVA: $F_{3,64} = 10.43$, p< 0.0001) and torso iridescence (GLM: $\chi^2_3 = 44.64$, p< 0.0001) were found to be significantly different across the four geographic locations, however, mean band iridescence was not significantly different (GLM: $\chi^2_3 = 7.61$, p = 0.055) (Table 1; Figure 4A). For both the overall iridescence and torso iridescence, pipefish from Anne's Beach, Florida expressed the highest levels of iridescence (Figures 4B, C respectively). *Post hoc* analysis *via* Tukey's HSD test (Table S1) showed the overall iridescence on pipefish from Anne's Beach was not significantly higher than the population from South Padre, Texas, but both populations had significantly higher overall iridescence than the other Florida population from Fort De Soto. The pipefish collected from Port Aransas, Texas showed no significant difference in overall iridescence when compared to any of the other populations (Table 1; Figure 4B). Torso iridescence did differ among populations, with the Anne's Beach population having the highest expression levels compared to the other populations with overwhelmingly positive estimates from the GLMM (2.07 ± 0.096) while iridescence from the other populations had estimates that were substantially negative (FL Fort de Soto: -0.98 ± 0.18, TX South padre: -0.87 ± 0.18, TX Port Aransas: -0.54 ± 0.16). This confirmed initial visual observations made from photographs of pipefish (Figure 2).

Independent of location, a weak correlation was found between mean permanent band area from Flanagan et al. (2014) and mean band iridescence measured using the IDIA, with the general positive trend of band area increasing as the iridescence increased (Pearson's correlation: r = 0.37, df = 49, p = 0.0068). When the three populations were analyzed separately, the strongest relationships between mean band area and mean band iridescence were found in the South Padre, Texas population (Pearson's correlation: r = 0.42, df = 15, p = 0.095) and the Fort de Soto, Florida population (Pearson's correlation: r = 0.40, df = 15, p = 0.12), but neither of the relationships were statistically significant (Figure 5). Female *S. scovelli* from Anne's Beach, Florida demonstrated the weakest relationship between the mean band area and mean band iridescence (Pearson's correlation: r = 0.31, df = 15, p = 0.23) (Figure 5).

3.3 An ecological application of the IDIA: Detection of iridescence as a biological indicator

The change in iridescence after an eighteen-day period was significantly higher for male pipefish exposed to synthetic estrogen compared to the control (Wilcoxon rank sum test: W=1, P= 0.0031)

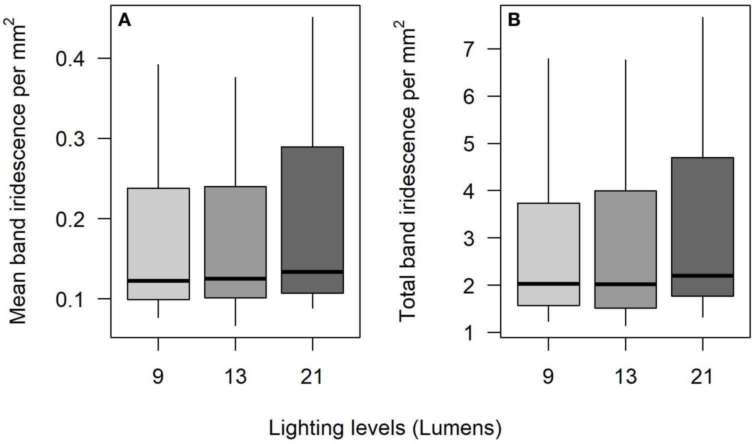

FIGURE 3
(A) Mean band iridescence does not significantly differ across the different lighting conditions (n=21), however, the **(B)** overall iridescence is nearly significant, likely due to the slightly higher levels measured at 21 lumens.

TABLE 1 Summary of morphometrics (mean ± S.E.) including body depth, number of bands, mean band iridescence, overall iridescence, and torso iridescence for female *S. scovelli* across the four geographic locations and results of statistical analyses conducted.

	Anne's Beach, FL	Fort De Soto, FL	Port Aransas, TX	South Padre, TX	Df	Statistical Value	P-value
Depth (mm)	7.13 ± 0.25	7.14 ± 0.28	5.69 ± 0.15	3.99 ± 0.16	3, 64	F = 48.43^{a}	<0.0001
Number of Bands	15.88 ± 0.44	15.29 ± 0.29	17.65 ± 0.58	18.94 ± 0.57	3, 64	F = 11.79^{a}	<0.0001
Mean Band Iridescence (mm^2)	0.0350 ± 0.0053	0.0334 ± 0.0039	0.0301 ± 0.0028	0.0441 ± 0.0026	1, 3	χ^2 = 7.61^{b}	0.055
Overall Iridescence (mm^2)	1.67 ± 0.15	0.898 ± 0.083	1.35 ± 0.10	1.66 ± 0.10	3, 64	F = 10.39^{a}	<0.0001
Torso Iridescence (mm^2)	7.95 ± 0.93	2.96 ± 0.55	4.63 ± 0.44	3.33 ± 0.38	1, 3	χ^2 = 44.64^{b}	<0.0001

^{a}= Analyzed using a One-Way ANOVA
^{b}= Analyzed using a GLM with underlying quasi-poisson distribution
Results considered significant at p<0.05.

(Figure 6A). The larger change in iridescence observed for *S. scovelli* exposed to estrogen was due to the high values of iridescence detected in exposed males on day eighteen compared with their initial measurements on day one (Figures 6B, C), while the unexposed males did not develop iridescence. Although the overall iridescence detected on exposed males is significantly lower than the overall iridescence reported for females collected in the same Port Aransas location (Wilcoxon rank sum test: W = 59, p = 0.0030), many of the iridescence values for exposed males (0.82-6.0) fall within the range of iridescence levels (2.3-13.0) of wild caught, non-exposed females.

4 Discussion

This study highlighted the effectiveness of the Iridescence Detection and Isolation Algorithm (IDIA) as a method to quantify iridescent signaling. After testing the validity of the program, IDIA was used to highlight geographic variation in the expression of the iridescent signal and to detect the development of iridescence resulting from exposure to contaminants. The use of the program successfully across several applications indicates the potential for this program to be evaluated in other systems. While *Syngnathus scovelli* served as a model system for testing the algorithm in the current study, the IDIA can be adapted to measure iridescence in a variety of taxa by optimizing the ratios of blue, red, and green that the IDIA distinguishes as iridescent for additional species.

4.1 Proof of concept: Testing the reliability and repeatability of the iridescence detection and isolation algorithm

To address the reliability of the IDIA individual pipefish were photographed at three different lighting levels and both the overall and mean band iridescence was measured. The different light intensities did not significantly affect the amount of the mean band iridescence that was recognized by the IDIA; however, when calculating overall iridescence, the results were nearly significant. Although using the polygon tool eliminates human biases, the program will acknowledge any iridescence not located on the bands within the area of interest, regardless of if it is true iridescence located on the pipefish or slight imperfections of the photo. This increases the sensitivity of the polygon tool and may be the leading cause of the nearly significant values for overall iridescence measurements. Therefore, users of the IDIA should determine the amount of background noise that could possibly be detected by the polygon tool before choosing between using the mean versus overall measurement methods. For example, in the current study, the photographs were taken through a glass aquarium where glare was detected in some of the images, resulting in false iridescence. Any photographs that contained a glare overlaying a significant portion of the torso were eliminated from the study as accurate measurements could not be taken.

The repeatability of the IDIA was additionally tested to ensure the program is a reliable resource. At each level of lighting three photos were taken of the same pipefish. The nine photos were then analyzed for measurements of iridescence for each individual pipefish. To investigate the overall effect of lighting on iridescence, the iridescence measurements for the three photos were averaged, however, when looking at them individually it helps to highlight the strength of repeatability for the IDIA (Figure S1). We detected variation in the iridescent measurements within each pipefish, but the magnitude of that variation differs considerably across the pipefish. Aside from a few cases, the degree of variation in the iridescent measurements was minimal, indicating the proximity of the three measurements and thus the repeatability of the IDIA (Figure S1). Additionally, the variation between the lighting conditions is highlighted with the majority of the three boxplots for each individual clustering together emphasizing through visual analysis the reliability of the program. The fish that show the highest magnitudes of variation have a light-colored background. It is possible that it is not the iridescence changing at the different lighting conditions but the background of the fish that is altering slightly therefore indirectly affecting the iridescence measurement that is calculated.

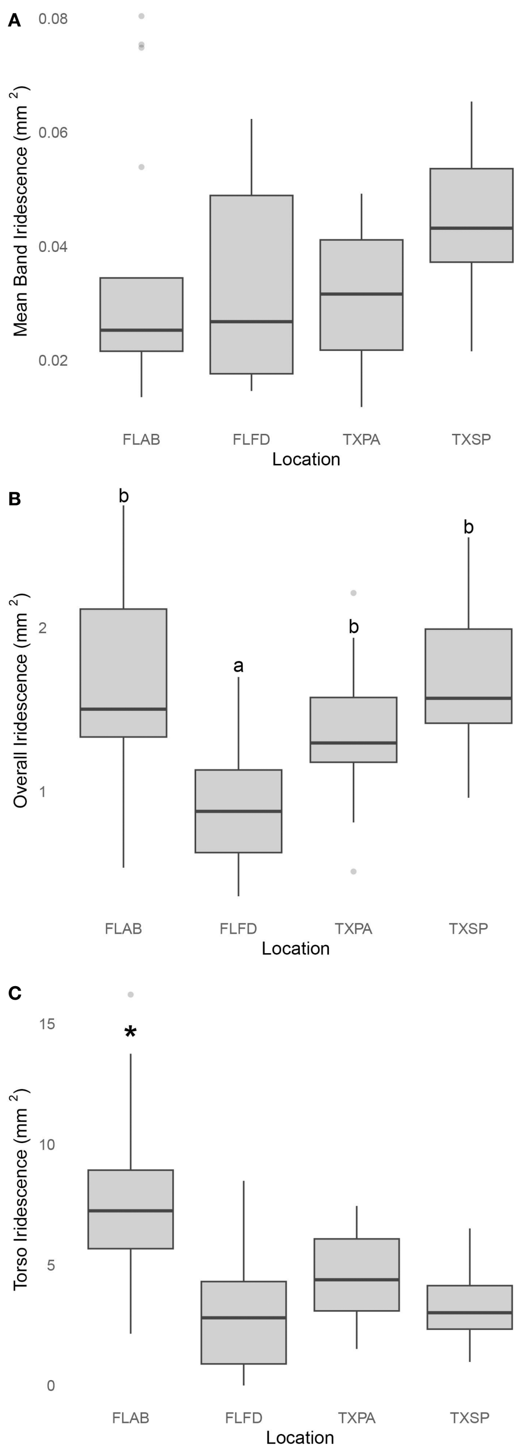

FIGURE 4
The variation in **(A)** depth-adjusted mean band iridescence, **(B)** depth-adjusted overall iridescence, and **(C)** torso iridescence across four distinct populations of female *S. scovelli*. These populations include two from Florida [Anne's Beach (FLAB) and Fort DeSoto (FLFD)] and two from the coast of Texas [Port Aransas (TXPA) and South Padre (TXSP)]. Mean band iridescence was not significant across the geographic locations and both overall iridescence and torso iridescence differed significantly between the different populations. Letters denote results from Tukey HSD *post-hoc* analysis; different letters represent significantly different populations ($P<0.05$). The asterisk above FLAB in **(C)** denotes the overwhelmingly positive estimates from the GLMM compared to the substantially negative estimates corresponding to the other three populations.

The IDIA was shown to be a reliable way to confidently assess iridescence allowing for it to be used in more applied methods. In terms of the different ways in which iridescence was quantified, physically tracing the iridescence showed more consistency than using the polygon tool in ImageJ to draw around the area of interest (Figures 3, S1). However, the different methods measure different aspects of the ornamentation that may be biologically significant and therefore both must be kept in mind for further analyses. When outside of a laboratory setting, ensuring fully stable conditions is near impossible. To increase confidence and consistency of iridescent measurements it is recommended that all photographs are taken in consistent conditions when possible, to prevent issues such as reflection of the camera or the sheen on containers, whether it be a glass container or plastic bags containing water. Additionally, images of lower resolution, or taken in considerably lower light conditions, will not work with the algorithm because of increased pixelation of the bands themselves, as the ornaments of interest are so narrow. If the IDIA is to be applied for analyses using photos with a wider range of lighting or pixel sizes a reoptimization of the program would be required.

4.2 An evolutionary application of the IDIA: Quantifying geographical variation in the iridescent signal

After verifying the capability of the IDIA, it was successfully used to measure variation in band, overall, and torso iridescence in female *S. scovelli* across several geographic locations. Investigating the mean band iridescence, overall iridescence, and torso iridescence of female pipefish from the four geographic locations highlighted differences in the way the iridescent signal may be used within each population. These differences were notably emphasized through the overall iridescence and torso iridescence as the mean band iridescence did not vary drastically across the distinct populations. For both the overall and torso iridescence the highest levels were found in the Anne's Beach populations, as expected from initial observations of the photos (Figure 2). The high overall iridescence found in South Padre pipefish was initially puzzling as they do not contain the significant background iridescence found of Anne's Beach pipefish. However, this can be explained by the fact that they have significantly more bands (Figure S2B), which in turn increases the overall iridescence values.

Both the iridescent signal detected through the IDIA and the physical area of the permanent bands contribute to the representation of the ornament on female *S. scovelli*. To further understand the role that ornamentation plays, both individual components and any interactions between them must be considered. Prior to measurements, it was observed that some of the bands located on the female pipefish were either not distinguished as iridescent through the IDIA or were found to be only partially iridescent. Therefore, just because the physical bands are present does not mean that just as much iridescence will be present. This was supported through investigating the strength of the relationship between mean band area and mean band iridescence. Overall, there is a positive trend, as the band area

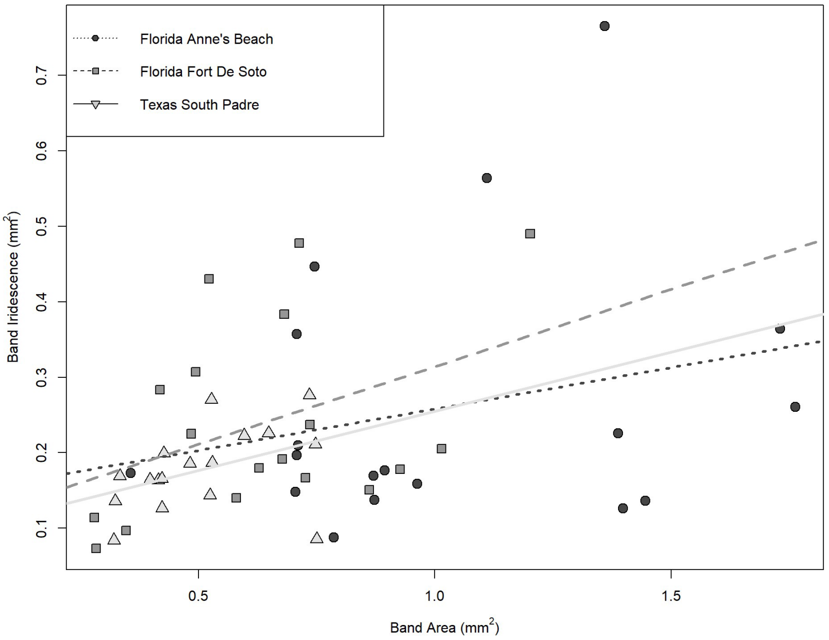

FIGURE 5
The relationship between mean band area and mean band iridescence for the two Florida populations (Anne's Beach and Fort De Soto) and the South Padre, Texas population. The strongest relationships between band area and iridescence were found for South Padre and Fort de Soto, while female pipefish from Anne's Beach showed the weakest correlation. Overall, none of the relationships were statistically significant (p>0.05).

increases the iridescence also increases, however, there is a weak, nonsignificant correlation. Geographically, the strongest correlations were seen in pipefish from South Padre, Texas and Fort De Soto, Florida. Both populations possess females with iridescence primarily on the bands. Female pipefish from Anne's Beach, Florida demonstrated the weakest relationship between the physical area of the bands and the iridescent signal, likely due to the high amount of torso iridescence they have.

Band area and band iridescence are represented differently in the ornamentation highlighting the potential for band area and iridescence to be responsible for different roles in the mating system of *S. scovelli* and for these roles to change and fluctuate geographically. To fully understand the ornamentation found on *S. scovelli*, all components, the geographic differences, the physical area of the permanent bands, and the iridescent signal, must be considered. Overall, the variability of this sexually selected signal is illustrated and prompts further questions about what is driving this variation. One large factor tied to the variation in this ornamentation is the difference in the environments of these pipefish. Components such as the density of the seagrass beds they reside in or turbidity levels and depth of the water may be affecting the signal expression. If the pipefish reside in particularly dense beds, such as the seagrass sampled in Anne's Beach, a higher level of iridescence may be beneficial for the females to be seen by potential mates through the blades of seagrass. Contrastingly, pipefish residing in areas of low visibility, such as the turbid

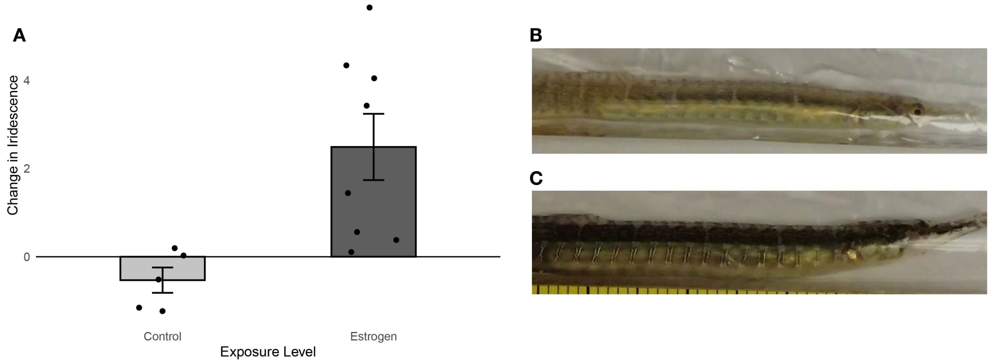

FIGURE 6
(A) Change in area of iridescence (± S.E.) for male pipefish exposed to synthetic estrogen (n=8) and male pipefish unexposed to estrogen (n=5). Exposed males showed a significant increase in iridescence. **(B)** Example of a male *S. scovelli* on Day 1 of the study and **(C)** that same male after 18 days of exposure to 5 ng/L of EE2.

waters of the Texas coast, may reallocate energy from visual sexual signals into something such as chemical signals (Ratterman et al., 2009; Sundin et al., 2010; Sundin et al., 2016; Sundin et al., 2017). While sampling depth did not vary greatly across the locations sampled for pipefish, previous studies have shown that the different wavelengths available at a certain depth may also impact iridescent ornamentation (Chae and Nishida, 1995). Additionally, sexual selection pressures themselves have been shown to vary geographically in other species of pipefish (Mobley and Jones, 2007; Monteiro and Lyons, 2012), providing another possible explanation for the variation in the iridescent signal.

Although it is currently unknown which of the iridescent traits may be used by male *S. scovelli* in mate selection, previous work has established that the number of permanent light-colored bands and their size increases the reproductive success of the female (Flanagan et al., 2014). Therefore, each of the three iridescent measurements could play an important role in mate selection, and these roles may differ between populations. However, there is the possibility that the iridescent ornamentation acts more as an amplifier trait. Amplifier traits are ones that do not serve to signify quality themselves, but rather to enhance the assessment of other traits that do signal quality (Doucet and Meadows, 2009). A study done by Anderson and Jones (2019) demonstrated the tight relationship between the area of the permanent bands and body depth, with deeper bodied females possessing larger bands, but found that males choose mates based on their depth rather than the ornamentation. The permanent light-colored bands and bluish iridescent signal may then be responsible for emphasizing the size of the females and therefore indirectly signaling the quality of potential mates as larger females have the capacity for more or larger eggs.

4.3 An ecological application of the IDIA: Detection of iridescence as a biological indicator

The IDIA was successfully used to detect morphological changes in male *S. scovelli* as a response to environmental contaminants, specifically synthetic estrogen (17α-ethinylestradiol, EE2). The most prominent source for this contamination is discharge from wastewater treatment plants into bodies of water. *Syngnathus scovelli* can potentially reside in seagrass beds located near the wastewater treatment plants discharge making them vulnerable to the negative effects of estrogen exposure. Previous laboratory studies have shown levels of EE2 exposure as low as 5 ng/L leads to the feminization of male pipefish, including the development of iridescent bands that are typically only found in sexually mature females, production of egg precursor proteins, and difficulty reproducing (Rose et al., 2013b, 2015). With the IDIA, the development of those iridescent bands was used as a morphological indicator of exposure to levels of 5-10 ng/L of sythetic estrogen.

Iridescence measurements calculated from the IDIA for the exposed male *S. scovelli* resulted in numbers within the range of the quantity of iridescence found on female *S. scovelli*. It should be noted that pipefish were exposed to higher concentrations of EE2 (5-10 ng/L) for the purpose of showing the development of iridescent bands. Lower levels of EE2 (<5 ng/L) do not induce as drastic of a change morphologically in male *S. scovelli* (Rose et al., 2013b, 2015). In aquatic systems EE2 has been detected in concentrations as high as 820 ng/L in US rivers and 35 ng/L in certain European locations (Kolpin et al., 2002; Pojana et al., 2007) and lower ranges of 0.5 to 10 ng/L in locations worldwide (Allen et al., 1999; Johnson et al., 2000). The IDIA can detect changes in morphology at levels of 5-10 ng/L, suggesting that *S. scovelli* has potential as an ecotoxicology model system for monitoring estrogen contamination in aquatic environments. By developing a standard curve with known levels of EE2 and corresponding levels of iridescence measured with the IDIA, future studies can link morphological changes in iridescence on fish to specific levels of synthetic estrogen exposure. This approach offers a simple and non-invasive way to estimate levels of estrogen in aquatic systems from iridescence quantified in photographs of male pipefish, rather than more invasive and time-consuming methods, such as sacrificing the animals to measure hormone levels using blood plasma or RNA-sequencing or testing the surrounding waters. However, further work is necessary to determine the efficacy of this technique for monitoring the presence of environmental contaminants, such as synthetic estrogen. It is worth noting that synthetic estrogen is not the only environmental contaminant found in aquatic environments, and other contaminants may also elicit morphological changes in iridescence. Therefore, additional research is required to evaluate the usefulness of iridescence-based monitoring for detecting a range of contaminants in aquatic systems.

4.4 Implementation and facilitation of the IDIA with other organisms

The combined applications of the Iridescence Detection and Isolation Algorithm (IDIA) in this study provide support that this program is a successful tool for measuring iridescence but highlight issues relating to measuring changing signals in live animals and in producing reliable photographs. *S. scovelli* have the ability to change their background coloration (i.e. increase or decrease contrast of torso color to the iridescent bands) in response to different circumstances. Changes in the contrast have been observed for the *S. scovelli* following death, in response to stress, and throughout courtship, as seen in other syngnathids (Lin et al., 2009; Sundin et al., 2016). Linked to this phenomenon is the possibility that these changes in background coloration are influencing the magnitude of the iridescent signal detected by the IDIA. While still unknown and unresearched, examples of this have been observed scattered throughout multiple laboratory experiments (Figure S3). The reproductive timing of the pipefish may also influence the quality of photographs taken in the aspect that observations of females across different reproductive stages highlight an increase in torso size and robustness at the later reproductive stages, likely due to the development of ovaries. This increase in size may influence the manner in which the pipefish lays against the material while photographing, possibly influencing the quality of the iridescent measurement.

Iridescence has been shown to play a multitude of important roles within an immense diversity of organisms. Because of this, it is important to understand why iridescence would have evolved as a signal to begin with. It can serve as a sexual signal to increase an organism's fitness, as a "weapon" for intraspecies competition, or a method for a species to find its own kind. However, to ever understand fully any of the functionality, there must be a way in which it can be quantified. The Iridescence Detection and Isolation Algorithm has the potential to be adapted and utilized across a variety of taxa. To do so, the specific biology of the organism of interest must be well understood and the program re-optimized in response. With programs such as the IDIA, fine-scale variation in iridescent signal is picked up, deepening the comprehension of why iridescence is present and how it is potentially evolving in populations experiencing selection.

Data availability statement

All code used to conduct the analysis and produce the figures is available on github (https://github.com/coley-tosto/Sscov_band_iri) and archived on zenodo (https://doi.org/10.5281/zenodo.7793993). Data are available at https://doi.org/10.5281/zenodo.7796404.

Author contributions

NT, ER, and HM contributed to study design and SF provided intellectual support. HM was involved in the original development and optimization of the IDIA with Gulf pipefish. All measurements of iridescence and subsequent analyses were performed by NT. Writing of the manuscript was done by NT, ER, SF, HM. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1127790/full#supplementary-material

References

Allen, Y., Scott, A. P., Matthiessen, P., Haworth, S., Thain, J. E., and Feist, S. (1999). Survey of estrogenic activity in united kingdom estuarine and coastal waters and its effects on gonadal development of the flounder *Platichthys flesus*. *Environ. Toxicol. Chem.* 18, 1791–1800. doi: 10.1002/etc.5620180827

Anderson, A. P., and Jones, A. G. (2019). Choosy gulf pipefish males ignore age but prefer active females with deeply keeled bodies. *Anim. Behav.* 155, 37–44. doi: 10.1016/j.anbehav.2019.05.018

Anderson, A. P., Rose, E., Flanagan, S. P., and Jones, A. G. (2020). The estrogen-responsive transcriptome of female secondary sexual traits in the gulf pipefish. *J. Hered.* 111, 294–306. doi: 10.1093/jhered/esaa008

Baldwin, J., and Johnsen, S. (2012). The male blue crab, *Callinectes sapidus*, uses both chromatic and achromatic cues during mate choice. *J. Exp. Biol.* 215, 1184–1191. doi: 10.1242/jeb.067512

Bebié, N., and McElligott, A. G. (2006). Female aggression in red deer: Does it indicate competition for mates? *Mamm. Biol.* 71, 347–355. doi: 10.1016/j.mambio.2006.02.008

Berglund, A. (2000). Sex role reversal in a pipefish: female ornaments as amplifying handicaps. *Ann. Zool. Fennici* 37, 1–13.

Berglund, A., Rosenqvist, G., and Bernet, P. (1997). Ornamentation predicts reproductive success in female pipefish. *Behav. Ecol. Sociobiol.* 40, 145–150. doi: 10.1007/s002650050327

Berglund, A., Rosenqvist, G., and Svensson, I. (1986). Mate choice, fecundity and sexual dimorphism in two pipefish species (Syngnathidae). *Behav. Ecol. Sociobiol.* 19, 301–307. doi: 10.1007/BF00300646

Bernet, P., Berglund, A., and Rosenqvist, G. (1998). Female-female competition affects female ornamentation in the sex-role reversed pipefish syngnathus typhle. *Behaviour* 135, 535–550. doi: 10.1163/156853998792897923

Bitton, P.-P., and Dawson, R. D. (2008). Age-related differences in plumage characteristics of male tree swallowsTachycineta bicolor: hue and brightness signal different aspects of individual quality. *J. Avian Biol.* 39, 446–452. doi: 10.1111/j.0908-8857.2008.04283.x

Chae, J., and Nishida, S. (1995). Vertical distribution and die1 migration in the iridescent copepods of the family sapphirinidae: a unique example of reverse migration? *Mar. Ecol. Prog. Ser.* 119, 111–124. doi: 10.3354/meps119111

Clutton-Brock, T. (2007). Sexual selection in males and females. *Science* 318, 1882–1885. doi: 10.1126/science.1133311

Clutton-Brock, T. (2009). Sexual selection in females. *Anim. Behav.* 77, 3–11. doi: 10.1016/j.anbehav.2008.08.026

Clutton-Brock, T. (2017). Reproductive competition and sexual selection. *Philos. Trans. R. Soc Lond. B Biol. Sci.* 372. doi: 10.1098/rstb.2016.0310

Cunha, M. A. B., Berglund, A., and Monteiro, N. M. (2017). Female ornaments signal own and offspring quality in a sex-role-reversed fish with extreme male parental care. *Mar. Ecol.* 38, e12461. doi: 10.1111/maec.12461

Darwin, C. (1871). *The descent of man, and selection in relation to sex* (London, England: Murray) Vol. 1.

Dijkstra, P. D., van Dijk, S., Groothuis, T. G. G., Pierotti, M. E. R., and Seehausen, O. (2009). Behavioral dominance between female color morphs of a lake Victoria cichlid fish. *Behav. Ecol.* 20, 593–600. doi: 10.1093/beheco/arp036

Doucet, S. M., and Meadows, M. G. (2009). Iridescence: a functional perspective. *J. R. Soc. Interface* 6 (Suppl 2), S115–S132. doi: 10.1098/rsif.2008.0395.focus

Doucet, S. M., Shawkey, M. D., Hill, G. E., and Montgomerie, R. (2006). Iridescent plumage in satin bowerbirds: structure, mechanisms and nanostructural predictors of individual variation in colour. *J. Exp. Biol.* 209, 380–390. doi: 10.1242/jeb.01988

Emlen, S. T., and Oring, L. W. (1977). Ecology, sexual selection, and the evolution of mating systems. *Science* 197, 215–223. doi: 10.1126/science.327542

Fisher, R. A. (1915). The evolution of sexual preference. *Eugen. Rev.* 7, 184–192.

Fitzstephens, D. M., and Getty, T. (2000). Colour, fat and social status in male damselflies, *Calopteryx maculata*. *Anim. Behav.* 60, 851–855. doi: 10.1006/anbe.2000.1548

Flanagan, S. P., Johnson, J. B., Rose, E., and Jones, A. G. (2014). Sexual selection on female ornaments in the sex-role-reversed gulf pipefish (*Syngnathus scovelli*). *J. Evol. Biol.* 27, 2457–2467. doi: 10.1111/jeb.12487

Flanagan, S. P., Rose, E., and Jones, A. G. (2016). Population genomics reveals multiple drivers of population differentiation in a sex-role-reversed pipefish. *Mol. Ecol.* 25, 5043–5072. doi: 10.1111/mec.13794

Fox, J., and Weisberg, S. (2019). *An r companion to applied regression*, *3rd ed.* (Thousand Oaks CA: Sage).

Gomez, D., and Théry, M. (2007). Simultaneous crypsis and conspicuousness in color patterns: Comparative analysis of a Neotropical rainforest bird community. *Am. Nat.* 169, S42–S61. doi: 10.1086/510138

Gruson, H., Andraud, C., Daney de Marcillac, W., Berthier, S., Elias, M., Gomez, D., et al (2019). Quantitative characterization of iridescent colours in biological studies: a novel method using optical theory. *J. R. Soc. Interface* 9 (1). doi: 10.1098/rsfs.2018.0049

Hare, R. M., and Simmons, L. W. (2019). Sexual selection and its evolutionary consequences in female animals. *Biol. Rev. Camb. Philos. Soc* 94, 929–956. doi: 10.1111/brv.12484

Johnson, A. C., Belfroid, A., and Di Corcia, A. D. (2000). Estimating steroid oestrogen inputs into activated sludge treatment works and observations on their removal from the effluent. *Sci. Total Environ.* 256, 163–173. doi: 10.1016/S0048-9697(00)00481-2

Jones, A. G., and Ratterman, N. L. (2009). Mate choice and sexual selection: what have we learned since Darwin? *Proc. Natl. Acad. Sci. U. S. A.* 106 (Suppl 1), 10001–10008. doi: 10.1073/pnas.0901129106

Jones, A. G., Rosenqvist, G., Berglund, A., and Avise, J. C. (2005). The measurement of sexual selection using bateman's principles: an experimental test in the sex-role-reversed pipefish *Syngnathus typhle*. *Integr. Comp. Biol.* 45, 874–884. doi: 10.1093/icb/45.5.874

Jones, A. G., Walker, D., and Avise, J. C. (2001). Genetic evidence for extreme polyandry and extraordinary sex-role reversal in a pipefish. *Proc. Biol. Sci.* 268, 2531–2535. doi: 10.1098/rspb.2001.1841

Kemp, D. J., Vukusic, P., and Rutowski, R. L. (2006). Stress-mediated covariance between nano-structural architecture and ultraviolet butterfly coloration. *Funct. Ecol.* 20, 282–289. doi: 10.1111/j.1365-2435.2006.01100.x

Kirkpatrick, M. (1982). Sexual selection and the evolution of female choice. *Evolution* 36, 1–12. doi: 10.2307/2407961

Kirkpatrick, M., Price, T., and Arnold, S. J. (1990). The Darwin-Fisher theory of sexual selection in monogamous birds. *Evolution* 44, 180–193. doi: 10.2307/2409533

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., et al. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams 1999-2000: a national reconnaissance. *Environ. Sci. Technol.* 36, 1202–1211. doi: 10.1021/es011055j

Lim, M. L. M., and Li, D. (2006). Behavioural evidence of UV sensitivity in jumping spiders (*Araneae: Salticidae*). *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 192, 871–878. doi: 10.1007/s00359-006-0126-5

Lim, M. L. M., and Li, D. (2007). Effects of age and feeding history on structure-based UV ornaments of a jumping spider (*Araneae: Salticidae*). *Proc. Biol. Sci.* 274, 569–575. doi: 10.1098/rspb.2006.0020

Lin, Q., Lin, J., and Huang, L. (2009). Effects of substrate color, light intensity and temperature on survival and skin color change of juvenile seahorses, *Hippocampus erectus*. *Aquaculture* 298, 157–161. doi: 10.1016/j.aquaculture.2009.10.015

Lipshutz, S. E. (2017). Divergent competitive phenotypes between females of two sex-role-reversed species. *Behav. Ecol. Sociobiol.* 71, 106. doi: 10.1007/s00265-017-2334-0

Loyau, A., Gomez, D., Moureau, B., Théry, M., Hart, N. S., and Jalme, M. S. (2007). Iridescent structurally based coloration of eyespots correlates with mating success in the peacock. *Behav. Ecol.* 18, 1123–1131. doi: 10.1093/beheco/arm088

Masonjones, H. D., Rose, E., McRae, L. B., and Dixson, D. L. (2010). An examination of the population dynamics of syngnathid fishes within Tampa bay, Florida,USA. *Curr. Zool.* 56, 118–133. doi: 10.1093/czoolo/56.1.118

McGraw, K. J., Mackillop, E. A., Dale, J., and Hauber, M. E. (2002). Different colors reveal different information: how nutritional stress affects the expression of melanin- and structurally based ornamental plumage. *J. Exp. Biol.* 205, 3747–3755. doi: 10.1242/jeb.205.23.3747

Mobley, K. B., and Jones, A. G. (2007). Geographical variation in the mating system of the dusky pipefish (*Syngnathus floridae*). *Mol. Ecol.* 16, 2596–2606. doi: 10.1111/j.1365-294X.2007.03337.x

Mobley, K. B., Morrongiello, J. R., Warr, M., Bray, D. J., and Wong, B. B. M. (2018). Female ornamentation and the fecundity trade-off in a sex-role reversed pipefish. *Ecol. Evol.* 8, 9516–9525. doi: 10.1002/ece3.4459

Monteiro, N. M., Carneiro, D., Antunes, A., Queiroz, N., Vieira, M. N., and Jones, A. G. (2017). The lek mating system of the worm pipefish (*Nerophis lumbriciformis*): a molecular maternity analysis and test of the phenotype-linked fertility hypothesis. *Mol. Ecol.* 26, 1371–1385. doi: 10.1111/mec.13931

Monteiro, N. M., and Lyons, D. O. (2012). Stronger sexual selection in warmer waters: the case of a sex role reversed pipefish. *PloS One* 7, e44251. doi: 10.1371/journal.pone.0044251

Moore, A. J. (1990). The evolution of sexual dimorphism by sexual selection: the separate effects of intrasexual selection and intersexual selection. *Evolution* 44, 315–331. doi: 10.2307/2409410

Muniz, D. G., and Machado, G. (2018). Mate sampling influences the intensity of sexual selection and the evolution of costly sexual ornaments. *J. Theor. Biol.* 447, 74–83. doi: 10.1016/j.jtbi.2018.03.026

Partridge, C., Boettcher, A., and Jones, A. G. (2010). Short-term exposure to a synthetic estrogen disrupts mating dynamics in a pipefish. *Horm. Behav.* 58, 800–807. doi: 10.1016/j.yhbeh.2010.08.002

Partridge, C., Boettcher, A., and Jones, A. G. (2013). The role of courtship behavior and size in mate preference in the sex-role-reversed gulf pipefish, *Syngnathus scovelli*. *Ethology* 119, 692–701. doi: 10.1111/eth.12110

Pojana, G., Gomiero, A., Jonkers, N., and Marcomini, A. (2007). Natural and synthetic endocrine disrupting compounds (EDCs) in water, sediment and biota of a coastal lagoon. *Environ. Int.* 33, 929–936. doi: 10.1016/j.envint.2007.05.003

Powell, D. M. (2008). Female–female competition or male mate choice? patterns of courtship and breeding behavior among feral horses (*Equus caballus*) on assateague island. *J. Ethol.* 26, 137–144. doi: 10.1007/s10164-007-0043-2

Pröhl, H., and Hödl, W. (1999). Parental investment, potential reproductive rates, and mating system in the strawberry dart-poison frog, dendrobates pumilio. *Behav. Ecol. Sociobiol.* 46, 215–220. doi: 10.1007/s002650050612

Ratterman, N. L., Rosenthal, G. G., and Jones, A. G. (2009). Sex recognition *via* chemical cues in the sex-role-reversed gulf pipefish (Syngnathus scovelli). *Ethology* 115, 339–346. doi: 10.1111/j.1439-0310.2009.01619.x

Rillich, J., Buhl, E., Schildberger, K., and Stevenson, P. A. (2009). Female crickets are driven to fight by the male courting and calling songs. *Anim. Behav.* 77, 737–742. doi: 10.1016/j.anbehav.2008.12.009

Rometsch, S. J., Torres-Dowdall, J., Machado-Schiaffino, G., Karagic, N., and Meyer, A. (2021). Dual function and associated costs of a highly exaggerated trait in a cichlid fish. *Ecol. Evol.* 11, 17496–17508. doi: 10.1002/ece3.8383

Rose, E., Flanagan, S. P., and Jones, A. G. (2015). The effects of synthetic estrogen exposure on the sexually dimorphic liver transcriptome of the sex-Role-Reversed gulf pipefish. *PloS One* 10, e0139401. doi: 10.1371/journal.pone.0139401

Rose, E., Paczolt, K. A., and Jones, A. G. (2013a). The contributions of premating and postmating selection episodes to total selection in sex-role-reversed gulf pipefish. *Am. Nat.* 182, 410–420. doi: 10.1086/671233

Rose, E., Paczolt, K. A., and Jones, A. G. (2013b). The effects of synthetic estrogen exposure on premating and postmating episodes of selection in sex-role-reversed gulf pipefish. *Evol. Appl.* 6, 1160–1170. doi: 10.1111/eva.12093

Rosenqvist, G., and Berglund, A. (2011). Sexual signals and mating patterns in syngnathidae. *J. Fish Biol.* 78, 1647–1661. doi: 10.1111/j.1095-8649.2011.02972.x

Sæther, S. A. (2002). Female calls in lek-mating birds: indirect mate choice, female competition for mates, or direct mate choice? *Behav. Ecol.* 13, 344–352. doi: 10.1093/beheco/13.3.344

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671–675. doi: 10.1038/nmeth.2089

Silberglied, R. E., and Taylor, O. R. (1978). Ultraviolet reflection and its behavioral role in the courtship of the sulfur butterflies *Colias eurytheme* and *C.*

philodice (Lepidoptera, pieridae). *Behav. Ecol. Sociobiol.* 3, 203–243. doi: 10.1007/BF00296311

Silva, K., Vieira, M. N., Almada, V. C., and Monteiro, N. M. (2010). Reversing sex role reversal: compete only when you must. *Anim. Behav.* 79, 885–893. doi: 10.1016/j.anbehav.2010.01.001

Sogabe, A., and Ahnesjö, I. (2011). The ovarian structure and mode of egg production in two polygamous pipefishes: a link to mating pattern. *J. Fish Biol.* 78, 1833–1846. doi: 10.1111/j.1095-8649.2011.02973.x

Sundin, J., Aronsen, T., Rosenqvist, G., and Berglund, A. (2017). Sex in murky waters: algal-induced turbidity increases sexual selection in pipefish. *Behav. Ecol. Sociobiol.* 71, 1-8. doi: 10.1007/s00265-017-2310-8

Sundin, J., Berglund, A., and Rosenqvist, G. (2010). Turbidity hampers mate choice in a pipefish. *Ethology* 8, 713-721. doi: 10.1111/j.1439-0310.2010.01787.x

Sundin, J., Rosenqvist, G., Myhren, S., and Berglund, A. (2016). Algal turbidity hampers ornament perception, but not expression, in a sex-Role-Reversed pipefish. *Ethology* 122, 215–225. doi: 10.1111/eth.12461

Számadó, S., and Penn, D. J. (2015). Why does costly signalling evolve? challenges with testing the handicap hypothesis. *Anim. Behav.* 110, e9–e12. doi: 10.1016/j.anbehav.2015.06.005

Trail, P. W. (1990). Why should lek-breeders be monomorphic? *Evolution* 44, 1837–1852. doi: 10.1111/j.1558-5646.1990.tb05254.x

Venables, W. N., and Ripley, B. D. (2002). *Modern applied statistics with s, 4th ed.* (New York: Springer).

Vincent, A., Ahnesjö, I., Berglund, A., and Rosenqvist, G. (1992). Pipefishes and seahorses: Are they all sex role reversed? *Trends Ecol. Evol.* 7, 237–241. doi: 10.1016/0169-5347(92)90052-D

West-Eberhard, M. J. (1979). Sexual selection, social competition, and evolution. *Proc. Am. Philos. Soc* 123, 222–234.

Wilson, A. B., Ahnesjö, I., Vincent, A. C. J., and Meyer, A. (2003). The dynamics of male brooding, mating patterns, and sex roles in pipefishes and seahorses (family syngnathidae). *Evolution* 57, 1374–1386. doi: 10.1111/j.0014-3820.2003.tb00345.x

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Fatty acid profiles of cultured *Hippocampus hippocampus* trunk muscles and potential nutritional value

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Syngnathids (seahorses, pipefishes and seadragons) are an attractive resource for Traditional Chinese Medicine (TCM). Despite few scientific studies supporting seahorse nutritional benefits, they are believed to possess medicinal properties that enhance human health. The European short-snout seahorse *Hippocampus hippocampus* is classified as Data Deficient by the IUCN Red List of Threatened Species. Nevertheless, there are increasing records of this species being illegally captured and traded to supply TCM. This study investigated the fatty acid (FA) profiles of the trunk muscles of cultured female and male *H. hippocampus*, to assess sex and intraspecific variation, as well as their potential nutritional value. The contents of crude lipid (4.05 ± 2.15% dry weight, DW in females and 2.82 ± 1.48% DW in males) and phospholipid (8.23 ± 3.34 µg mg^{-1} DW in females and 7.91 ± 2.36 µg mg^{-1} DW in males) were not significantly different between the two sexes. The absolute FA compositions of *H. hippocampus* trunk muscles revealed higher mean values for FA 16:0, 18:0, 18:1 *n*-9 and 22:6 *n*-3 (DHA), in both female (2.82 ± 1.11, 1.81 ± 0.89, 0.90 ± 0.41 and 0.93 ± 0.35 µg mg^{-1} DW, respectively) and male specimens (1.99 ± 0.95, 1.52 ± 0.78, 0.74 ± 0.44 and 0.80 ± 0.41 µg mg^{-1} DW, respectively). In terms of FA classes, saturated fatty acids (SFA) showed the highest absolute value of the total pool of FA, for both sexes (4.73 ± 1.94 µg mg^{-1} DW in females and 3.58 ± 1.76 µg mg^{-1} DW in males). Males tended to exhibit a more suitable profile for human nutrition, displaying a lower atherogenic index (AI) and thrombogenic index (TI). The relative composition of *H. hippocampus* trunk muscles followed the patterns of seahorse species valued in TCM, with DHA ranking amongst the PUFA with higher mean relative abundances (12.0% of total FA). While seahorse FA profiles may be of interest in terms of their nutritional value for humans, only specimens originating from sustainable production practices should be traded and the conservation of their populations in the wild should continue to be a global priority.

KEYWORDS

DHA, GC-MS, lipids, nutritional value, omega-3 fatty acids, syngnathids, seahorse

1 Introduction

Seahorse species within genus *Hippocampus* are vulnerable to illegal, unregulated and unreported (IUU) trade (Foster et al., 2019) mostly due to their demand by Traditional Chinese Medicine (TCM) and, to a lesser extent, to the marine ornamental and curio trades (Cohen et al., 2017). Global seahorse extraction is substantial, rounding tens of millions of specimens per year (Vincent et al., 2007; Vincent et al., 2011; Foster et al., 2019; Vaidyanathan et al., 2021). One way to address the pressure on wild populations is the implementation of sustainable aquaculture practices with the upside of supporting small communities that are dependent on seahorses as a source of income (Cohen et al., 2017). In 2002, the increase recorded in the live trade of farmed seahorses somehow signaled the growing international effort towards their aquaculture (Koldewey and Martin-Smith, 2010; Olivotto et al., 2011; Planas et al., 2017). There are, however, several seahorse species that by being caught in large numbers from the wild, highlight the pressing need to diversify the scope of seahorse aquaculture (Koldewey and Martin-Smith, 2010).

Most seahorse specimens supplying TCM are obtained from the wild to be consumed dried (Foster et al., 2019), as they are believed to have nutritional properties that are beneficial for human health (Kim et al., 2019). Dried seahorses are placed whole in beverages or consumed in the form of powder to treat several illnesses such as respiratory diseases and arteriosclerosis (Vincent, 1996; Vincent et al., 2011). Biomolecules with nutritional relevance already reported from *Hippocampus* spp. include lipids and amino acids, as well as minerals, namely some trace elements (Lin et al., 2009; Kim et al., 2019). Among the health promoting benefits that have been described to date from pharmacological studies addressing natural derivatives from seahorse species valued in TCM one can highlight the anti-oxidant activity reported from *H. abdominalis* (Kim et al., 2019) and its, as well as *H. comes*, anti-cancer potential (Xu et al., 2020; Ghasemi et al., 2021). Additionally, the phenolic compound paeonol isolated from *H. kuda* has been reported to display activity against neuro-inflammation (Himaya et al., 2012).

Marine fish are commonly recognized as a rich source of polyunsaturated fatty acids (PUFA), especially omega-3 fatty acids (FA), namely docosahexaenoic acid (DHA, 22:6 *n*-3) and eicosapentaenoic acid (EPA, 20:5 *n*-3) (Woods, 2003; Huang et al., 2010). Docosahexaenoic acid and EPA are abundant FA in fish cell membranes (Johnston et al., 2020), being the first a relevant component of cell membranes involved in the synthesis of muscle fibers (Planas et al., 2020). These omega-3 FA, as well as some omega-6 FA (arachidonic acid, ARA, 20:4 *n*-6), are known to be of major importance in human metabolism, for contributing to the prevention of multiple non-communicable diseases (e.g., cardiovascular disorders) and modulate immune responses (Crawford et al., 1999; Nestel, 2000; Sonnweber et al., 2018). Nevertheless, the biosynthesis pathway that allow *de novo* synthesis of DHA and EPA from its precursor, the essential α-linoleic acid (18:3 *n*-3, ALA) that needs to be obtained from dietary sources and cannot be biosynthesized, is very limited in humans (Burdge and Calder, 2005; Scaioli et al., 2017). For this reason, consumers and nutritionists value marine fish and seafood as important sources of omega-3 FA (Tocher, 2015).

The European short-snout seahorse *H. hippocampus* and long-snout seahorse *H. guttulatus* co-occur in sympatry in shallow European coastal habitats (Curtis et al., 2017). In Ria Formosa coastal lagoon (south of Portugal), the populations of these two species have suffered pronounced declines associated to multiple factors, such as habitat degradation, by-catch, natural phenomena (e.g., silting events) and illegal fishing; yet the direct causes for this decline are still being assessed (Caldwell and Vincent, 2012; Correia et al., 2018). *Hippocampus hippocampus* is classified, at global level, as Data Deficient by the IUCN Red List of Threatened Species (The IUCN Red List of Threatened Species, 2017a) and it is not extensively traded (Curtis et al., 2017), as other seahorse species that are targeted for TCM. Nevertheless, in the Mediterranean region, this species has been classified as Near Threatened (The IUCN Red List of Threatened Species, 2017b) and there are a few records of its illegal capture and trade to supply TCM (Planelles, 2017).

Nowadays, consumers are more concerned about environmental and food security matters and, as a result, are interested in knowing the origin and production processes of seafood (Leal et al., 2015; Doubleday et al., 2022). As such, seahorse aquaculture can potentially enhance consumers' safety by providing a legal product to traditional and emerging markets. The FA profiles of edible muscles of farmed fish are influenced by the diet supplied during grow-out (Bordignon et al., 2020) and culture conditions, ultimately affecting the quality of the fillets that will reach the final consumer (Monge-Ortiz et al., 2018). As wild and cultured marine fish experience contrasting feeding regimes, their biochemical and geochemical profiles may be used to discriminate farmed seahorses from wild ones (Leal et al., 2015). Indeed, a geochemical tool has already been successfully applied to confirm the geographic origin of cultured *H. guttulatus* (Cabral et al., 2021), confirming the potential of these approaches to help fighting IUU fishing, as well as promoting the certification of origin for sustainably farmed seahorses.

The present study aimed to determine the FA profiles of cultured specimens of *H. hippocampus* and investigate the existence of intraspecific variation. While seahorses are usually consumed whole (muscle, viscera, bones and skin), the present study only analyzed the FA profile of trunk muscle. Muscle is an important lipid storage tissue in fish (Jobling et al., 1998) and while the FA profile of trunk muscle does not reflect the full FA pool of seahorses, it displays a more stable profile than that of gonads (e.g., ovaries) and reserve organs (e.g., liver) which are more susceptible to short-term shifts promoted by diet and abiotic factors (Planas et al., 2020; Zhang et al., 2022). Therefore, when aiming to perform intraspecific comparisons (e.g., male *vs.* female specimens), a more stable biological matrix will be less prone to bias in the pool of FA promoted by diet or different stages of sexual maturation (Zhang et al., 2022). The FA profiles of cultured short-snout seahorse's trunk muscles are compared, from a nutritional point of view, to those of the whole-body of other cultured *Hippocampus* species described in the literature, acknowledging that the pool of FA of *H. hippocampus* is only partly represented by trunk muscle. Valuing the nutritional and functional potential of cultured seahorses is of main importance, as this may represent a potential pathway for relieving the ongoing fishing pressure over wild populations. This goal can be more easily achieved if a product with a more stable FA profile is presented to consumers, namely by encouraging the use of seahorse trunk muscle alone, rather than whole dried specimens featuring a much more variable FA profile.

2 Materials and methods

2.1 Seahorse samples

Fourteen specimens of captive bred *H. hippocampus* were sourced from Centro de Ciências do Mar (CCMAR, University of Algarve, Faro, Portugal). The captive breeding program for *H. hippocampus* (Project HIPPONUTRE, reference 16-02-01-FMP-54) was approved by the ethics committee from the Veterinary Medicines Directorate, Ministry of Agriculture, Rural Development and Fisheries, Portugal. Under this approval, the program is conducted in accordance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals. These short-snout seahorses (6 males and 8 females) were cultured using natural seawater from Ria Formosa coastal lagoon, located in Portugal's southern Atlantic coast (36°59' N; 7°51' W). Temperature and salinity followed natural seasonal patterns with annual mean values of 20 °C and 35.7, respectively. During grow-out seahorses were fed on wild mysids *Mesopodopsis slabberi* and/or *Leptomysis* spp., depending on daily and/or seasonal availability.

The seahorse samples considered for FA analysis died from natural causes, during the same time frame (during the year of 2020). In the laboratory, frozen seahorse samples, maintained at -20 °C, were washed with distilled water and freeze-dried (Labogene CoolSafe 55-9L PRO), prior to the extraction of their trunk muscle. Seahorses' whole body dry weight (DW) was registered, presenting mean values (± standard deviation, SD) of 1.41 ± 0.23 g in females and 1.49 ± 0.67 g in males. Seahorse sex was determined through the presence of brood pouch in the specimens, a trait exclusive to males.

Seahorses' trunk was considered to be the extension from the cleithral ring to the last trunk ring, as described by Lourie (2003). This body part was separated from the rest of the whole body (98.34 ± 0.41% in females and 98.18 ± 0.53% in males) using ceramic coated blades, with dorsal trunk muscles (Figure 1) being removed with the help of stainless-steel tweezers. Tweezers and blades were cleaned with 99% (w/v) ethanol between the replicates of each sex group, to avoid cross contamination. Trunk muscle samples were stored at -20 °C until further analysis.

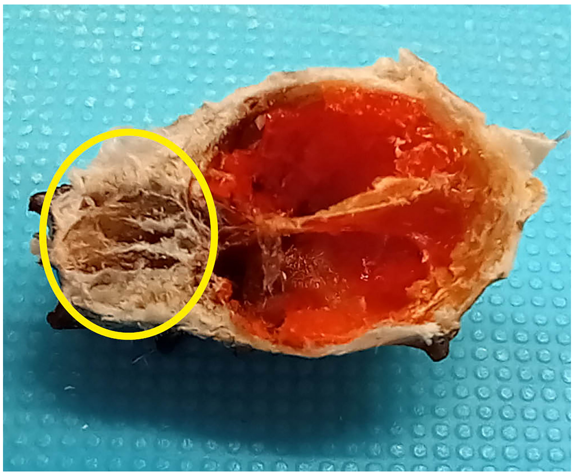

FIGURE 1
Trunk cross-section of a female short-snout seahorse *Hippocampus hippocampus* cultured at Centro de Ciências do Mar (CCMAR, University of Algarve, Faro, Portugal). Trunk muscles were extracted from the dorsal region highlighted by the yellow circle.

2.2 Biochemical analysis

2.2.1 Lipid extraction

Freeze-dried samples with a mean DW of 23.99 ± 7.43 mg were macerated in the extraction tube using a stainless-steel spatula. Lipid extraction was performed using a modified Bligh and Dyer (1959) method. Briefly, 2500 µL of methanol (MeOH) (HPLC grade, Fisher Scientific, UK) and 1250 µL of dichloromethane (CH_2Cl_2) (HPLC grade, Fisher Scientific, UK) were added to each sample, which were then sonicated for 1 min and incubated on ice in an orbital shaker at 150 rpm (Stuart SSL2 Reciprocating Shaker) for 30 min. After a second addition of 1250 µL CH_2Cl_2, samples were centrifuged at 1207 g force for 10 min (Centurion Scientific Pro Analytical C4000R with a BRK5324 rotor, Stoughton, UK) and the organic phase was collected. Biomass residue was re-extracted by adding another 2500 µL of MeOH and 2500 µL of CH_2Cl_2, followed by centrifugation. A volume of 2250 µL of Mili Q water (Synergysup®, Millipore Corporation, Billerica, MA, USA) was added in the organic phases to promote phase separation and after a new centrifugation for 10 min at 537 g force, the organic phase was collected. The aqueous phase was re-extracted with 1880 µL of CH_2Cl_2 and centrifuged. The combined organic phases were dried under a nitrogen stream and total lipid content was determined by gravimetry.

2.2.2 Phospholipid quantification

Phospholipid (PL) amount in total lipid extracts were determined spectrophotometrically through the phosphorus assay, as described by Bartlett and Lewis (1970). Briefly, the lipid extracts were re-suspended in 300 µL of CH_2Cl_2 and 10 µL of the samples were transferred to glass tubes. The samples were dried under a nitrogen stream and 125 µL of perchloric acid ($HClO_4$) (70% w/v) were posteriorly added. The tubes were then placed in a heating block (SBH200D/3, Stuart, Bibby Scientific Ltd., Stone, UK) at 180 °C for 60 min. A volume of 825 µL of Milli Q water and 125 µL sodium molybdate ($NaMoO_4.H_2O$) (2.5% w/v) were added to the tubes containing the samples. After homogenization, 125 µL of ascorbic acid ($C_6H_8O_6$) (10% w/v) were added and the samples were incubated in a water bath (Precisterm, JP Selecta, Barcelona, Spain) at 100 °C for 10 min. The standard solutions of 0.1–2.0 µg of phosphate (solution of 100 µg. mL^{-1} $NaH_2PO_4{\cdot}2H_2O$), were subjected to the same treatment as the samples, except for the placement in the heating block. A volume of 200 µL of the samples and standards was transferred to a 96-well plate and the absorbance was measured at 797 nm, using a microplate ultraviolet-visible spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Vantaa, Finland). The conversion factor 775/31 (25) was applied in order to obtain the amount of PL in the lipid extracts.

2.2.3 Fatty acid analysis by gas chromatography mass spectrometry

Fatty acid methyl esters (FAME) were prepared from total lipid extracts of the trunk muscle of *H. hippocampus* by alkaline transmethylation. An amount of total lipid extract corresponding to 10 µg of PL were transferred to tubes previously washed with *n*-hexane (95%). After drying the lipid extract under a nitrogen stream, 1 mL of internal standard 19:0 FA (1.08 µg mL^{-1}, CAS number1731-94-8, Merck) in *n*-hexane (99%) was added to the tube containing the lipids, followed by the addition of 200 µL of a methanolic potassium hydroxide (KOH) solution (2 M). After sample homogenization, 2 mL of a saturated sodium chloride solution (NaCl) (10 mg mL^{-1}) were added and the sample tubes were centrifuged for 5 min at 537 g force. A volume of 600 µL of the organic phase containing the FAME was extracted and dried under a nitrogen stream. For gas chromatography mass spectrometry (GC-MS) analysis, FAME were re-suspended in 100 µL of *n*-hexane (99%). A volume of 2.0 µL of the FAME solution was injected in the GC-MS equipment (Agilent Technologies 5977 B GC/MSD, Santa Clara, CA, USA) with a DB-FFAP column (123-3232, J and W Scientific, Folsom CA, USA) presenting the following specifications: 30 m in length, an internal diameter of 320 µm and a film thickness of 0.25 µm. The equipment was connected to a Mass Selective Detector operating with an electron impact mode at 70 eV and a scanning mass range of *m/z* 50–550 (1 s cycle in a full scan mode). Concerning the system conditions, helium was used as the carrier gas (constant flow 1.4 mL min^{-1}), inlet temperature 220 °C and detector temperature 230 °C. Oven temperature was programmed as follows: initial temperature of 58 °C for 2 min; linear increase to 160 °C (25 °C min^{-1}); linear increase to 210 °C (2 °C min^{-1}); linear increase to 225 °C (20 °C min^{-1}) and maintenance at 225 °C for 15 min. The data acquisition software employed was the GCMS 5977B/Enhanced MassHunter.

Fatty acid peaks were identified using Agilent MassHunter Qualitative Analysis 10.0 software, through retention time values and comparison of MS spectra with the NIST chemical database library and MS spectra of a FAME certified standard mixture (Supelco 37 Component FAME Mix, Sigma-Aldrich, Darmstadt, Germany). The absolute composition of FA (µg mg^{-1} DW) was determined using calibration curves of the FAME certified standard mixture under the same instrumental conditions and the FA 19:0 as the internal standard, while the relative abundances (%) were obtained from absolute values.

2.3 Lipid indexes

The assessment of nutritional quality of *H. hippocampus* trunk muscle, in terms of FA relative abundance (% of total fatty acids, TFA) was determined according to four lipid indexes. The atherogenic (AI), thrombogenic (TI) and hypocholesterolemic/hypercholesterolemic indexes (HH), were obtained from the formulas described by Ulbricht and Southgate (1991), while the polyene index (PI) was determined according to Lubis and Buckle (1990). The FA 12:0 and 14:0 were not included in the equations, as they were not detected in the trunk muscles of *H. hippocampus*. The equations for these lipid indexes are as follows (MUFA refers to monounsaturated FA and PUFA to polyunsaturated FA):

$$\mathrm{AI} = \frac{12{:}0+4\mathrm{x}14{:}0+16{:}0}{\Sigma\,\mathrm{MUFA} + \Sigma\,\mathrm{PUFA}\;n-6 + \Sigma\,\mathrm{PUFA}\;n-3} \qquad \text{equation (1)}$$

$$\mathbf{TI} = \frac{14{:}0+16{:}0+18{:}0}{(0.5\;\;\Sigma\mathbf{MUFA}) + (0.5\;\;\Sigma\;\;\mathbf{PUFA}\;\;n-6) + (3\;\;\Sigma\;\;\mathbf{PUFA}\;\;n-3) + \Sigma\;\frac{\mathbf{PUFA}\;\;n-3}{\mathbf{PUFA}\;\;n-6}} \qquad \text{equation (2)}$$

$$\mathbf{HH} = \frac{18{:}1\,n-9+18{:}2\,n-6+20{:}4\,n-6+18{:}3\,n-3+20{:}5\,n-3+22{:}5\,n-3+22{:}6\,n-3}{14:0+16:0} \qquad \text{equation (3)}$$

$$\mathbf{PI} = \frac{\mathbf{EPA}+\mathbf{DHA}}{16:0} \qquad \text{equation (4)}$$

2.4 Statistical analysis

To assess the existence of significant differences (p-value ≤ 0.05) in the absolute abundance (µg mg^{-1} DW) of FA composition between *H. hippocampus* males and females, both groups were compared. Although the absolute abundance of some FA exhibited a normal distribution (determined by Shapiro-Wilk test), due to the low number of replicates, a non-parametric version of the t-test for unpaired samples (Mann -Whitney U) was performed. The same approach was applied to assess if both groups were significantly different, in terms of their pool of saturated FA (SFA), MUFA and PUFA, in terms of their lipid indexes and for lipid and PL contents. Bonferroni's correction was applied whenever multiple comparisons were performed.

All statistical analyses were performed using RStudio v4.0.2 (RStudio Team, 2019), as well as the graphical representations which were obtained with *ggplot2 package* (Wickham, 2016).

3 Results

The crude lipid content of *H. hippocampus* trunk muscles was 4.05 ± 2.15% DW in females and 2.82 ± 1.48% DW in males (Supplementary Table S1). Regarding PL content, the mean values were 8.23 ± 3.34 µg mg^{-1} DW in females and 7.91 ± 2.36 µg mg^{-1} DW in males. Lipid and PL contents did not present significant differences between females and males (Mann-Whitney U, *p*-value = 0.18 for lipid content and *p*-value = 0.95 for PL content).

A total of 16 FA were identified in the trunk muscle of cultured adult males and females of *H. hippocampus*. The absolute FA compositions revealed high mean values for 16:0, 18:0, 18:1 *n*-9 and 22:6 *n*-3 (DHA), in both female (2.82 ± 1.11, 1.81 ± 0.89, 0.90 ± 0.41 and 0.93 ± 0.35 µg mg^{-1} DW, respectively) and male specimens (1.99 ± 0.95, 1.52 ± 0.78, 0.74 ± 0.44 and 0.80 ± 0.41 µg mg^{-1} DW, respectively) (Table 1). Concerning lipid classes, SFA displayed the highest absolute values in both sexes, with females tending to exhibit higher mean values (4.73 ± 1.94 µg mg^{-1} DW) than males (3.58 ± 1.76 µg mg^{-1} DW). The amount of PUFA followed SFA in terms of absolute composition (2.25 ± 0.98 µg mg^{-1} DW in females and 1.88 ± 0.98 µg mg^{-1} DW in males), with MUFA displaying the lowest values (1.15 ± 0.55 µg mg^{-1} DW in females and 0.85 ± 0.53 µg mg^{-1} DW in males). Concerning *n*-3 and *n*-6 PUFA, the former exhibited the highest mean absolute values (1.46 ± 0.62 µg mg^{-1} DW in females and 1.09 ± 0.49 µg mg^{-1} DW in males) (Table 1). The *n*-6/*n*-3 ratio exhibited values lower than 1, in both females and males (Table 1).

Table 2 summarizes the lipid indexes (AI, TI, HH and PI) recorded for cultured females and males of *H. hippocampus*. Females tended to present higher AI (0.87 ± 0.18) and TI (0.91 ± 0.17) than males (AI: 0.75 ± 0.14; TI: 0.84 ± 0.18), while males showed a tendency for higher HH (1.24 ± 0.25) and PI (0.52 ± 0.13) than females (HH: 1.06 ± 0.23; PI: 0.40 ± 0.07). Females and males did not present significant differences (Mann-Whitney U, *p*-value > 0.05) in lipid indexes.

TABLE 1 Absolute fatty acid composition (µg mg^{-1} DW) of trunk muscle from cultured female (n = 8) and male (n = 6) short snout seahorses *Hippocampus hippocampus*.

Fatty Acid	*Hippocampus hippocampus*		*p*-value
	Absolute composition (µg mg $^{-1}$ DW)		
	Female	Male	
15:0	0.010 ± 0.01	0.007 ± 0.012	1
16:0	2.819 ± 1.110	1.988 ± 0.953	1
17:0	0.048 ± 0.029	0.038 ± 0.017	1
18:0	1.807 ± 0.887	1.517 ± 0.780	1
20:0	0.035 ± 0.017	0.033 ± 0.011	1
16:1 *n*-7	0.101 ± 0.071	0.038 ± 0.023	0.948
17:1 *n*-7	0.038 ± 0.020	0.027 ± 0.015	1
18:1	0.115 ± 0.064	0.054 ± 0.053	1
18:1 *n*-9	0.896 ± 0.408	0.735 ± 0.440	1
18:2 *n*-6	0.154 ± 0.092	0.115 ± 0.060	1
20:4 *n*-6	0.486 ± 0.234	0.515 ± 0.358	1
20:5 *n*-3	0.347 ± 0.213	0.185 ± 0.057	1
22:4 *n*-6	0.061 ± 0.023	0.056 ± 0.031	1
22:5 *n*-6	0.093 ± 0.040	0.095 ± 0.047	1
22:5 *n*-3	0.177 ± 0.078	0.111 ± 0.055	1
22:6 *n*-3	0.933 ± 0.346	0.797 ± 0.405	1
n-3	1.457 ± 0.623	1.094 ± 0.494	0.568
n-6	0.793 ± 0.384	0.781 ± 0.493	1
n-6/*n*-3	0.544 ± 0.115	0.742 ± 0.137	0.282
ΣSFA[1]	4.728 ± 1.940	3.581 ± 1.759	0.847
ΣMUFA[2]	1.149 ± 0.551	0.853 ± 0.528	0.847
ΣPUFA [3]	2.251 ± 0.984	1.875 ± 0.984	1

Values are expressed as mean ± standard deviation. SFA[1]- saturated fatty acids: 15:0, 16:0, 17:0, 18:0 and 20:0; MUFA[2] - monounsaturated fatty acids: 16:1 *n*-7, 17:1 *n*-7; 18:1 and 18:1 *n*-9; PUFA[3]- polyunsaturated fatty acids: 18:2 *n*-6, 20:4 *n*-6, 20:5 *n*-3, 22:4 *n*-6, 22:5 *n*-6, 22:5 *n*-3 and 22:6 *n*-3; *n*-3 and *n*-6 correspond to PUFA (*n*-3) and PUFA (*n*-6). *p*-values (significant differences when *p*-value ≤ 0.05) from Mann-Whitney U, with Bonferroni corrections for multiple comparisons.

4 Discussion

4.1 Fatty acid profiles and lipid indexes of *Hippocampus hippocampus* trunk muscle

Marine fish are usually rich in highly unsaturated FA (HUFA), especially DHA and EPA (Woods, 2003; Lin et al., 2008; Huang et al., 2010). In the case of *H. hippocampus* specimens surveyed in the present study, both females and males displayed higher absolute values of 16:0, 18:0, 18:1 *n*-9 and DHA, in their trunk muscles. These findings are in accordance with available literature, in which 16:0 is the SFA with highest expression in fish tissues and 18:1 *n*-9 is one of the most abundant MUFA (Amoussou et al., 2022). The HUFA DHA, one of the FA displaying a higher absolute abundance in the trunk muscles of *H. hippocampus* females and males, is an important component of cell membranes involved in the synthesis of muscle fibers in fish (Planas et al., 2020; Sushchik et al., 2020). In the work developed by Planas et al. (2020), DHA and EPA were two of the most dominant FA in mysidaceans (*Siriella armata* and *Leptomysis* sp.) commonly used as prey for adult *H. hippocampus* and *H. guttulatus*. The absolute abundance of DHA in the trunk muscles of male and female *H. hippocampus* may therefore be a consequence of their diet in captivity. Nevertheless, in line with the work developed by Xu et al. (2018), the absolute value of EPA in trunk muscles apparently did not reflect as linearly the dietary regime experienced by seahorses in captivity.

TABLE 2 Lipid indexes of cultured female and male *Hippocampus hippocampus*.

Lipid Index	Female	Male	*p-value*
AI	0.87 ± 0.18	0.75 ± 0.14	1
TI	0.91 ± 0.17	0.84 ± 0.18	1
PI	0.40 ± 0.07	0.52 ± 0.13	0.17
HH	1.06 ± 0.23	1.24 ± 0.25	1

Atherogenic index (AI), thrombogenic index (TI), polyene index (PI) and hypocholesterolemic/hypercholesterolemic index (HH). Mean values ± standard deviation (SD);*p*-values (significant differences when *p*-value ≤ 0.05), from Mann-Whitney U, with Bonferroni corrections.

Trunk muscles of both females and males demonstrated a higher content of *n*-3 PUFA than *n*-6 PUFA. A high content of *n*-3 PUFA in the muscles from males and females results in an *n*-6/*n*-3 ratio lower than 1, highlighting the nutritional potential of *H. hippocampus* (Table 1). Although it has been suggested a minimum *n*-6/*n*-3 ratio of 1:1 for balanced diets, since modern human diets are rich in *n*-6 PUFA, which increases the probability of developing health disorders, such as cardiovascular, inflammatory, and autoimmune diseases (Simopoulos, 2002; Scaioli et al., 2017), an increase in *n*-3 PUFA consumption will contribute to balance the *n*-6/*n*-3 ratio towards a healthier diet (Vallecillos et al., 2021).

The trunk muscles from *H. hippocampus*, which, to our best knowledge, had not yet been described in terms of the FA profile, presented higher absolute compositions of SFA in both sex groups (Table 1). While diet influences the FA composition of the fillets in cultured fish (Vallecillos et al., 2021), Ballester-Lozano et al. (2011) highlight that one must also account for the lipid content of the fillet (neutral and polar fractions), as this is also a source of variability in FA profiles. Triacylglycerols (TAG) are rich in MUFA and SFA, while polar lipids present a high proportion of PUFA (Cowey and Sargent, 1977; Rey et al., 2018; Sushchik et al., 2020; Vallecillos et al., 2021). An increase in fillet lipid content is related to an increase in fat deposits, usually rich in TAG, resulting in higher MUFA and lower PUFA relative abundances (%TFA) (Ballester-Lozano et al., 2011; Vallecillos et al., 2021). Additionally, the study developed by Osako et al. (2003) addressing horse-mackerel (*Trachurus japonicus*) described that the relative abundances of SFA in the muscle's total lipid content decreased with starvation, which was metabolized for energy production. Faleiro and Narciso (2013) reported that SFA abundance was linearly transferred from prey to juvenile *H. guttulatus*, while the transferring of MUFA and PUFA was not straightforward. Since *H. hippocampus* were fed with mysidacea, one of the main prey consumed by seahorses in the wild and that is known to exhibit high relative abundances of PUFA and SFA (Planas et al., 2020), the predominance of these two FA classes in seahorse trunk muscles may reflect their dietary regime.

According to Jobling et al. (1998), the liver and skeletal muscles are important sites for lipid storage, with some differences between fish species. In terms of fat content, fish can be grouped into four categories: lean (< 2%), low fat (2-4%), medium fat (4-8%) and high fat (> 8%) (in terms of wet weight) (Tonial et al., 2014). Since the lipid value of *H. hippocampus* trunk muscle ranged between 4.05 ± 2.15% DW (females) and 2.82 ± 1.48% DW (males) (Supplementary Table S1), this species ranks in the lean category, as our data refer to dried specimens and average moisture content in seahorses represent 75% of their wet weight (Lin et al., 2008; Lin et al., 2009).

Differences in reproductive investment of male and female fish, may lead to different nutritional profiles (Lin et al., 2009). However, this likely depends on the type of breeding strategy exhibited by fishes. Capital breeding is based on the buildup of reserves by breeders, which are allocated for the spawning season, while in the income strategy breeders use the food ingested, directly for reproductive purposes (Sainmont et al., 2014). Studies carried out in the temperate *H. guttulatus* and the tropical *H. reidi* suggested that seahorses display a mixed capital-income strategy, which might be modulated by species-specific differences on inter-batch intervals and temperature thresholds (Planas et al., 2020; Planas et al., 2021). Accordingly, an initial mixed capital-income period is followed by an income breeding period with progressive exhaustion of body reserves. It is likely, however, that most lipid and FA changes on muscle tissue (i.e., a rather conservative tissue) due to dietary resources occur to a much lower extent than in gonads, especially in females. In the case of the trunk muscle, females and males of *H. hippocampus* did not exhibit significant differences between the absolute abundance of FA and FA classes (i.e., SFA, MUFA and PUFA). Lean fish store their lipids in the liver (Njinkoué et al., 2002; Calder, 2013) and, as such, the FA composition of trunk muscles of *H. hippocampus* may be more stable, between sexes. However, additional studies should be performed during the reproductive season, for a better understanding of how FA are mobilized from different tissues during this stage.

The lipid indexes considered in the present study, AI, TI, PI and HH, provide important information on the nutritional value and suitability of nutritional resources for human health (Marques et al., 2019; Conde et al., 2021). Low values of AI and TI are predictors of a low risk for developing cardiovascular diseases in humans (Tonial et al., 2014; Conde et al., 2021). The AI and TI of *H. hippocampus* trunk muscles were higher than those recorded for the muscles of other marine fish species (Rueda et al., 2001). However, male seahorses tended to present lower values for both indexes than females (Table 2). In the case of HH, the higher the value, the more adequate is the fish fat for human nutrition (Tonial et al., 2014). In the present study, males exhibited a tendency for higher HH values, than females. The PI is considered a proxy of lipid oxidation (Marques et al., 2019), expressing the retention of PUFA in relation to more stable FA 16:0 (Chaula et al., 2019). The higher mean values in HH and PI, as well as lower values for AI and TI in males, may suggest that trunk muscles of male seahorses are more suitable for human consumption than those from females. Nonetheless, it is worth highlighting that there were no significant differences between the lipid indexes recorded for both males and females *H. hippocampus* and future analysis should be performed to support these findings.

4.2 Interspecific variability of fatty acid profiles of cultured *Hippocampus* spp.

Fish present different lipid contents according to species-specific factors, their geographical origin and diet, as well as variable intra-specific features, such as size and sexual maturity (Rueda et al., 2001). The relative abundances (%) of FA common to the trunk muscles of *H. hippocampus* and to the whole body of cultured adults *H. kuda*, *H. trimaculatus*, *H. comes* and *H. erectus* described in the literature, were compared. As no significant differences were observed between the FA profiles recorded in trunk muscles of female and male *H. hippocampus*, data from the two sexes were pooled. The seahorse species considered for the interspecific comparison performed in the present study are amongst the most valued syngnathids in TCM (Lin et al., 2008; Chang et al., 2013). Furthermore, they are believed to have several beneficial effects on human health, such as in reducing fatigue and caducity or enhancing immunity (Lin et al., 2008; Lin et al., 2009; Kang et al., 2017), even though there are few scientific studies that have investigated the nutritional value and healthy benefits of *Hippocampus* spp. (Shen et al., 2016).

The essential DHA has been described to play a relevant role in humans' nervous tissues (Crawford et al., 1999; Ahmmed et al., 2020). This FA was amongst the most abundant FA in the whole body of cultured *H. kuda* (27.2% TFA) and *H. trimaculatus* (22.4% TFA) (Figures 2A, B, respectively and Supplementary Table S1). In line with these two species, the DHA levels in *H. hippocampus* trunk muscles (12% TFA) were amongst those of FA with a higher relative abundance, which may indicate that cultured specimens of this species could indeed be an interesting source of DHA for humans. The other two seahorse species considered, revealed lower relative abundances of DHA (5.5% TFA for *H. comes* and 5.6% TFA for *H. erectus*) than *H. hippocampus* trunk muscles (Figures 2C, D, respectively and Supplementary Table S1). Additionally, 18:1 *n*-9 was also one of the most abundant FA, in *H. hippocampus* (11% TFA), *H. comes* (11.4% TFA) and *H. erectus* (20.4% TFA) (Figures 2C, D, respectively and Supplementary Table S1). This FA has modulatory effects in human physiological activities and has been suggested to have a protective role in autoimmune and inflammatory diseases (Sales-Campos et al., 2013). Other important FA to human health that were common in the FA pool of *H. hippocampus*, as well as other seahorse species already farmed, were EPA and ARA (information only available to date for *H. comes* (Buen-Ursua et al., 2015) and *H. erectus* (Vargas-Abúndez et al., 2021)) (Figures 2C, D, respectively and Supplementary Table S1). The relative abundance of EPA was always higher in the four species of seahorses considered than in *H. hippocampus* trunk muscles, while ARA was higher in *H. erectus* (7.5% TFA). Eicosapentaenoic acid has been pointed as potentially having a preventive effect in cardiac diseases (Nestel, 2000; Schunck et al., 2018), while ARA has an important role in the fostering of immunity (Sonnweber et al., 2018).

Total SFA was the most abundant FA class in *H. hippocampus* trunk muscles (58.5% TFA), as well as in three of the four species identified in the literature (*H. kuda*, with 47.8% TFA; *H. trimaculatus*, 47.8% TFA and *H. erectus*, 45.2% TFA). Polyunsaturated fatty acids followed SFA in terms of their mean relative abundances (*H. hippocampus*, 28.1% TFA; *H. kuda*, 33.7% TFA and *H. trimaculatus*, 31.9% TFA), with the exception of *H. erectus*, which presented a high relative abundance of MUFA (31.0% TFA) (Figure 3; Supplementary Table S1). Four of the species considered, including *H. hippocampus* from the present study, were fed with mysids, while *H. erectus* was fed with amphipods. The higher relative abundance of MUFA than PUFA in *H. erectus*, may be related to the diet in captivity, since the fatty acid profile of captured amphipods was richer in SFA, followed by MUFA (Vargas-Abúndez et al., 2021). There were no data for FA classes (% TFA) of *H. comes*. Some of these inter-specific differences might rely, however, on both the effect of temperature and the reproductive stage of the specimens analyzed, factor that deserve further investigation.

Values of PUFA/SFA ratio below 0.45 have been described as not ideal for human health, as they express the potential of increasing cholesterol levels in the blood (Tonial et al., 2014). The trunk muscles of *H. hippocampus* presented a PUFA/SFA ratio of 0.49, while cultured *H. kuda* and *H. trimaculatus* presented values of 0.91 and 0.87, respectively (Supplementary Table S1). Nevertheless, according to Chen and Liu (2020), the protective role of some MUFA, such as 18:1 *n*-9, should also be taken into account, as they contribute to the activity of protein receptors that decrease cholesterol in the serum.

The absolute and relative compositions of *H. hippocampus* trunk muscles may indicate that cultured specimens of this species have the potential to be an interesting source of health-promoting FA, such as DHA, a feature shared with other seahorse species already valued by TCM. While seahorses FA profiles composition may be of interest in terms of their nutritional value, it is important to underline the need to develop sustainable seahorse production practices and foster the conservation of wild populations.

5 Conclusions

Hippocampus hippocampus is a species, for which aquaculture practices are still being established (Planas et al., 2013) and, although it is apparently not targeted for human consumption, there are some reports of illegal capture in the southern coast of mainland Portugal (Planelles, 2017). This preliminary study provided some clues

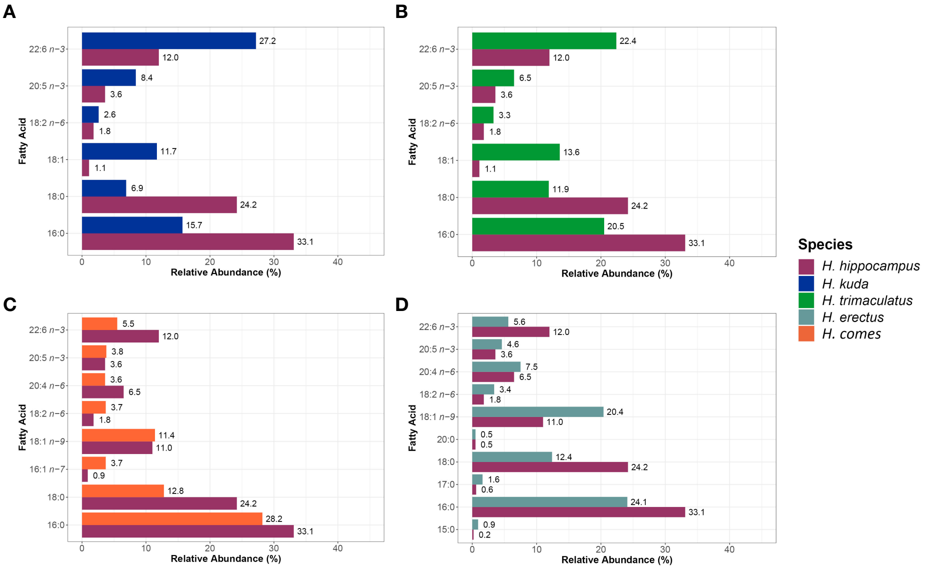

FIGURE 2
Mean fatty acid relative abundances (% of total fatty acids) of trunk muscle of cultured adult short snout seahorses *Hippocampus hippocampus* (n = 14) and whole body of cultured adults of *H. kuda* (n = 8, as described by Lin et al., 2009) **(A)**, *H. trimaculatus* (n = 8, as described by Lin et al., 2009) **(B)**, *H. comes* (n = 6, as described by Buen-Ursua et al., 2015) **(C)** and *H. erectus* (n = 16, as described by Vargas-Abúndez et al., 2021) **(D)**. The mean relative values of fatty acids *from H. kuda* and *H. trimaculatus*, with the exception of 22:6 *n*-3, are estimated values retrieved from bar graphs.

concerning the nutritional potential of the trunk muscles of cultured *H. hippocampus*, with male specimens tending to present the best results in terms of the lipid indexes considered (AI, TI, HH and PI), even though there were no significant differences between the two sexes. Furthermore, this species exhibited some trends already highlighted for other cultured seahorse species valued in TCM, such as high relative abundances of DHA and 18:1 *n*-9 and, to a smaller extent, of EPA and ARA. Cultured *H. hippocampus* may be a sustainable component of functional foods, as long as its production is framed within strict good practices and can be reliably traced from farm to fork to contribute towards the conservation of wild populations of these flagship marine species.

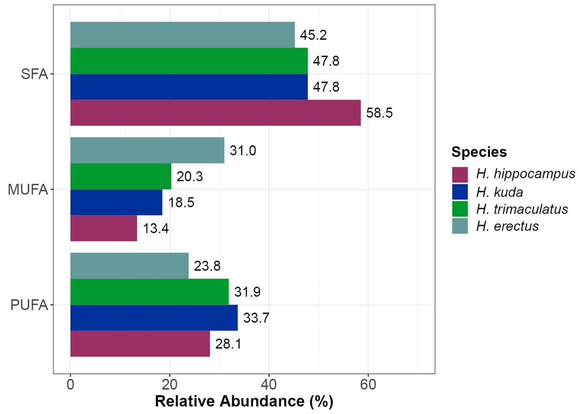

FIGURE 3
Mean relative abundance (% of total fatty acids) of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) of trunk muscle of cultured adult short snout seahorses *Hippocampus hippocampus* (n = 14) and whole body of cultured adults of *H. kuda* (n = 8, as described by Lin et al., 2009), *H. trimaculatus* (n = 8, as described by Lin et al., 2009) and *H. erectus* (n = 16, as described by Vargas-Abúndez et al., 2021). *Hippocampus kuda* and *H. trimaculaus* SFA relative compositions are estimated values retrieved from the data available in Lin et al. (2009).

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical review and approval was not required for the animal study because The seahorses considered in this study were not euthanized and died from natural causes. The captive breeding program for *H. hippocampus* (Project HIPPONUTRE, reference 16-02-01-FMP-54) was approved by the ethics committee from the Veterinary Medicines Directorate, Ministry of Agriculture, Rural Development and Fisheries, Portugal. Under this approval, the program is conducted in accordance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals.

Author contributions

Conceptualization, FR, MRD, and RC; methodology, FR, MRD, and MC; validation, FR, MRD, and MC; formal analysis, AEC, FR, and MC; investigation, AEC, FR, and RC; resources, MRD, JP, MP, and RC; data curation AEC, FR, and MC; writing—original draft preparation, AEC; writing—review and editing, All authors; visualization, AEC; supervision, FR, MP, MRD, and RC; funding acquisition, MRD and RC. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1135250/full#supplementary-material

References

Ahmmed, M. K., Ahmmed, F., Tian, H., Carne, A., and Bekhit, A. E. D. (2020). Marine omega-3 (n-3) phospholipids: a comprehensive review of their properties, sources, bioavailability, and relation to brain health. *Compr. Rev. Food Sci. Food Saf.* 19 (1), 64–123. doi: 10.1111/1541-4337.12510

Amoussou, N., Marengo, M., Iko Afé, O. H., Lejeune, P., Durieux, É.D.H., Douny, C., et al. (2022). Comparison of fatty acid profiles of two cultivated and wild marine fish from Mediterranean Sea. *Aquaculture. Int.* 30, 1435–1452. doi: 10.1007/s10499-022-00861-3

Ballester-Lozano, G. F., Benedito-Palos, L., Navarro, J. C., Kaushik, S., and Pérez-Sánchez, J. (2011). Prediction of fillet fatty acid composition of market-size gilthead sea bream (*Sparus aurata*) using a regression modelling approach. *Aquaculture* 319 (1-2), 81–88. doi: 10.1016/j.aquaculture.2011.06.015

Bartlett, E. M., and Lewis, D. H. (1970). Spectrophotometric determination of phosphate esters in the presence and absence of orthophosphate. *Anal. Biochem.* 36, 159–167. doi: 10.1016/0003-2697(70)90343-X

Bligh, E. G., and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911–917. doi: 10.1139/o59-099

Bordignon, F., Tomás-Vidal, A., Trocino, A., Milián Sorribes, M. C., Jover-Cerdá, M., and Martínez-Llorens, S. (2020). Fatty acid signatures in different tissues of mediterranean yellowtail, *Seriola dumerili* (Risso 1810), fed diets containing different levels of vegetable and fish oils. *Animals* 10 (2), 198. doi: 10.3390/ani10020198

Buen-Ursua, S. M. A., Azuma, T., Arai, K., and Coloso, R. M. (2015). Improved reproductive performance of tiger tail seahorse, *Hippocampus comes*, by mysid shrimp fed singly or in combination with other natural food. *Aquacult. Int.* 23 (1), 29–43. doi: 10.1007/s10499-014-9795-1

Burdge, G. C., and Calder, P. C. (2005). Conversion of α linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reprod. Nutr. Dev.* 45 (5), 581–597. doi: 10.1051/rnd:2005047

Cabral, A. E., Ricardo, F., Patinha, C., Silva, E. F. D., Correia, M., Palma, J., et al. (2021). Successful use of geochemical tools to trace the geographic origin of long-snouted seahorse *Hippocampus guttulatus* raised in captivity. *Animals* 11 (6), 1534. doi: 10.3390/ani11061534

Calder, P. C. (2013). “Nutritional benefits of omega-3 fatty acids,” in *Food enrichment with omega-3 fatty acids*. Eds. C. Jacobsen, N. S. Nielsen, A. F. Horn and A. D. M. Sørensen (Cambridge, GB: Woodhead Publishing), 3–26.

Caldwell, I. R., and Vincent, A. C. J. (2012). Revisiting two sympatric European seahorse species: apparent decline in the absence of exploitation. *Aquat. Conserv: Mar. Freshw. Ecosyst.* 22, 427–435. doi: 10.1002/aqc.2238

Chang, C. H., Jang-Liaw, N. H., Lin, Y. S., Fang, Y. C., and Shao, K. T. (2013). Authenticating the use of dried seahorses in the traditional Chinese medicine market in Taiwan using molecular forensics. *J. Food Drug Anal.* 21 (3), 310–316. doi: 10.1016/j.jfda.2013.07.010

Chaula, D., Laswai, H., Chove, B., Dalsgaard, A., Mdegela, R., and Hyldig, G. (2019). Fatty acid profiles and lipid oxidation status of sun dried, deep fried, and smoked sardine (*Rastrineobola argentea*) from lake Victoria, tanzania. *J. Aquat. Food Prod. Technol.* 28 (2), 165–176. doi: 10.1080/10498850.2019.1570992

Chen, J., and Liu, H. (2020). Nutritional indices for assessing fatty acids: A mini-review. *Int. J. Mol. Sci.* 21 (16), 5695. doi: 10.3390/ijms21165695

Cohen, F. P., Valenti, W. C., Planas, M., and Calado, R. (2017). Seahorse aquaculture, biology and conservation: knowledge gaps and research opportunities. *Rev. Fish. Sci. Aquac.* 25 (1), 100–111. doi: 10.1080/23308249.2016.1237469

Conde, T. A., Neves, B. F., Couto, D., Melo, T., Neves, B., Costa, M., et al. (2021). Microalgae as sustainable bio-factories of healthy lipids: evaluating fatty acid content and antioxidant activity. *Mar. Drugs* 19 (7), 357. doi: 10.3390/md19070357

Correia, M., Koldewey, H. J., Andrade, J. P., Esteves, E., and Palma, J. (2018). Identifying key environmental variables of two seahorse species (*Hippocampus guttulatus* and *Hippocampus hippocampus*) in the ria Formosa lagoon, south Portugal. *Environ. Biol. Fishes* 101 (9), 1357–1367. doi: 10.1007/s10641-018-0782-7

Cowey, C. B., and Sargent, J. R. (1977). Lipid nutrition in fish. *Comp. Biochem. Physiol.* 57 (4), 269–273. doi: 10.1016/0305-0491(77)90051-7

Crawford, M. A., Bloom, M., Broadhurst, C. L., Schmidt, W. F., Cunnane, S. C., Galli, C., et al. (1999). Evidence for the unique function of docosahexaenoic acid during the evolution of the modern hominid brain. *Lipids* 34, S39–S47. doi: 10.1007/BF02562227

Curtis, J. M. R., Santos, S. V., Nadeau, J. L., Gunn, B., Bigney Wilner, K., Balasubramanian, H., et al. (2017). Life history and ecology of the elusive European short-snouted seahorse *Hippocampus hippocampus*. *J. Fish Biol.* 91 (6), 1603–1622. doi: 10.1111/jfb.13473

Doubleday, Z. A., Martino, J. C., and Trueman, C. (2022). Harnessing universal chemical markers to trace the provenance of marine animals. *Ecol. Indic.* 144, 109481. doi: 10.1016/j.ecolind.2022.109481

Faleiro, F., and Narciso, L. (2013). Prey–predator dynamics in seahorses (*Hippocampus guttulatus*): deciphering fatty acid clues. *Aquac. Res.* 44 (4), 618–633. doi: 10.1111/j.1365-2109.2011.03067.x

Foster, S. J., Kuo, T. C., Wan, A. K. Y., and Vincent, A. C. (2019). Global seahorse trade defies export bans under CITES action and national legislation. *Mar. Policy* 103, 33–41. doi: 10.1016/j.marpol.2019.01.014

Ghasemi, A., Ghavimi, R., Momenzadeh, N., Hajian, S., and Mohammadi, M. (2021). Characterization of antitumor activity of a synthetic moronecidin-like peptide computationally predicted from the tiger tail seahorse *Hippocampus comes* in tumor-bearing mice. *Int. J. Pept. Res. Ther.* 27, 2391–2401. doi: 10.1007/s10989-021-10260-6

Himaya, S. W. A., Ryu, B., Qian, Z. J., and Kim, S. K. (2012). Paeonol from *Hippocampus kuda* bleeler suppressed the neuro-inflammatory responses *in vitro via* NF-κB and MAPK signaling pathways. toxicol. *In Vitro* 26 (6), 878–887. doi: 10.1016/j.tiv.2012.04.022

Huang, X., Yin, Y., Shi, Z., Li, W., Zhou, H., and Lv, W. (2010). Lipid content and fatty acid composition in wild-caught silver pomfret (*Pampus argenteus*) broodstocks: effects on gonad development. *Aquaculture* 310 (1-2), 192–199. doi: 10.1016/j.aquaculture.2010.10.015

Jobling, M., Johansen, S. J. S., Foshaug, H., Burkow, I. C., and Jørgensen, E. H. (1998). Lipid dynamics in anadromous Arctic charr, *Salvelinus alpinus* (L.): seasonal variations in lipid storage depots and lipid class composition. *Fish. Physiol. Biochem.* 18 (3), 225–240. doi: 10.1023A:1007747201521

Johnston, T. A., Wiegand, M. D., Szmadyla, R. L., Porteous, L. R., Casselman, J. M., and Leggett, W. C. (2020). Sex-based differences in fatty acid composition of adult walleye. *Ecol. Freshw. Fish* 29 (4), 654–664. doi: 10.1111/eff.12541

Kang, N., Kim, S. Y., Rho, S., Ko, J. Y., and Jeon, Y. J. (2017). Anti-fatigue activity of a mixture of seahorse (*Hippocampus abdominalis*) hydrolysate and red ginseng. *Fish Aquat. Sci.* 20, 1–8. doi: 10.1186/s41240-017-0048-x

Kim, H. S., Kim, S. Y., Fernando, I. S., Sanjeewa, K. A., Wang, L., Lee, S. H., et al. (2019). Free radical scavenging activity of the peptide from the alcalase hydrolysate of the edible aquacultural seahorse (*Hippocampus abdominalis*). *J. Food Biochem.* 43 (7), e12833. doi: 10.1111/jfbc.12833

Koldewey, H. J., and Martin-Smith, K. M. (2010). A global review of seahorse aquaculture. *Aquaculture* 302 (3-4), 131–152. doi: 10.1016/j.aquaculture.2009.11.010

Leal, M. C., Pimentel, T., Ricardo, F., Rosa, R., and Calado, R. (2015). Seafood traceability: current needs, available tools, and biotechnological challenges for origin certification. *Trends Biotechnol.* 33 (6), 331–336. doi: 10.1016/j.tibtech.2015.03.003

Lin, Q., Lin, J., Lu, J., and Li, B. (2008). Biochemical composition of six seahorse species, hippocampus sp., from the Chinese coast. *J. World Aquac. Soc* 39 (2), 225–234. doi: 10.1111/j.1749-7345.2008.00159.x

Lin, Q., Lin, J., and Wang, C. (2009). Biochemical composition of the wild and cultured seahorses, *Hippocampus kuda* bleeker and *Hippocampus trimaculatus* leach. *Aquac. Res.* 40 (6), 710–719. doi: 10.1111/j.1365-2109.2008.02149.x

Lourie, S. (2003). "Measuring seahorses," in *[Project seahorse technical report No.4]*, vol. 15. (University of British Columbia Fisheries Centre).

Lubis, Z., and Buckle, K. A. (1990). Rancidity and lipid oxidation of dried-salted sardines. *Int. J. Food Sci. Technol.* 25, 295–303. doi: 10.1111/j.1365-2621.1990.tb01085.x

Marques, B., Lillebø, A. I., Domingues, M. D. R. M., Saraiva, J. A., and Calado, R. (2019). Effect of high-pressure processing (HPP) on the fatty acid profile of different sized ragworms (*Hediste diversicolor*) cultured in an integrated multi-trophic aquaculture (IMTA) system. *Molecules* 24 (24), 4503. doi: 10.3390/molecules24244503

Monge-Ortiz, R., Tomás-Vidal, A., Rodriguez-Barreto, D., Martínez-Llorens, S., Pérez, J. A., Jover-Cerdá, M., et al. (2018). Replacement of fish oil with vegetable oil blends in feeds for greater amberjack (*Seriola dumerili*) juveniles: effect on growth performance, feed efficiency, tissue fatty acid composition and flesh nutritional value. *Aquac. Nutr.* 24 (1), 605–615. doi: 10.1111/anu.12595

Nestel, P. J. (2000). Fish oil and cardiovascular disease: lipids and arterial function. *Am. J. Clin. Nutr.* 71 (1), 228S–231S. doi: 10.1093/ajcn/71.1.228S

Njinkoué, J. M., Barnathan, G., Miralles, J., Gaydou, E. M., and Samb, A. (2002). Lipids and fatty acids in muscle, liver and skin of three edible fish from the Senegalese coast: *Sardinella maderensis*, *Sardinella aurita and cephalopholis taeniops*. comp. *Biochem. Physiol. - B Biochem. Mol. Biol.* 131 (3), 395–402. doi: 10.1016/S1096-4959(01)00506-1

Olivotto, I., Planas, M., Simões, N., Holt, G. J., Avella, M. A., and R. Calado, R. (2011). Advances in breeding and rearing marine ornamentals. *J. World Aquacult. Soc* 42, 135–166. doi: 10.1111/j.1749-7345.2011.00453.x

Osako, K., Kuwahara, K., Saito, H., Hossain, M. A., and Nozaki, Y. (2003). Effect of starvation on lipid metabolism and stability of DHA content of lipids in horse mackerel (*Trachurus japonicus*) tissues. *Lipids* 38 (12), 1263–1267. doi: 10.1007/s11745-003-1187-5

Planas, M., Burhans, R., and Simões, N. (2017). "Seahorses and pipefish," in *Marine ornamental species aquaculture*. Eds. R. Calado, I. Olivotto, M. Planas and G. J. Holt (West Sussex, UK: Wiley Blackwell), 299–326.

Planas, M., Olivotto, I., González, M. J., Laurà, R., Angeletti, C., Amici, A., et al. (2021). Pre-breeding diets in the seahorse *Hippocampus reidi*: how do they affect fatty acid profiles, energetic status and histological features in newborn? *Front. Mar. Sci.* 8, 688058. doi: 10.3389/fmars.2021.688058

Planas, M., Olivotto, I., González, M. J., Laurà, R., and Zarantoniello, M. (2020). A multidisciplinary experimental study on the effects of breeders diet on newborn seahorses (*Hippocampus guttulatus*). *Front. Mar. Sci.* 7, 638. doi: 10.3389/fmars.2020.00638

Planas, M., Quintas, P., and Chamorro, A. (2013). Maturation of *Hippocampus guttulatus* and *Hippocampus hippocampus* females by manipulation of temperature and photoperiod regimes. *Aquaculture* 388, 147–152. doi: 10.1016/j.aquaculture.2013.01.030

Planelles, M. (2017). *El Lucrativo contrabando con caballitos de mar* (El País). Available at: https://elpais.com/politica/2017/01/16/actualidad/1484595017_603568.html.

Rey, F., Alves, E., Domingues, P., Domingues, M. R. M., and Calado, R. (2018). A lipidomic perspective on the embryogenesis of two commercially important crabs, *Carcinus maenas* and *Necora puber*. bull. *Mar. Sci.* 94 (4), 1395–1411. doi: 10.5343/bms.2017.1140

RStudio Team (2019). *RStudio: integrated development for r*. Ed. M. A. Boston (RStudio, Inc.). Available at: http://www.rstudio.com/.

Rueda, F. M., Hernández, M. D., Egea, M. A., Aguado, F., García, B. , and Martínez, F. J. (2001). Differences in tissue fatty acid composition between reared and wild sharpsnout sea bream, *Diplodus puntazzo* (Cetti 1777). *Brit. J. Nutr.* 86 (5), 617–622. doi: 10.1079/BJN2001438

Sainmont, J., Andersen, K. H., Varpe, Ø., and Visser, A. W. (2014). Capital versus income breeding in a seasonal environment. *Am. Nat.* 184 (4), 466–476. doi: 10.1086/677926

Sales-Campos, H., Reis de Souza, P., Crema Peghini, B., Santana da Silva, J., and Ribeiro Cardoso, C. (2013). An overview of the modulatory effects of oleic acid in health and disease. mini-rev. *Med. Chem.* 13 (2), 201–210. doi: 10.2174/1389557511313020003

Scaioli, E., Liverani, E., and Belluzzi, A. (2017). The imbalance between n-6/n-3 polyunsaturated fatty acids and inflammatory bowel disease: a comprehensive review and future therapeutic perspectives. *Int. J. Mol. Sci.* 18 (12), 2619. doi: 10.3390/ijms18122619

Schunck, W. H., Konkel, A., Fischer, R., and Weylandt, K. H. (2018). Therapeutic potential of omega-3 fatty acid-derived epoxyeicosanoids in cardiovascular and inflammatory diseases. *Pharmacol. Ther.* 183, 177–204. doi: 10.1016/j.pharmthera.2017.10.016

Shen, Q., Dai, Z., Huang, Y. W., and Cheung, H. Y. (2016). Lipidomic profiling of dried seahorses by hydrophilic interaction chromatography coupled to mass spectrometry. *Food Chem.* 205, 89–96. doi: 10.1016/j.foodchem.2016.02.151

Simopoulos, A. P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed. Pharmacother.* 56 (8), 365–379. doi: 10.1016/S0753-3322(02)00253-6

Sonnweber, T., Pizzini, A., Nairz, M., Weiss, G., and Tancevski, I. (2018). Arachidonic acid metabolites in cardiovascular and metabolic diseases. *Int. J. Mol. Sci.* 19 (11), 3285. doi: 10.3390/ijms19113285

Sushchik, N. N., Makhutova, O. N., Rudchenko, A. E., Glushchenko, L. A., Shulepina, S. P., Kolmakova, A. A., et al. (2020). Comparison of fatty acid contents in major lipid classes of seven salmonid species from Siberian Arctic lakes. *Biomolecules* 10 (3), 419. doi: 10.3390/biom10030419

The IUCN Red List of Threatened Species. (2017a). *Hippocampus hippocampus (Global assessment)*. Available at: https://www.iucnredlist.org/species/10069/67618259 (Accessed November 14, 2022).

The IUCN Red List of Threatened Species. (2017b). *Hippocampus hippocampus (Mediterranean assessment)*. Available at: https://www.iucnredlist.org/species/10069/90866381 (Accessed December 1, 2022).

Tocher, D. R. (2015). Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. *Aquaculture* 449, 94–107. doi: 10.1016/j.aquaculture.2015.01.010

Tonial, I. B., Oliveira, D. F., Coelho, A. R., Matsushita, M., Coró, F. A. G., De Souza, N. E., et al. (2014). Quantification of essential fatty acids and assessment of the nutritional quality indexes of lipids in tilapia alevins and juvenile tilapia fish (*Oreochromis niloticus*). *J. Food Res.* 3 (3), 105–114. doi: 10.5539/jfr.v3n3p105

Ulbricht, T. L. V., and Southgate, D. A. T. (1991). Coronary heart disease: seven dietary factors. *Lancet* 338, 985–992. doi: 10.1016/0140-6736(91)91846-M

Vaidyanathan, T., Zhang, X., Balakrishnan, R., and Vincent, A. (2021). Catch and trade bans for seahorses can be negated by non-selective fisheries. *Aquat. Conserv.: Mar. Freshw. Ecosyst.* 31 (1), 43–59. doi: 10.1002/aqc.3419

Vallecillos, A., Marín, M., Bortoletti, M., López, J., Afonso, J. M., Ramis, G., et al. (2021). Genetic analysis of the fatty acid profile in gilthead seabream (*Sparus aurata* l.). *Animals* 11 (10), 2889. doi: 10.3390/ani11102889

Vargas-Abúndez, J. A., Martínez-Moreno, G. L., Simões, N., Noreña-Barroso, E., and Mascaró, M. (2021). Marine amphipods (*Parhyale hawaiensis*) as an alternative feed for the lined seahorse (*Hippocampus erectus*, perri 1810): nutritional value and feeding trial. *PeerJ* 9, e12288. doi: 10.7717/peerj.12288

Vincent, A. C. (1996). “The international trade in seahorses,” in *[Traffic network report]* (Cambridge, UK: Traffic International).

Vincent, A. C., Foster, S. J., and Koldewey, H. J. (2011). Conservation and management of seahorses and other syngnathidae. *J. Fish Biol.* 78 (6), 1681–1724. doi: 10.1111/j.1095-8649.2011.03003.x

Vincent, A. C., Meeuwig, J. J., Pajaro, M. G., and Perante, N. C. (2007). Characterizing a small-scale, data-poor, artisanal fishery: seahorses in the central Philippines. *Fish. Res.* 86 (2-3), 207–215. doi: 10.1016/j.fishres.2007.06.006

Wickham, H. (2016). *Ggplot2: elegant graphics for data analysis. 2nd ed* (New York, USA: Springer International Publishing).

Woods, C. M. (2003). Effects of varying artemia enrichment on growth and survival of juvenile seahorses, *Hippocampus abdominalis*. *Aquaculture* 220 (1-4), 537–548. doi: 10.1016/S0044-8486(02)00639-7

Xu, H., Cao, L., Wei, Y., Zhang, Y., and Liang, M. (2018). Lipid contents in farmed fish are influenced by dietary DHA/EPA ratio: a study with the marine flatfish, tongue sole (*Cynoglossus semilaevis*). *Aquaculture* 485, 183–190. doi: 10.1016/j.aquaculture.2017.11.054

Xu, Y., Ryu, S., Lee, Y. K., and Lee, H. J. (2020). Brassicasterol from edible aquacultural *Hippocampus abdominalis* exerts an anti-cancer effect by dual-targeting AKT and AR signaling in prostate cancer. *Biomedicines* 8 (9), 370. doi: 10.3390/biomedicines8090370

Zhang, F., Liu, J., Bi, Q., Ma, Q., Wei, Y., Liang, M., et al. (2022). Sex difference in fatty acid composition of six marine teleosts. *J. Fish Biol* 101 (6), 1606–1610. doi: 10.1111/jfb.15220

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Local ecological knowledge provides important conservation guidelines for a threatened seahorse species in mangrove ecosystems

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Local Ecological Knowledge and social perceptions of human communities that depend on natural resources can provide important baseline information on local threats and impacts at a fine scale for conservation management. Seahorses play important economic role in Brazilian mangroves, being sought-after for seahorse-watching tours. This touristic activity is not yet formally regulated and our work is a contribution to seahorse-watching management at three study sites located in two Marine Protected Areas (MPAs) – Jericoacoara National Park, and Delta do Parnaíba Environmental Protection Area – in the Brazilian northeast states of Ceará, Piauí, and Maranhão. Through semi-structured interviews, we investigated the perception of seahorse-watching operators regarding seahorse abundance, local threats, and conservation insights in mangrove ecosystems. We interviewed all 38 existing informants at the study sites. Approximately half of the informants rely exclusively on seahorse-watching as an income source. Overall, 55.3% highlighted declines in seahorse abundance, 100% believed that seahorses are threatened with extinction. Silting (57.9%), fishing (55.3%), and pollution (37%) were cited as main threats to seahorses and mangroves. Among the proposed conservation actions, environmental surveillance (57.9%), zoning (26.3%), education (26.3%) were the most cited. Respondents support the MPAs but would like to see greater engagement of management institutions towards the communities, considering their local needs. Some differences in perception between communities reflect local realities and social contexts, which should be considered for conservation and management effectiveness. Our study contributes supporting this approach, as local scale studies on social perceptions can improve conservation and local management, especially for data-poor species.

KEYWORDS

Syngnathidae, *Hippocampus reidi*, tourism, management, threats, Jericoacoara National Park, Delta do Parnaíba Environmental Protection Area, Brazil

1 Introduction

Local Ecological Knowledge (LEK) and social perceptions from human communities that depend on the exploitation of natural resources for their subsistence can provide important baseline information when there is a lack of scientific data, informing threats and impacts at a finer scale that can help inform management activities (Bennett, 2016; Cortés-Avizanda et al., 2018; Cullen-Unsworth et al., 2018). Mangroves are sensitive coastal ecosystems that play a critical social and ecological role through the provisioning of vital ecosystem services, alongside being an important nursery environment for marine life (Ferreira and Lacerda, 2016). Among numerous species inhabiting mangroves, seahorses are considered a threatened flagship species for marine conservation. Human activities such as fishing, pollution and habitat degradation are responsible for seahorse declines worldwide (Foster and Vincent, 2004; Vincent et al., 2011; Zhang and Vincent, 2019). Seahorse conservation is urgent given the species depletion, besides human economic activities that depend on their presence in suitable habitats (Foster and Vincent, 2004; Vincent et al., 2011).

The long-snout seahorse (*Hippocampus reidi*) is a threatened species (MMA, Ministério do Meio Ambiente 2014) which plays an important economic role being sought-after for seahorse-watching tours on Brazilian mangroves (Ternes et al., 2016). Local communities offer boat tours inside mangrove areas for tourists to observe seahorses which are commonly found attached to mangrove roots. When found, seahorses are manually captured and transferred with water into a transparent glass recipient to be shown to tourists, and then they are returned to their natural environment. This activity is not yet formally regulated, and our study is a contribution for seahorse-watching management in communities of two Marine Protected Areas (MPAs) in Brazil: Jericoacoara National Park (JNP) and Delta do Parnaíba Environmental Protection Area (DPEPA). Both MPAs are the home habitat of the long-snout seahorse, *Hippocampus reidi* (Mai and Rosa, 2009; Martins et al., 2022), and are equally extremely popular tourist destinations where local human communities depend on this species and the mangrove for touristic activities as an income source. Our study aimed to provide conservation guidelines for a threatened seahorse species in ecologically important mangrove ecosystems from the Northeastern Brazilian coast, investigating the perceptions of seahorse-watching operators regarding seahorse abundance trends, local threats, and conservation insights.

2 Method

2.1 Study site

This study focused on three sites on the northeastern Brazilian Atlantic coast where seahorse-watching takes place (states of Ceará, Piauí, and Maranhão), located within two MPAs: Jericoacoara National Park (JNP) and Delta do Parnaíba Environmental Protection Area (DPEPA) (Figure 1). JNP is a no-take protected area created in 2002, surrounding the Guriú River estuary (02°52’S; 40°34’W), bordering the municipalities of Jijoca de Jericoacoara and Camocim, in Ceará. DPEPA is a partially protected area created in 1996, encompassing the region of Cajueiro da Praia municipality in Piauí (02°55’S, 41°24’W), and Tutóia municipality in Maranhão (2°45’S, 42°16’W).

Both MPAs are 160km apart, but share common features, including mangroves, dunefields and sandy beaches with a tidal

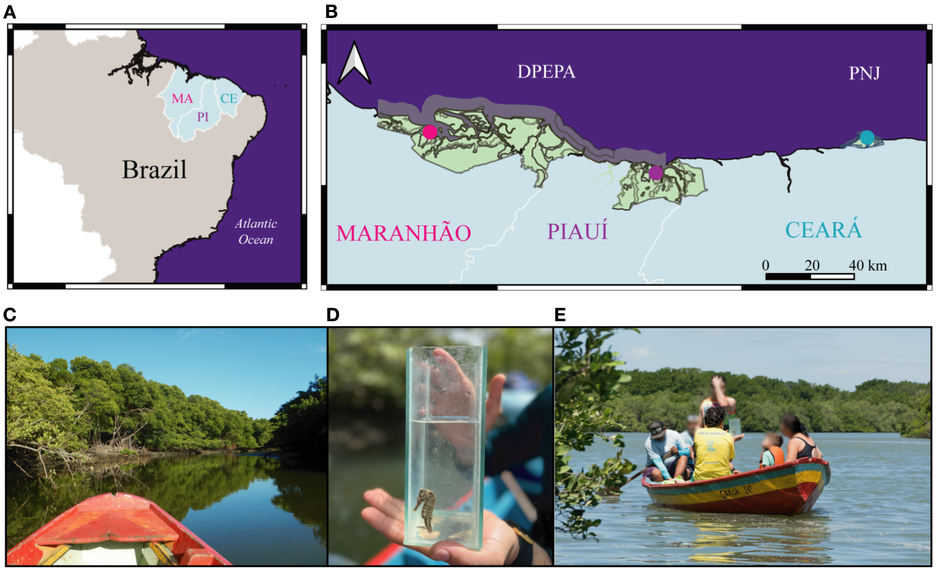

FIGURE 1
(A, B) Study areas, northeastern Brazil. PNJ, Jericoacoara Nacional Park. DPEPA, the Delta do Parnaíba Environmental Protection Area. Communities surveyed are represented by colored dots: Mangue Seco in the state of Ceará (turquoise), Cajueiro da Praia, in Piauí (violet), and Tutóia in Maranhão (pink). **(C–E)** seahorse-watching activities on mangroves.

amplitude of up to 3 meters. Wind velocities are high, driving erosion, transportation and deposition of sediment by strong wind (mean = 7.8 $m.s^{-1}$), with a dry season between August and December, and a rainy season from January through July (Hesp et al., 2009). The main economic activities are tourism, artisanal fishing, shrimp farming, and livestock.

2.2 Data collection

This ethnoecological study was approved by the Research Ethics Committee of Universidade Federal do Pará (CAAE 99615418.1.0000.0018) and authorized by Sistema de Autorização e Informação em Biodiversidade - SISBIO permits #56811-1 and #67298-1. Semi-structured interviews were applied individually under previous consent of each seahorse-watching operator in Ceará, Piauí and Maranhão. Following Ternes et al. (2016) our questionnaire encompassed the interviewee perceptions on seahorse abundance, local threats, and conservation insights.

2.3 Data analysis

We used word cloud to present the perception of threats to seahorses and their environment, as well as conservation insights by seahorse-watching operators for each locality. The method is a visual presentation distinguishing words more and less frequently used (McNaught and Lam, 2010), herein representing a pattern of shared ideas in response to the following questions: (1) What are the local threats to seahorses and their environment? and (2) What should be done to conserve seahorses and their environment? Responses were tabulated, and non-informative elements such as "stop words" were removed. Words were ranked by frequency of use. Analyses were carried out using the R software, v 4.0.3, packages "tm", "wordcloud" (R Core Team, 2020). Responses were further summarized and fitted into categories to create a Venn Diagram for an integrative comparison, illustrating intersections and particularities between the study sites.

3 Results

3.1 Socioeconomic profile

We interviewed 38 informants encompassing 100% of the total 12 seahorse-watching operators in JNP in 2016 and 100% of the total 26 operators in DPEPA in 2019–2020. Seahorse-watching is a community-based tourism activity held in small and isolated coastal villages, carried out for just a few local operators, despite being situated in extremely popular tourist destinations. Interviewees were all male, age ranging from 16 to 50 years (mean = 32 ± 8.9 years ± s.d.), with seahorse-watching experience of 6–11 years in JNP (mean= 8.3 ± 3.0 years) and three months to 15 years in DPEPA (mean= 6.6 ± 4.6 years). Approximately half of the informants rely exclusively on seahorse-watching as an income source, while others even depending on this tourism have also complementary economic activities (Table S1, Supplementary Material).

3.2 Perceptions on seahorse abundance trends

All informants believe seahorses are threatened and can become extinct. In general, most informants (55.3%, n = 21/38) highlighted population decline. This trend was reported by all Ceará and the majority of Piauí informants. Each estuary has its own particularities regarding natural dynamics and anthropogenic stressors, influencing abundance trend perceptions (Table S2, Supplementary Material).

3.3 Perceptions on local threats to seahorses and mangroves

The most cited threats among all informants (Figures 2, 3) were: silting in the mangrove (58% n= 22/38); anthropogenic impacts, such as fishing (55% n= 21/38, mostly illegal fishing); trash, mostly plastic (37% n= 14/38); motor boat impacts such as erosion from wake wash, oil, and noise (34% n= 13/38); shrimp farming (21% n= 8/38).

Regarding exclusive local perceptions (Figures 2A–C), Ceará reported fire in mangrove forests, seahorse population decline and lack of environmental surveillance as local threats. Piauí quoted uncontrolled urbanization, jet skis and diving in seahorse-watching area as also having impacts. Maranhão reported specific threats related to illegal/destructive fishing practices, including trawling and a regional, non-selective type of net, known as *zangaria*, which is used as a weir, with mesh size smaller than permitted by law (SUDEPE, 1988).

3.4 Conservation insights

Environmental surveillance (58% n= 22/38), zoning (26% n= 10/38), educational approaches towards conservation (26% n= 10/38), seahorse-watching tourism regulation (18% n= 7/38), and enhancement of seahorse populations and habitats (16% n= 6/38), were overall consensus categories of recommendation at all sites (Figure 4). Considering each study site reality, the need for scientific research through seahorse monitoring was highlighted in Ceará, actions against plastics pollution in Piauí, and the request for alternative income sources in Maranhão (Figures 2B, D, F).

4 Discussion

4.1 Perceptions on seahorse abundance trends

All informants believe seahorses are threatened and can go extinct, matching national and international red lists where

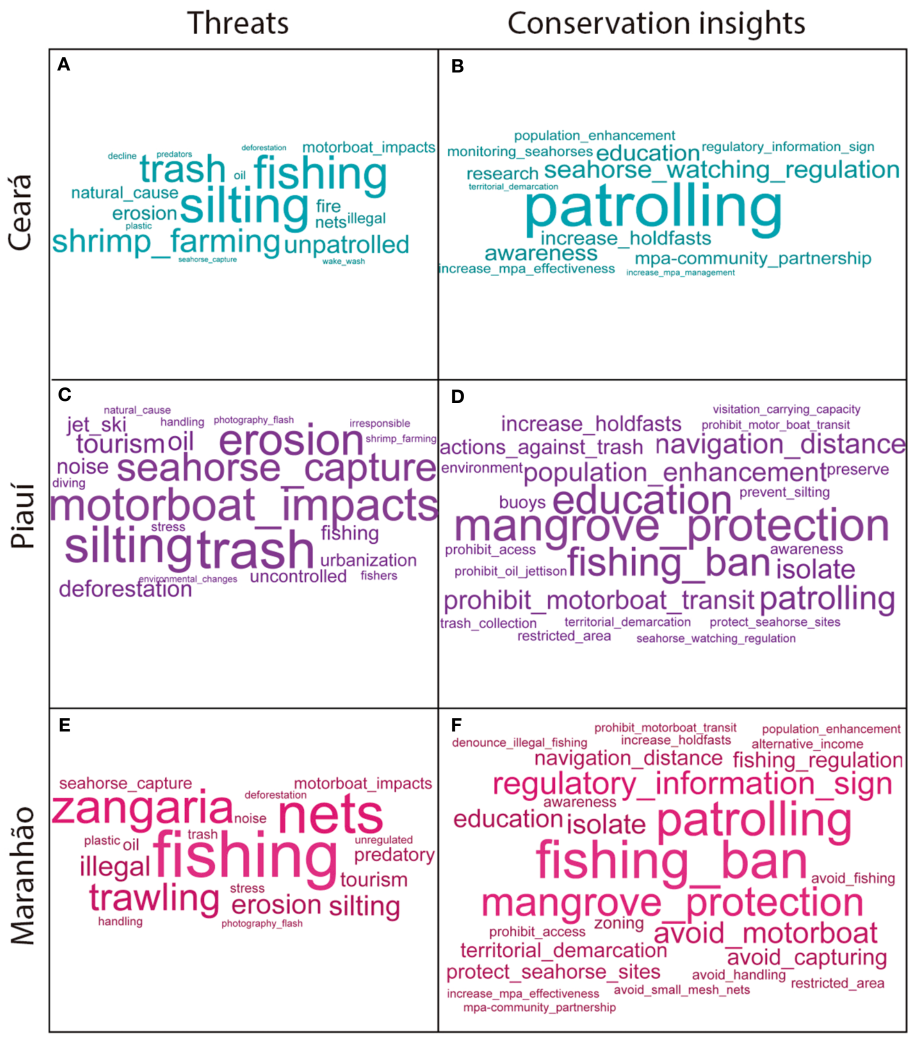

FIGURE 2
Word clouds based on interviewees' perception of seahorse local threats **(A)** Ceará; **(C)** Piaui; **(E)** Maranhão) and insights for conservation **(B)** Ceará; **(D)** Piauí; **(F)** Maranhão). The word size represents its frequency of occurrence, larger words express main concerns.

Hippocampus reidi is under Vulnerable and Near Threatened categories, respectively (MMA ordinance 445/2014; IUCN 2017). For our study site, population assessments have been ongoing (Ternes et al. *in prep*.) to compare current population parameters with past biological data available from 2006 and 2007 (Mai and Rosa, 2009), suggesting current abundance declines, besides short-term variations also perceived by Piauí and Maranhão informants (Table S2).

4.2 Perceptions on local threats to seahorses and mangroves

The impacts perceived by the informants represent threats globally reported for seahorses (Foster and Vincent, 2004; Vincent et al., 2011). Brazil is the third country in mangrove extension on Earth, but aquaculture, urbanization, industrial development among others, have devastated more than 50,000 ha of Brazilian mangroves over the past three decades (Ferreira and Lacerda, 2016). Seahorses are sedentary fish that rely on mangrove vegetation structures, such as roots and branches, as anchoring points (Foster and Vincent, 2004; Mai and Rosa, 2009). Habitat loss is herein represented by geological processes such as sand sedimentation onto mangrove areas, interpreted by interviewees as a coastal dynamics' natural phenomenon ("natural cause" Figure 2), besides other anthropic impacts such as deforestation. Erosion of mangrove margins caused by wake wash from motor boat traffic, includes consequent noise perturbation and occasional oil spill that can negatively impact seahorses causing physiological/behavioral stress, and site abandonment (e.g., Palma et al., 2019; Delunardo et al., 2020).

The three most cited threats in our study - silting (habitat alteration), fishing, and pollution (trash) - correspond to the top three public perceptions of marine threats around the world (Lotze

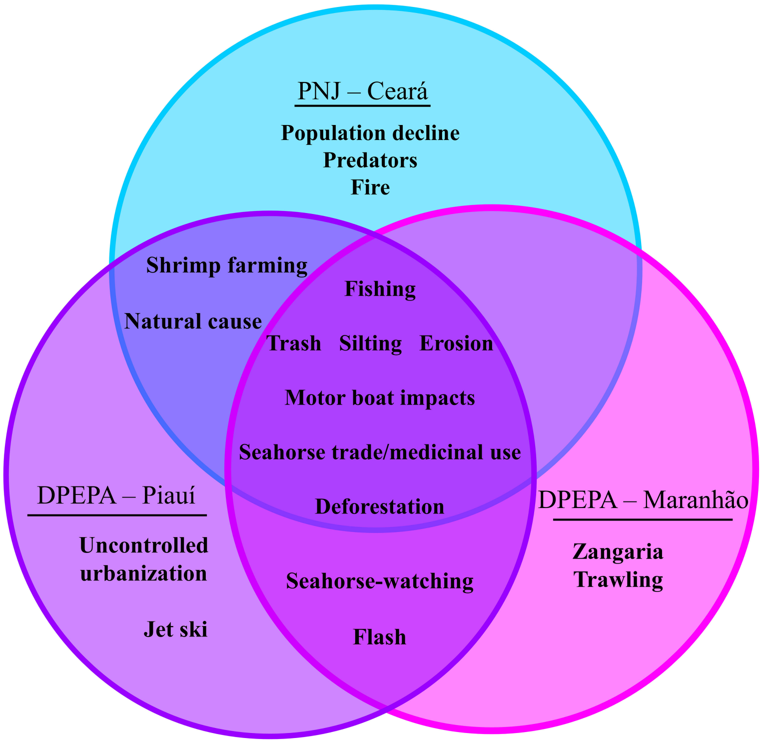

FIGURE 3
Venn diagram of principal categories of threat perceived by the informants, illustrating intersections and particularities between study sites.

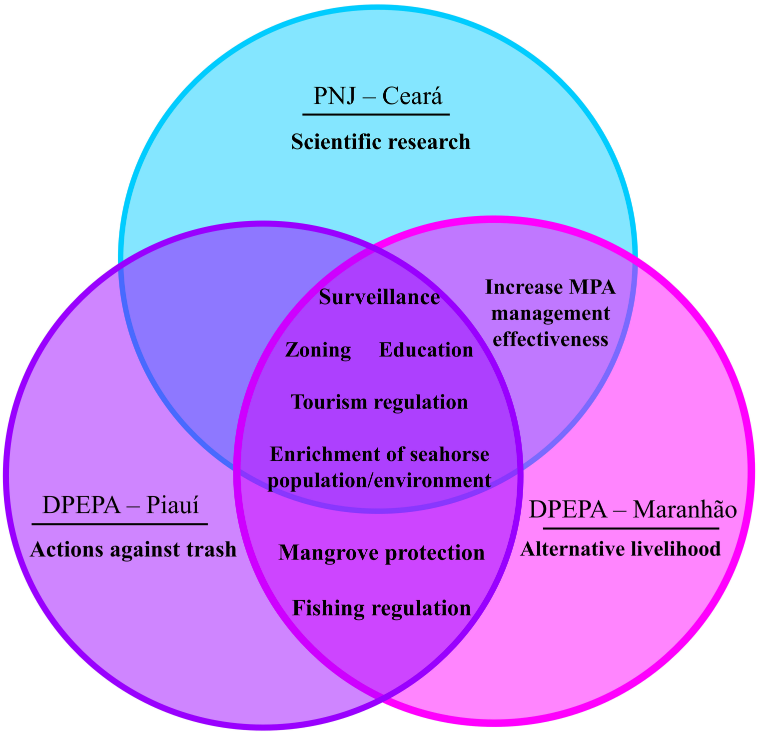

FIGURE 4
Venn Diagram of principal categories of conservation insights provided by the informants, illustrating intersections and particularities between study sites.

et al., 2018), reflecting global threats being locally reproduced. A cumulative human-impact study also highlighted fishing and pollution as most important anthropogenic stressors affecting seahorses worldwide (Zhang and Vincent, 2019).

In our study sites, there was a conflict related to fishing practices, mostly illegal and destructive ("predatory" Figure 2E), denunciating lack of both environmental surveillance ("unpatrolled" Figure 2A) and community compliance. In addition, all sites reported illegal seahorse capture for commercial or medicinal use, despite being prohibited by federal law since 2014 (MMA ordinance 445/2014). This could be driven by socioeconomic vulnerability or even unawareness towards fishing regulations in the two protected areas. In MPAs in developing countries such as Brazil, socioeconomic risks (i.e.,poverty, social inequality, lack of basic rights such as education, health) are linked to dependency on natural resources, which exacerbates environmental threats (Oliveira-Júnior et al., 2021).

Marine pollution by plastics is an extremely visible environmental impact also emphasized by the media and environmental campaigns (Lotze et al., 2018). Seahorses are particularly vulnerable to an invisible consequence of that: microplastic pollution. Since newborn on early developing stages, seahorses can ingest microplastics, accumulating particles that may cause physical obstructions in their guts (Domínguez-López et al., 2022).

Shrimp farming is a sensitive issue on Ceará and Piauí (Figures 2A, C). Ceará informants witnessed massive mortality of fish in 2014 and during our data collection, when the estuary water became "milky", and many fish, including seahorses, were found dead (authors' pers. obs.). Informants linked this mortality to effluent discharge by an upstream shrimp farm. Major shrimp farming environmental impacts include effluents pollution, loss of ecosystem services, carbon storage depletion, biodiversity loss, also threatening the survival of local human populations that rely on mangrove ecosystem for subsistence (Lacerda et al, 2021). Many Brazilian shrimp farms were established prior to the creation of environmental regulations. The inefficiency of legislation compliance and lack of participatory environmental management including local communities, have aggravated the socio-environmental impacts of shrimp farming in Brazil (Silva-Júnior et al., 2020).

Mangrove deforestation in Brazil has already devastated 20% of this ecosystem (Souza et al., 2020) and this threat was highlighted in Piauí and Maranhão, both located in the same partially-protected MPA, reinforcing the need for management actions. Tourism and seahorse stress while being captured/handled were also addressed. Seahorse-watching impacts are still unclear, but it is likely that capture and confinement leads to stress and behavioral disruptions. For scuba diving tourism, divers touching *H. Reidi* or approaching them to within 36 cm caused the seahorses to abandon their holdfasts and escape (Giglio et al., 2018). Not all informants saw seahorse watching as a threat, as the case of Ceará interviewees that were the most poorly literate and dependent group on seahorse-watching as an income source (Table S1), which may influence them not to question the sustainability of their activity. Maybe this could explain different perceptions, while Maranhão and Piauí share the highest scholarships and awareness. From a conservationist perspective, it is important that seahorse-watching operators are concerned about the sustainability of their activities, which can be explored as a universal concern, stimulating dialog among operators, researchers, and managers towards seahorse conservation (Ternes et al., 2016). Additionally, seahorse-watching impacts need to be investigated and mitigated, informing management actions.

4.3 Conservation insights

Environmental surveillance and educational interventions, claimed by all surveyed communities, are urgent to minimize conflicts and accomplish management and conservation goals (Figures 3B, D, F, 4). Enforcement of regulations is a priority frequently requested by MPA users around the world (Abecasis et al., 2013) and constant surveillance is an ostensive strategy to ensure this goal,while punishing illegalities. Environmental education programs (addressing seahorse conservation, mangrove protection, alternative livelihoods etc.) should be prioritized given that they can increase knowledge and engagement, contributing to compliance in MPAs (e.g., Leisher et al., 2012; Arias et al., 2015).

The need for actions towards management and regulation of seahorse-watching tourism were quoted in all sites, which is positive once these actions are mandatory for the exploitation of Vulnerable species, according to the Brazilian list of threatened species (MMA ordinance 445/2014). The Brazilian federal environmental agency – Instituto Chico Mendes de Conservação da Biodiversidade, ICMBio – is willing to regulate seahorse-watching in MPAs where this touristic activity previously exists, and our pioneer research is an important contribution. As a result, our study marked ICMBio's rapprochement with seahorse-watching stakeholders in JNP and regulation is already in progress in this MPA (Martins et al., 2022).

Linked to regulation, informant from all sites highlighted the need for spatial zoning to safeguard their livelihoods and protect seahorses, through the demarcation of seahorse-watching sites as exclusive no-take areas for tourism, with warning signs informing regulations and prohibitions. Zoning is critical for management, delimiting areas for specific purposes, controlling human activities, mitigating conflicts between different uses (Herrera-Montes, 2018). For example, establishment of navigation distance and exclusion areas may be a practicable strategy for mitigating motor boat impacts (e.g., Palma et al., 2019).

Environmental enhancement (Figure 4) through artificial habitat structures can be effective for animal conservation against habitat loss and degradation impacts (Watchorn et al., 2022). Given the life history traits of seahorses (e.g., site fidelity, reduced mobility, small home range), this strategy, has shown to successfully support populations of the endangered seahorse *H. whitei* in the absence of natural habitats (Simpson et al., 2020). Informants' suggestion of increasing holdfasts (Figures 2, 4) could be a suitable strategy facing habitat alterations. On the other hand, attempts to enhance seahorse stocks by releasing captive-bred individuals into the wild have revealed potential risks, including ecological impacts and loss of genetic diversity (e.g., Fraser, 2008; Luo et al., 2022). Therefore, its applicability should be cautiously discussed and investigated before any intervention. Most importantly, the causes of population decline must be addressed and mitigated as a priority (Fraser, 2008) and our study provides valuable information on local threats at a fine scale.

Fishing ban and mangrove protection were cited in Piauí and Maranhão (Figure 4). Both sites belong to DPEPA, a partially-protected MPA which is 35 times larger than PNJ, lacking no-take zones in the surveyed communities. Recently, a destructive fishing highlighted in Maranhão ("*zangaria*"), was officially banned in all DPEPA territories. This advance was supported by our study, which amplified stakeholders' voices, informing the MPA managers about *zangaria* impacts.

Ceará informants demonstrated awareness and interest in scientific research, such as seahorse population assessments, which is promising and should be implemented at all seahorse-watching sites, once the Brazilian list of threatened species requires it as mandatory for evaluating the sustainable exploitation of Vulnerable species (MMA ordinance 445/2014).

5 Conclusions and recommendations

Our study demonstrates seahorse-watching operators have a clear understanding that seahorses are threatened species, and that habitat alterations, fishing, and pollution are major threats shared among other local anthropogenic impacts. The informants` conservation strategies were comprehensive, addressing multiple threats, involving integrated management actions including zoning, surveillance and educational approaches as viable tools for achieving seahorse and mangrove conservation. In general, respondents support the MPAs and want more engagement from the management institutions towards the communities, considering local needs and thus increasing conservation effectiveness. Certain differences in the perception of threats between communities reflect the particularity of local realities and, possibly, also different levels of awareness, education, LEK, and dependence on seahorse-watching as an income source (c.f. Cortés-Avizanda et al., 2018). It is important to consider social perceptions in conservation planning, once they are often spatially heterogeneous.

Local scale studies on social perceptions from a LEK perspective can improve conservation and environmental management at a fine scale in large countries, such as Brazil, especially for data-poor species. Broad-scale management is unlikely to succeed when based on a single local data. Each locality has its own specifications to be addressed to ensure effective conservation of vulnerable species and habitats, as well as ensuring continued livelihood benefits. Our study has shown that local scale studies are worthwhile for supporting such an approach, more adjusted to the local realities. It also indicates that social perceptions should be employed to monitor threats and evaluate management and conservation strategies, integrating social and ecological indicators.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Universidade Federal do Pará. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

MLFT conceived the ideas, designed this study, collected data and wrote the original manuscript. RLN completed data analysis. MLFT, NVFM, RLN, and TG discussed the results. MLFT, NVFM, RLN, MDV, and TG revised and improved the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1139368/full#supplementary-material

References

Abecasis, R. C., Schmidt, L., Longnecker, N., and Clifton, J. (2013). Implications of community and stakeholder perceptions of the marine environment and its conservation for MPA management in a small azorean island. *Ocean Coast. Manag.* 84, 208–219. doi: 10.1016/j.ocecoaman.2013.08.009

Arias, A., Cinner, J. E., Jones, R. E., and Pressey, R. L. (2015). Levels and drivers of fishers' compliance with marine protected areas. *Ecol. Society.* 20 (4), 19. doi: 10.5751/ES-07999-200419

Bennett, N. J. (2016). Using perceptions as evidence to improve conservation and environmental management. *Conserv. Biol.* 30, 3. doi: 10.1111/cobi.12681

Cortés-Avizanda, A., Martín-López, B., Ceballos, O., and Pereira, H. M. (2018). Stakeholders perceptions of the endangered Egyptian vulture: insights for conservation. *Biol. Conserv.* 218, 173–180. doi: 10.1016/j.biocon.2017.09.028

Cullen-Unsworth, L. C., Jones, B. L., Seary, R., Newman, R., and Unsworth, R. K. F. (2018). Reasons for seagrass optimism: local ecological knowledge confirms presence of dugongs. *Mar. Pollut. Bull.* 134, 118–122. doi: 10.1016/j.marpolbul.2017.11.007

Delunardo, F. A. C., Paulino, M. G., Medeiros, L. C. C., Fernandes, M. N., Scherer, R., and Chippari-Gomes, A. R. (2020). Morphological and histopathological changes in seahorse (Hippocampus reidi) gills after exposure to the water-accommodated fraction of diesel oil. *Mar. Pollut. Bull.* 150, 110769. doi: 10.1016/j.marpolbul.2019.110769

Domínguez-López, M., Bellas, J., Sánchez-Ruiloba, L., Planas, M., and Hernández-Urcera, J. (2022). First evidence of ingestion and retention of microplastics in seahorses (Hippocampus reidi) using copepods (Acartiatonsa) as transfer vectors. *Sci. Total Environ.* 818, 151688. doi: 10.1016/j.scitotenv.2021.151688

Ferreira, A. C., and Lacerda, L. D. (2016). Degradation and conservation of Brazilian mangroves, status and perspectives. *Ocean Coast. Manag.* 125, 38–46. doi: 10.1016/j.ocecoaman.2016.03.011

Foster, S. J., and Vincent, A. C. J. (2004). Life history and ecology of seahorses: implications for conservation and management. *J. Fish Biol.* 65 (1), 1–61. doi: 10.1111/j.0022-1112.2004.00429.x

Fraser, D. J. (2008). How well can captive breeding programs conserve biodiversity? *A Rev. salmonids. Evol. Appl.* 1 (4), 2147483647–0. doi: 10.1111/j.1752-4571.2008.00036.x

Giglio, V. J., Ternes, M. L. F., Kassuga, A. D., and Ferreira, C. E. L. (2018). Scuba diving and sedentary fish watching: effects of photographer approach on seahorse behavior. *J. Eco.* 2, 142–151. doi: 10.1080/14724049.2018.1490302

Herrera-Montes, M. I. (2018). Protected area zoning as a strategy to preserve natural soundscapes, reduce anthropogenic noise intrusion, and conserve biodiversity. *Trop. Conserv. Sci.* 11 (11), 194008291880434. doi: 10.1177/1940082918804344

Hesp, P. A., Maia, L. P., and Claudino-Sales, V. (2009). "The Holocene barriers of maranhão, piauí and ceará states, northeastern Brazil," in *Geology and geomorphology of Holocene coastal barriers of brazil. lecture notes in earth sciences*, vol. 107. (Berlin, Heidelberg: Springer). doi: 10.1007/978-3-540-44771-9_10

Lacerda, L. D., Ward, R. D., Godoy, M. D. P., Meireles, A. J. A., Borges, R., and Ferreira, A. C. (2021). 20-years cumulative impact from shrimp farming on mangroves of northeast Brazil. *Front. For. Glob. Change.* 4. doi: 10.3389/ffgc.2021.653096

Leisher, C., Mangubhai, S., Hess, S., Widodo, H., Soekirman, T., Tjoe, S., et al. (2012). Measuring the benefits and costs of community education and outreach in marine protected areas. *Mar. Pol.* 36 (5), 1005–1011. doi: 10.1016/j.marpol.2012.02.022

Lotze, H. K., Guest, H., O'Leary, J., Tuda, A., and Wallace, D. (2018). Public perceptions of marine threats and protection from around the world. *Ocean Coast. Management.* 152, 14–22. doi: 10.1016/j.ocecoaman.2017.11.004

Luo, W., Wu, Q., Zhang, X., Wei, Y., Liao, M., Gao, T., et al. (2022). Potential risks for seahorse stock enhancement: insight from the declivity of genetic levels with hatchery management. *Front. Genet.* 12. doi: 10.3389/fgene.2021.830626

Mai, A. C. G., and Rosa, I. M. L. (2009). Ecological aspects of the seahorse hippocampus reidi in the Camurupim/Cardoso estuary, piauí state, Brazil, as subsidies for the implementation of an environmental protection area. *Biota Neotrop.* 9 (3), 85–89. doi: 10.1590/S1676-06032009000300007

Martins, J. C., Fontana, A., Silveira, R., and Vidal, M. D. (2022). Trajetória e perspectivas do turismo com cavalos-marinhos no parque nacional de jericoacoara, ceará. *BioBrasil. Gestão do Uso Público: Turismo e Lazer em Áreas Protegidas.* 3, 234–248. doi: 10.37002/biobrasil.v12i3.1988

McNaught, C., and Lam, P. (2010). Using wordle as supplementary research tool. *Qual. Rep.* 15 (3), 630–643. doi: 10.26536/FSR.2016.21

MMA, Ministério do Meio Ambiente (2014). "Ordinance 445/2014. lista nacional oficial de espécies da fauna ameaçadas de extinção," in *Peixes e invertebrados aquáticos* (Brazil: Diário Oficial da União). Available at: https://www.icmbio.gov.br/cepsul/images/stories/legislacao/Portaria/2014/p_mma_445_2014_lista_peixes_amea%C3%A7ados_extin%C3%A7%C3%A3o.pdf.

Oliveira-Júnior, J. G. C., Campos-Silva, J. V., Santos, D. T. V., Ladle, R. J., and Batista, V. S. (2021). Quantifying anthropogenic threats affecting marine protected areas in developing countries. *J. Environ. Management.* 279, 111614. doi: 10.1016/j.jenvman.2020.111614

Palma, J., Magalhães, M., Correia, M., and Andrade, J. P. (2019). Effects of anthropogenic noise as a source of acoustic stress in wild populations of hippocampus guttulatus in the ria Formosa, south Portugal. *Aquat. Conserv: Mar. Freshw. Ecosyst.* 29, 751–759. doi: 10.1002/aqc.3056

R Core Team. (2020). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Available at: https://www.R-project.org/.

Silva-Júnior, J. J., Nicacio, G., and Rodrigues, G. G. (2020). Carciniculture in the mangroves of northeastern Brazil: socioenvironmental problems in traditional communities. *Revista Movimentos Sociais e Dinâmicas Espaciais* 9, 70–84. doi: 10.46802/rmsde.v9i2.245816

Simpson, M., Coleman, R. A., Morris, R. L., and Harasti, D. (2020). Seahorse hotels: use of artificial habitats to support populations of the endangered white's seahorse hippocampus whitei. *Mar. Environ. Res.* 157, 104861. doi: 10.1016/j.marenvres.2019.104861

Souza, C. M., Shimbo, J. Z., Rosa, M., Parente, L., Alencar, A. A., Rudorff, B. F. T., et al. (2020). Reconstructing three decades of land use and land cover changes in Brazilian biomes with landsat archive and earth engine. *Remote Sens.* 12 (17), 1–27. doi: 10.3390/rs12172735

SUDEPE. (1988). *Portaria N°17, de 19 de agosto 1988, art. 2°. diário oficial [da] república federativa do brasil de 25.08.88, seção 1.* Diário oficial da República Federativa do Brasil.

Ternes, M. L., Gerhardinger, L. C., and Schiavetti, A. (2016). Seahorses in focus: local ecological knowledge of seahorse-watching operators in a tropical estuary. *J. ethnobiology ethnomedicine* 12 (1), 1–12. doi: 10.1186/s13002-016-0125-8

Vincent, A. C., Foster, S., and Koldewey, H. (2011). Conservation and management of seahorses and other syngnathidae. *J. Fish Biol.* 78, 1681–1724. doi: 10.1111/j.1095-8649.2011.03003.x

Watchorn, D. J., Cowan, M. A., Driscoll, D. A., Nimmo, D. G., Ashman, K. R., Garkaklis, M. J., et al. (2022). Artificial habitat structures for animal conservation: design and implementation, risks and opportunities. *Front. Ecol. Environment.* 20 (5), 301–309. doi: 10.1002/fee.2470

Zhang, X., and Vincent, A. C. J. (2019). Using cumulative human-impact models to reveal global threat patterns for seahorses. *Conserv. Biol.* 33, 1380–1391. doi: 10.1111/cobi.13325

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eDNA metabarcoding vs metagenomics: an assessment of dietary competition in two estuarine pipefishes

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Understanding the dietary preferences of endangered species can be useful in implementing conservation strategies, including habitat restoration, translocation, and captive breeding. Environmental DNA (eDNA) from feces provides a non-invasive method for analysing animal diets. Currently, metabarcoding, a PCR-based approach, is the method of choice for analysing such data. However, this method has limitations, specifically PCR bias, which can result in the overestimation of the importance of certain taxa and failure to detect other taxa because they do not amplify. The present study compared metabarcoding with metagenomics, a PCR-free method, to assess the diversity of prey items in the feces of a critically endangered South African estuarine pipefish, *Syngnathus watermeyeri*, and its widely distributed congener *S. temminckii* to investigate potential dietary competition. The metabarcoding results showed a distinct difference between the diets of *S. watermeyeri* and *S. temminckii*, with the former mainly consuming calanoid copepods and the latter preferring caridean shrimp. In each case, a single species dominated the sequences generated by metabarcoding. Metagenomics produced more species identifications, and although the same trend was found regarding the preference of *S. watermeyeri* for copepods and that of *S. temminckii* for shrimp, this approach identified additional, albeit yet unidentified, copepod species as being important in the diet of *S. watermeyeri*. We conclude that the lower number of species identified using metabarcoding was most likely a result of amplification bias, resulting in key copepod species missing from the dietary analysis. These findings suggest that metagenomics is not only a useful complementary method for molecular dietary analysis, but may in some cases outperform metabarcoding. However, metagenomics is even more strongly affected by the lack of reference sequences than is metabarcoding, as the majority of sequences originate from genomic regions that have not yet been sequenced for the putative prey species in question.

KEYWORDS

DNA barcoding, environmental DNA (eDNA), fecal DNA, diet analysis, endangered species, metabarcoding, metagenomics, pipefish

Introduction

Estuaries are amongst the most threatened aquatic habitats in the world, many of which have become functionally degraded due to anthropogenic pressures (Edgar et al., 2000; Turpie et al., 2002; Kaselowski and Adams, 2013; Kajee et al., 2018). These include water abstraction for agricultural and industrial activities, pollution, and urban development (Orth et al., 2006). As such, some storage reservoirs in water-scarce countries such as South Africa now have the potential to retain more than 50% of the freshwater that the estuaries would receive under normal conditions (Wooldridge and Callahan, 2000). Several endemic estuarine species in South Africa are threatened, including the Endangered Knysna seahorse, *Hippocampus capensis* (Lockyear et al., 2006; Mkare et al., 2017), the Critically Endangered limpet *Siphonaria compressa* (Allanson and Herbert, 2005) and the Critically Endangered estuarine pipefish, *Syngnathus watermeyeri* (Whitfield, 1995). All three species are associated with submerged macrophyte beds mainly dominated by the eelgrass *Zostera capensis*, which is itself listed as vulnerable by the IUCN because it is sensitive to the current level of anthropogenic pressure and experiences widespread degradation as a result of increased coastal development (Payne et al., 1998; Adams, 2016). With declines in their natural habitat, ecosystem restoration, translocation, and captive breeding need to be considered as a means to conserve the remaining populations of endangered species (Strum, 2005; Gumm et al., 2011; Landa et al., 2017). As such, a thorough knowledge of what these species consume in the wild is required to provide ecosystem managers with the information necessary to manage these populations better.

Reconstruction of diet in wild populations is critical in ecology because it reveals important details about a species' feeding habits (Pompanon et al., 2012), how a species uses its surroundings, and if there is resource competition with other members of the same community (Klare et al., 2011; Mumma et al., 2016). Animal diets have traditionally been determined by morphological examination of the gastrointestinal and fecal contents or by direct observation of their feeding habits (Pompanon et al., 2012; Sousa et al., 2019; Harper et al., 2020). However, the hard-part remains of some prey items can be difficult to identify using morphological analysis since they are usually damaged beyond recognition (Hawlitschek et al., 2018; Harper et al., 2020). In addition, studying the diet of endangered species can be challenging, particularly when the species of interest is rare and elusive, making field studies especially difficult (Ang et al., 2010).

A recently developed alternative method of assessing animal diets is eDNA metabarcoding (Kartzinel et al., 2015; Srivathsan et al., 2015; Emami-Khoyi et al., 2016; Boukhdoud et al., 2021). This PCR-based approach amplifies short DNA fragments of specified genetic markers that can then be identified using known reference sequences (Shehzad et al., 2012). However, metabarcoding has shortcomings, including PCR bias (Ferravante et al., 2021); this may occur due to irregular primer binding, thus resulting in some species amplifying less readily than others, or not at all (Alberdi et al., 2018; Mata et al., 2019).

Metagenomics is an alternative approach that involves the direct random sequencing of the entire genomic DNA rather than a small number of genetic markers (Bohmann et al., 2014; Bovo et al., 2018; Piñol, 2021). One of its drawbacks is that the bulk of the DNA that has been sequenced cannot be reliably assigned taxonomic rank due to a lack of comprehensive reference sequences since, for most species, only small portions of the genome have so far been sequenced (Bovo et al., 2018; Piñol, 2021).

Here, we compared the use of metagenomics and metabarcoding in identifying the prey species found in the feces of the estuarine pipefish and compared the prey items identified with corresponding data from its sister species, the longsnout pipefish *S. temminckii*, which is more abundant and widely distributed. Since the two pipefish share the same habitats and both capture small prey items by expanding their buccal cavity and suctioning prey through their tubular snouts (van Wassenbergh et al., 2008), it was hypothesised that dietary competition might exist between the two (Whitfield et al., 2017).

Materials and methods

Study sites and sample collection

Collection of *Syngnathus watermeyeri* and *S. temminckii* was approved by the Department of Agriculture, Forestry and Fisheries of South Africa (permit: RES2018/107), and ethical clearance was granted by the SAIAB Animal Ethics Committee (reference number: 25/4/1/5_2018-07) and the University of Johannesburg Faculty of Science Ethics Committee (reference number: 2021-10-05/Serite_Teske). Samples were collected from the only two South African estuaries where the estuarine pipefish still occurs, the Bushmans and Kariega (Claassens et al., 2022; Weiss et al., 2022). Pipefishes were collected at three sites; two in the Bushmans Estuary (site 1: 33°40'43.9 "S 26°39'12.2 "E; site 2: 33°40'21.6"S 26°38'46.1"E; data from these sites was subsequently pooled) and the third site in the Kariega Estuary (33°39'10.4"S 26°39'04.6"E). Both estuaries are permanently open, freshwater deprived, and have extensive macrophyte beds that constitute ideal habitat for pipefishes (Grange et al., 2000; Whitfield et al., 2017).

Sampling was conducted between March 30th and April 6th, 2019. In each location, all the pipefishes that could be collected within a period of 2 hours using a 5 mm stretch mesh seine net were placed into 5ℓ plastic tanks containing estuarine water for 3 h, after which they were released back into the estuaries. A total of 13 *S. watermeyeri* and 29 *S. temminckii* specimens were collected and kept in tanks in small groups of 2-3 individuals per species. The tanks were kept in the shade and aerated using portable air pumps, and the water was replaced every 30 min. Fecal pellets were dropped by the pipefishes in all these tanks throughout the 3 h period and were immediately collected using a sterile Pasteur pipette for each species, and subsequently blotted dry by placing them on paper towels before preserving them into 2 ml screw-cap microcentrifuge tubes containing RNAlater stabilization and storage reagent (QIAGEN GmbH, Hilden, Germany). Fecal pellets from all the individuals of a particular species and estuary were pooled. The tubes were kept frozen for up to two days and then stored at -70°C upon returning to the laboratory.

To control for the presence of any remaining DNA present in the estuarine water after blotting dry the fecal pellets, we collected 5 liters of surface water per estuary adjacent to the seagrass beds, using sterile plastic bottles. The water was filtered through two 100 ml Pall MicroFunnel^{TM} filter funnels with 0.2 mm Supor$^{®}$ membrane per estuary, using a vacuum pump. Filtering was performed at a location where no previous DNA extractions or PCR reactions had ever been conducted, following the cleaning of all surfaces, the vacuum manifold, and all tubes with 1% bleach solution. The filters were then kept frozen until further processing.

Laboratory analysis

Prior to DNA extraction, the fecal pellets were thawed at room temperature and then transferred to new 1.5 ml microcentrifuge tubes, which were placed on a heat block for 2 h at 37°C. To ensure that ample genomic DNA was extracted from the samples, DNA extraction was done in triplicate for each fecal sample using three different extraction protocols: the CTAB procedure (Doyle, 1991), as well as two extraction kit methods, NucleoSpin and Qiagen, following manufacturers' instructions. The quality of the extractions was compared by assessing them on a NanoDrop 2000c spectrophotometer, and by running them on a 1% agarose gel containing GelRed nucleic acid gel stain. As no differences in sample quality were evident for the three extraction methods, the extractions for each fecal sample were pooled. Metabarcoding was performed at AIT Austrian Centre of Technology in a laboratory that complies with the requirements of standard ISO 9001:2015. All workbenches and equipment were cleaned with bleach, the PCR setup was performed in a physically separated laboratory and within a HEPA-filtered laminar flow chamber, and no-template controls (NTCs) were incorporated into the workflow. The mitochondrial cytochrome oxidase c subunit I (COI) gene was amplified using forward primer mlCOIintF and reverse primer jgHCO2198 (Leray et al., 2013) as described in Ntuli et al. (2020). The COI gene used for metabarcoding is a genetic marker commonly used in the sequencing of animal DNA (Hebert et al., 2003), and this marker has also been proven to be suitable for zooplankton DNA barcoding (Clarke et al., 2017). The primer combination used here has amplified more prey species from fecal DNA than any other primer combination (Leray et al., 2013), and has subsequently been used in numerous similar studies (Morrill et al., 2021; Tran et al., 2022). The PCR products were purified using the AMPure XP system (Beckman Coulter), and a NEBNext Ultra DNA Library Prep Kit (New England BioLabs, United States) was used for the preparation of genomic libraries. The resulting libraries were screened for size distribution using a 2100 Bioanalyzer (Agilent) and quantified using real-time PCR. The libraries were then sequenced on an Illumina HiSeq 4000 platform (Illumina Inc., San Diego, California, United States) at Novogene (Hong Kong), using 2×250 bp paired-end chemistry according to the manufacturer's instructions.

Metagenomic sequencing was performed at Novogene Europe, using 0.4 µg of genomic DNA for library preparations. The libraries were generated using a NEBNext DNA Library Prep Kit (New England BioLabs, United States), and indices were then added to each sample. The genomic DNA was randomly sheared into fragments of 350 bp. The fragments were end-polished, A-tailed, and ligated using the NEBNext adapter for Illumina sequencing, and the fragments were PCR enriched by P5 and indexed P7 oligos. The PCR products were purified using the AMPure XP system (Beckman Coulter), and the resulting libraries were screened for size distribution using a 2100 Bioanalyzer (Agilent) and quantified using real-time PCR. Genomic libraries were sequenced on an Illumina Novaseq6000 platform (Illumina Inc., San Diego, California, United States) at Novogene (Hong Kong), using 2 × 150 bp paired-end chemistry according to the manufacturer's instructions.

Sequence assembly and analysis

For the metabarcoding, quality control was carried out using FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) and sequencing adapters, all sequences with length less than 150 bp, and low-quality sequences, which were defined as those sequences with a quality Phred Score of less than 25 in a five bp sliding window, were removed using Trimmomatic v0.36 (Bolger et al., 2014). Cutadapt v4.1 (Martin, 2011) was then used to trim both forward and reverse amplification primer sequences. When only the forward or reverse read of a particular sequence passed the quality filtering step, the expected error rate for “unpaired forward” and “unpaired reverse” was estimated in VSEARCH v2.17.0 (Rognes et al., 2016), and since the forward sequences consistently showed a lower error rate compared to reverse sequences, only a subset of full length (250 bp) forward sequences were selected for downstream analysis, together with sequences produced by merging forward and reverse reads.

Metabarcoding sequences were merged using VSEARCH v2.17.0 pipeline. Briefly, all pair-end sequences were merged based on their overlaps. Chimeric amplicons were removed using a denovo method implemented in the same package, and all non-chimeric sequences with a minimum of 98% similarity were clustered into distinct groups, also known as operational taxonomic units (or OTUs). The consensus sequence for each cluster and the number of sequences that formed each cluster were extracted for the taxonomic rank assignment step.

The metagenomic sequences from each location were separately assembled into longer contigs using MEGAHIT v1.1.1 (Li et al., 2015) by selecting the "meta-large" preset, which is most appropriate for complex metagenomic assemblies (https://github.com/voutcn/megahit). When possible, assembled sequences were dereplicated using VMATCH (Kurtz, 2003), and the quality of the assemblies was assessed with QUAST v4.6.3 (Gurevich et al., 2013). To estimate the number of sequences that were assembled to form each contig, the raw sequences from each sample were mapped against the metagenomic assemblies using Bowtie2 v.2.5 (Langmead et al., 2021), and the number of mapped sequences for each contig was quantified using Samtools v.1.9 (Li et al., 2009).

Taxonomic rank assignment

To assign a taxonomic rank to consensus sequences, metabarcoding sequences were blast-searched (Altschul et al., 1990) against a local non-redundant COI database, using a minimum similarity score of 95% , a minimum query coverage of 150 bp, and an e-value of 10^{-5}. Assembled contigs from metagenomics were then blast-searched against the complete NCBI nucleotide database ftp://ftp.ncbi.nlm.nih.gov/blast/db/nt, using the same parameters that were used for metabarcoding.

For both metabarcoding and metagenomics a consensus taxonomic rank was assigned to each sequence based on the Last Common Ancestor (LCA) of the five best matches, using BASTA (Kahlke and Ralph, 2019). In cases where no five matches exist that satisfy the requirements of 95% similarity and minimum query coverage of 150 bp, only the best matches were reported, as far as the percentage identify was not below 90% and the coverage was no less than 100 bp. Excel was then used to remove non-target taxa from the dataset (i.e., contaminants, pipefish DNA, and DNA from taxa that are too large or small to constitute prey, including mammals, bacteria, and algae). Subsets of the OTUs were then created based on those that contributed more than 1% to the overall read counts. The OTU counts for each putative species were agglomerated into the taxonomic rank of family, and visualised in Microsoft Excel with some additional annotations in Inkscape (https://inkscape.org/). A list of the potential prey items identified by metabarcoding and metagenomics was compiled. Information about the presence or absence of the identified species in South African estuaries was checked using the Global Biodiversity Information Facility (GBIF) database (https://www.gbif.org) and the World Register of Marine Organisms (WoRMS, https://marinespecies.org). In cases where the species does not occur in this region, we suggested which local species may be represented by the sequences in question based on taxonomic information. As estuaries have low diversity because few species can tolerate fluctuations in environmental conditions (Gray et al., 1997), the number of candidate species representing a particular genus or family tends to be low.

Results

The metabarcoding sequencing run generated 6 442 764 sequences from the Bushmans Estuary and 8 706 470 sequences from the Kariega Estuary for *S. watermeyeri*. For *S. temminckii*, 7 803 100 sequences and 8 540 218 sequences were recovered for the Bushmans Estuary and the Kariega Estuary, respectively. Post-quality filtering, the number of paired-end sequences kept per fecal sample ranged from 1 001 147 to 1 911 402. For the control samples, 538 422 and 93 920 sequences were generated for the Bushmans and Kariega estuaries, respectively. Of these, 32 197, and 351 were kept post-filtering (Supplementary Table 1).

Of the metabarcoding samples, the control samples from the Bushmans Estuary contained the highest numbers of unknowns (86.7%), followed by the controls from the Kariega Estuary, where 68.6% of the taxonomic assignments were unknown. The fecal samples of *S. temminckii* from the Kariega sample had 80 892 taxonomic assignments, and the Bushmans fecal sample had 153 925 taxonomic assignments, of which 12.5% and 48.5% of all consensus taxonomy rank assignments were classified as unknown, and therefore only the best matches were reported. The *S. watermeyeri* fecal samples had 26 964 and 266 754 taxonomic assignments for the Kariega and Bushmans estuaries, respectively, with 61.5% and 51.6% being reported as unknown based on the five best matches, and only best matches were reported (Supplementary Table 2).

The metagenomic assemblies comprised 611 473 (N50 = 707) and 183 631 (N50 = 2 952) singleton contigs for the *S. watermeyeri* samples from the Bushmans and Kariega estuaries, respectively. For *S. temminckii*, 467 665 (N50 = 650) singleton contigs were generated for the Kariega Estuary samples and 514 739 (N50 = 720) for the Bushmans Estuary samples. On average, 91.6 % of the raw sequences were successfully mapped against the assembled metagenomic contig. The control samples comprised 776 081 (N50 = 1 039) and 345 030 (N50 = 1 171) contigs for the Bushmans Estuary and the Kariega Estuary, respectively (Supplementary Table 3). More than 88% of the metagenomic taxonomic assignments for each fecal sample consisted of unknowns, host DNA and non-target DNA, and a similar trend was seen for the metabarcoding control samples (Supplementary Table 4).

Both the metabarcoding and the metagenomic datasets included a large proportion of pipefish DNA. For metabarcoding, this included 31% and 34% of the total number of sequences for *S. watermeyeri* from the Bushmans and Kariega estuaries, respectively, and 20% and 14% for the *S. temminckii* samples from the Bushmans and Kariega estuaries, respectively. The proportion of pipefish DNA was even greater for the metagenomic data, with 54% and 95% for *S. watermeyeri* from the Bushmans and Kariega, respectively, and 69% and 76% for *S. temminckii* from the Bushmans and Kariega estuaries, respectively.

Metabarcoding results showed a clear preference of each pipefish for a specific type of prey (Figure 1A). The estuarine pipefish data were numerically dominated by a single species of calanoid copepod, *Pseudodiaptomus hessei* (at both sites, this number exceeded 600 000 sequences), whereas sequences from the shrimp *Palaemon peringueyi* dominated the feces of *S. temminckii*. Other species that comprised at least 1% of the reads included two gastropods, *Assiminea capensis* (Assimineidae) and the *Hydrobia knysnaensis* (Hydrobiidae). The control samples mostly contained non-target DNA originating from bacteria, algae and larger vertebrates. A single exception was the presence of macroinvertebrate DNA from the gastropod *Haminoea alfredensis* in the Bushmans control sample. This species was not found in any of the fecal samples.

Metagenomics recovered more diversity of species than metabarcoding (Table 1), although the majority of these were rare, and were excluded from Figure 1B because they were not visible (<1% of the total number of reads). In addition to the copepod *Pseudodiaptomus hessei*, this method also identified three additional but unidentified copepods (each in a different higher taxon, i.e. Calanidae, Cyclopidae and Harpactoida) as being important in the diet of S. *watermeyer*i. Similar to metabarcoding, metagenomics identified the gastropod *A. capensis*, and other reasonably common taxa identified included members of the Ostracoda (several distinct species were grouped here because of taxonomic uncertainty), Thecostraca and Oppiidae. The control samples included DNA from several species of gastropods that were either absent or present at very low concentrations in the fecal samples (*Afrolittorina africana*), and some ostracods.

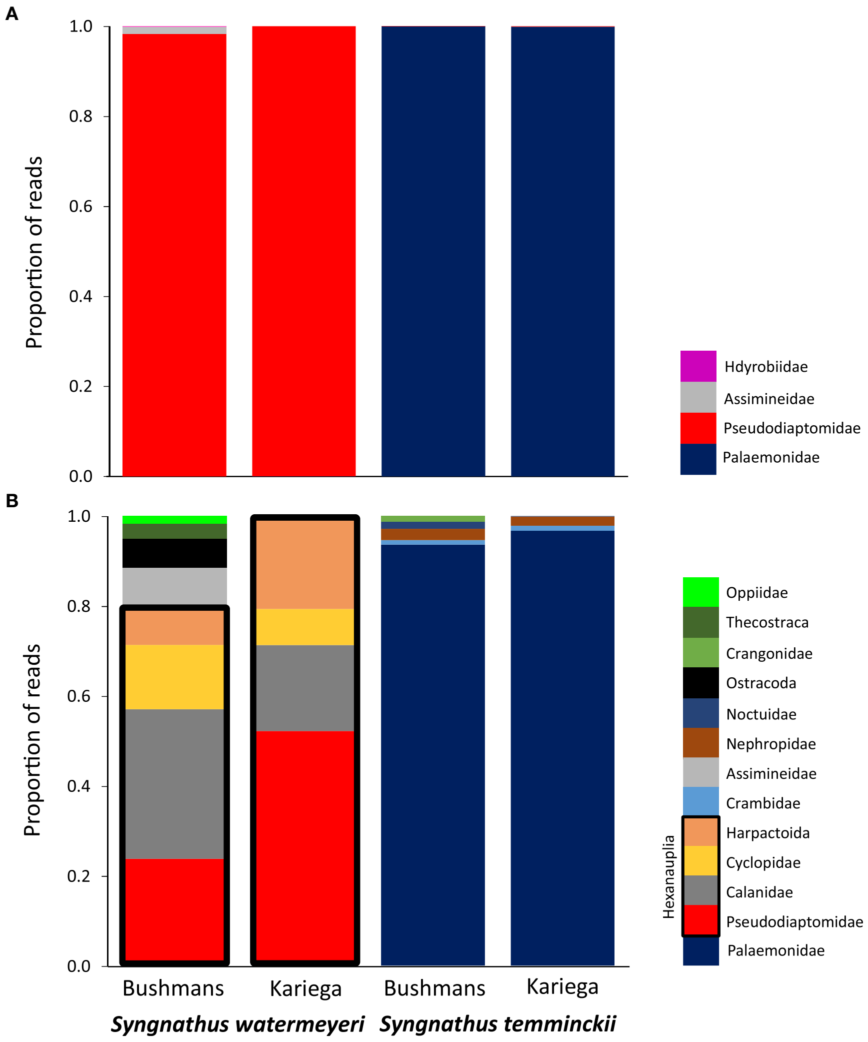

FIGURE 1
A comparison of the proportion of reads from putative prey families found in the feces of the two pipefish species; **(A)** metabarcoding data; **(B)** metagenomic data. In each case, species with a number of reads <1% of the total number of reads per fecal samples were excluded. The Hexanauplia (copepod) families found in the metagenomic data of *S. watermeyeri* are grouped together by black boxes.

A total of 24 species of Palaemonidae were found in the feces of *S. temminckii*, compared to only one (*Palaemon peringueyi*) that was identified by metabarcoding. Most of these do not occur in South Africa or in the study area, suggesting that they most likely all represent the same species, *Palaemon peringueyi*, and were identified because no complete genome has yet been sequenced for this species. This is not only true for the species of *Palaemon*, but also for *Macrobrachium*. Although the genus occurs in subtropical South Africa, its range does not extend to the Bushmans and Kariega estuaries. The metagenomic results for this pipefish are thus largely congruent as those of based on metabarcoding in that caridean shrimp are particularly important in its diet (Figure 1B).

Discussion

This study used metabarcoding and metagenomic analysis of eDNA collected non-intrusively from fecal samples to compare the dietary preferences of the Critically Endangered estuarine pipefish, *Syngnathus watermeyeri*, and its more abundant congener, *S. temminckii*. The research aimed to provide information that contributes towards improving the conservation management of *S. watermeyeri*, particularly in captive breeding and releases of captive-bred progeny into estuaries within the species' historical distribution range.

Due to the high conservation status of the estuarine pipefish, fecal samples could only be collected from a few individuals in a single season, and, fecal material from each species and estuary were pooled to minimise the cost of sequencing. Therefore, this study can only provide a snapshot of diet composition in each population rather than information on the preferences of individual pipefishes. However, the fact that all individuals from the same site were captured on the same day indicates that both species had access to the same prey species, thus rejecting the idea that the differences in diet composition could be due to small sample sizes or spatial and temporal separation of the captured individuals.

TABLE 1 Putative prey taxa found in the feces of *Syngnathus watermeyeri* (W) and *S. temminckii* (T), compared to species from the same classes found in the control (C) samples, based on metabarcoding and metagenomics.

Class	Family	Species	Metabarcoding					Metagenomics						South African taxa
			W		T		C	W		T		C		
Actinopterygii	Atherinidae	*Atherina breviceps*	■	B										
	Clupeidae	*Gilchristella aestuaria*	■	K										
Arachnida	Oppiidae	*Medioppia subpectinata*						■	B					
	Tydeidae	Tydeidae sp.	■	K										Tydeidae sp.
	Digamasellidae	Digamasellidae sp.			■	K								Digamasellidae sp.
	Oppiidae	*Oppiella nova*						■	B					*Oppiella* sp.
Bivalvia	Mytilidae	*Limnoperna fortunei*								■				*Perna perna*
	Veneridae	*Mercenaria mercenaria*								■	B			
	Cardiidae	*Tridacna gigas*								■				*Tridacna* sp.
Cephalopoda	Octopodidae	*Octopus bimaculoides*								■				
Gastropoda	Littorinidae	*Afrolittorina africana*	■	B										*Afrolittorina africana*
	Assimineidae	*Assiminea capensis*	■	B				■	B					*Assiminea capensis*
	Haminoeidae	*Haminoea alfredensis*					■							*Haminoea alfredensis*
	Littorinidae	*Littorina saxatilis*										■	B	*Afrolittorina africana*
	Aplysiidae	*Bursatella leachii*										■	B	*Bursatella leachii*
		Aplysia californica										■	B	
	Trochidae	*Gibbula magus*								■				*Gibbula cicer*
	Hydrobiidae	*Hydrobia knysnaensis*	■	B										*Hydrobia knysnaensis*
	Pomatiopsidae	*Oncomelania hupensis*						■	B					
	Patellidae	*Patella pellucida*								■				*Patella* sp.
		Patella vulgata								■				*Patella* sp.
	Buccinidae	*Penion sulcatus*						■	B					
	Hydrobiidae	*Peringia ulvae*						■	B					*Hydrobia knysnaensis*
		Laevicaspia caspia						■	B					
	Trochidae	*Steromphala cineraria*								■				*Steromphala cineraria*
Hexanauplia	Cyclopidae	*Halicyclops* sp.						■						*Halicyclops dedeckeri*
	Calanidae	*Calanus finmarchicus*						■	B					*Calanus agulhensis*
	Nannopodidae	*Nannopus* sp.						■	B					
	Canuellidae	*Canuella perplexa*						■	B					
	Paracalanidae	*Paracalanus parvus*			■	B								
	Pseudodiaptomidae	*Pseudodiaptomus hessei*	■		■			■						*Pseudodiaptomus hessei*
		Pseudodiaptomus euryhalinus						■	K					
		Pseudodiaptomus sp.								■	K			
		Pseudodiaptomus marinus						■	K					
		Pseudodiaptomus nihonkaiensis						■						
	Cyclopidae	Cyclopidae sp.						■						*Paracyclops* sp.
	Harpacticoida	Harpacticoida sp.						■	K					

(Continued)

TABLE 1 Continued

Class	Family	Species	Metabarcoding					Metagenomics						South African taxa
			W		T		C	W		T		C		
	Temoridae	*Eurytemora affinis*						■	K					
	Diaptomidae	*Eudiaptomus* sp.						■	K					
		Diaptomidae sp.						■	K					
Insecta	Liposcelididae	*Liposcelis brunnea*			■	B								*Liposcelis brunnea*
	Aphididae	*Tuberculatus annulatus*			■	K								*Tuberculatus annulatus*
	Hesperiidae	*Lucida lucia*	■	B	■	K								
	Dermestidae	Dermestidae sp.	■	B	■									
Malacostraca	Crangonidae	*Crangon franciscorum*								■	B			*Crangon crangon*
	Sphaeromatidae	*Exosphaeroma hylecoetes*			■	B								*Exosphaeroma hylecoetes*
	Nephropidae	*Homarus americanus*								■				*Homarinus capensis*
	Palaemonidae	*Creaseria morleyi*								■	B			*Palaemon peringueyi*
		Zenopontonia soror								■	B			
		Macrobrachium nipponense								■				
		Macrobrachium rosenbergii								■				
		Macrobrachium olfersii								■	K			
		Palaemon adspersus								■	K			
		Palaemon carinicauda								■				
		Palaemon elegans								■				
		Palaemon paucidens								■				
		Palaemon modestus								■				
		Palaemon paludosus								■				
		Palaemon peringueyi	■	K	■			■	B	■				
		Palaemon pugio								■				
		Palaemon serenus								■				
		Palaemon sinensis								■	B			
		Palaemon serratus								■				
		Palaemon varians								■				
	Penaeidae	*Penaeus chinensis*								■				*Penaeus* sp.
		Penaeus japonicus								■				
		Penaeus vannamei								■	B			
		Penaeus indicus			■	K								
	Cambaridae	*Procambarus clarkii*								■				*Procambarus clarkii*
	Portunidae	*Portunus trituberculatus*								■				*Portunus sanguinolentus*
	Pandalidae	Pandalidae sp.								■	K			*Palaemon peringueyi*
Ostracoda	Cyprididae	*Cyprideis torosa*						■		■	B	■		*Cyprideis torosa*
	Darwinulidae	*Darwinula stevensoni*						■	B			■		*Darwinula stevensoni*
	Leptocytheridae	*Ishizakiella miurensis*						■	K					
Thecostraca	Sacculinidae	*Sacculina carcini*						■	B	■	B			*Sacculina carcini*

The column on the right indicates which South African taxa are most likely represented by species that do not occur in the sampling area. Squares indicate the presence of a taxon and the letters to the right of the squares represent: K, Kariega Estuary only; B, Bushmans Estuary only.

The results showed clear differences in dietary preferences between the two pipefish species. Although some species were found in the feces of both species, these were rare, and each pipefish had a preference for different types of prey. Only two invertebrate classes (Malacostraca and Hexanauplia) were consistently found in the diet of the two pipefish species. In each case, these were limited to a few species. The estuarine pipefish has a preference for copepods (class Hexanauplia). The calanoid copepod *Pseudodiaptomus hessei* dominated the metabarcoding sequences, while the metagnomic dataset included three additional species, each in a different family. In contrast, although the feces of *S. temminckii* also contained a small number of sequences from *P. hessei*, this pipefish showed a preference for the caridean shrimp *Palaemon peringueyi*. In addition to crustaceans, both metabarcoding and metagenomics confirmed the importance of the gastropod *Assiminea capensis* in the diet of the estuarine pipefish. As the adults of Palaemonidae and *Assiminea* are too large to fit through the mouths of pipefishes, it is likely that the *S. watermeyeri* preys upon their larvae.

Metabarcoding is presently the primary molecular method to reconstruct the diet and assess the importance of different food items based on the number of retrieved sequences. As is the case in all PCR-based approaches, our results concerning the importance of these main prey items based on metabarcoding may have been impacted by amplification bias (Tedersoo et al., 2015; Krehenwinkel et al., 2017). Metagenomics is predicted to provide superior taxonomic resolution compared to metabarcoding because of its capacity to incorporate information from multiple markers across the genome, and to assemble longer contigs for more accurate species identification (Srivathsan et al., 2016; Chua et al., 2021), and its power was clearly demonstrated by the fact that it identified several more important copepod species, in addition to *P. hessei*, that metabarcoding failed to identify.

The efficiency of taxonomic rank assignment in metabarcoding depends strongly on how comprehensive the available reference database is regarding the species represented and the markers used to amplify them (Chua et al., 2021), but this is even more so the case with metagenomics. In this study, most metagenomic identifications were for species that do not occur in the study area; this was particularly evident in the family Palaemonidae. In contrast to the metabarcoding sequences, most of which originated from *Palaemon peringueyi*, several additional caridean shrimps of the family Palaemonidae (which includes the genera *Palaemon* and *Macrobrachium*). This is likely an artifact of gene regions being represented in this dataset that have not yet been sequenced for *P. peringueyi*. Consequently, the taxonomic rank reported was often from species for which complete genomes have been published, including *M. nipponense* and *P. carinicauda*. Hence, both sequencing methods used in this study display bias that can be attributed to incomplete databases.

Despite these discrepancies, this study provides important information on differences in prey selection between the two pipefish species. The choice of prey items likely depends on clear differences in snout length and gape size and variations in hunting tactics. The estuarine pipefish's shorter snout size may restrict the size of prey species on which it can feed, which would explain its preference for small copepods (and potentially also gastropod veliger larvae). It is also possible that *S. watermeyeri* may detect small zooplankton more effectively than *S. temminckii* because of the position of its eyes, which are located closer to the tip of the snout. In addition to stronger suction and greater gape size due to a larger and longer snout, *S. temminckii* has been observed to hunt its prey actively, whereas *S. watermeyeri* is more passive and waits to ambush prey animals that swim within its reach (Sven-Erick Weiss, pers. obs.). Together, these factors likely enable *S. temminckii* to stalk prey that might otherwise escape, such as the larvae of *P. peringueyi*.

The finding that the Critically Endangered *S. watermeyeri* relies to a large extent on relatively small zooplankters (particularly copepods) supports the hypothesis that significant reductions in zooplankton abundance in response to reduced freshwater influx (Grange et al., 2000) can result in estuarine pipefish population declines (Whitfield et al., 2017). Excessive freshwater abstraction has transformed both estuaries inhabited by *S. watermeyeri* from systems with well-developed salinity gradients to homogeneously marine-dominated systems. The Bushmans River, for example, has approximately 30 impoundments in its upper reaches that have significantly reduced freshwater inflow (Bornman and Klages, 2004). While this would have resulted in a decrease in phytoplankton biomass (and, by extension, zooplankton biomass) (Hilmer and Bate, 1990), the resulting increase in water clarity would also have facilitated the formation of the current extensive submerged macrophyte beds (Bornman and Klages, 2004). Because of this contradiction (reduced food availability but increased habitat availability), it cannot be ruled out that the two estuaries in their current marine-dominated state have a higher carrying capacity for *S. watermeyeri* than they would have had under natural conditions, at least during periods of moderate rainfall.

This study suggests that resource competition between the two pipefish species is likely negligible as they mostly consume a different diet. Thus, it is not an important factor that could explain why *S. watermeyeri* is such a rare species. Although the estuarine pipefish prefers copepods, results from both sequencing methods indicated that its diet could be supplemented in captivity with gastropod veliger larvae to resemble how they would eat in their natural environment. Other taxa may also be opportunistically consumed, including ostracods.

Overall, eDNA sequencing methods have offered a way of identifying soft-bodied prey that would have been difficult or even impossible to detect through traditional morphological analysis of fecal samples, such as the copepods identified here. Moreover, the identification of veliger shells in the feces would have been insufficient to identify the species from which they originated. However, the results also document how the lack of reference sequences can greatly impact the efficiency of metabarcoding and metagenomics as diet analysis methods. Our study highlights the critical need for a more comprehensive reference database of South African estuarine macroinvertebrates to improve the power of both metabarcoding and metagenomics in identifying the prey species present in fecal eDNA samples.

Data availability statement

The data presented in the study are deposited in the NCBI Sequence Read Archive (SRA) repository BioProject ID PRJNA911940.

Ethics statement

The animal study was reviewed and approved by the SAIAB Animal Ethics Committee (reference number: 25/4/1/5_2018-07) and the University of Johannesburg Faculty of Science Ethics Committee (reference number: 2021-10-05/Serite_Teske).

Author contributions

PC, AW, and PT conceived the research. PC and PT generated funding and obtained permits. ON, AE-K, PC, NJ, TB, and PT participated in sampling and/or laboratory work. CS and AE-K analyzed the data. CS and PT created figures and tables. AE-K, BJ, and PT supervised the students. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1116741/full#supplementary-material

References

Adams, J. B. (2016). Distribution and status of *Zostera capensis* in South African estuaries — a review. *S. Afr. J. Bot.* 107, 63–73. doi: 10.1016/j.sajb.2016.07.007

Alberdi, A., Aizpurua, O., Gilbert, M. T. P., and Bohmann, K. (2018). Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods Ecol. Evol.* 9, 134–147. doi: 10.1111/2041-210X.12849

Allanson, B. R., and Herbert, D. G. (2005). A newly discovered population of the critically endangered false limpet *Siphonaria compressa* Allanson, 1958 (Pulmonata: Siphonariidae), with observations on its reproductive biology. *S. Afr. J. Sci.* 101, 95–97. doi: 10.10520/EJC96338

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Ang, A., Ismail, M. R. B., and Meier, R. (2010). Reproduction and infant pelage colouration of the banded leaf monkey (Mammalia: Primates: Cercopithecidae) in Singapore. *Raffles Bull. Zool.* 58, 411–415.

Bohmann, K., Evans, A., Gilbert, M. T. P., Carvalho, G. R., Creer, S., Knapp, M., et al. (2014). Environmental DNA for wildlife biology and biodiversity monitoring. *Trends Ecol. Evol.* 29, 358–367. doi: 10.1016/j.tree.2014.04.003

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Bornman, T., and Klages, N. T. W. (2004). *Upgrade of Kenton-on-Sea/Bushmans River Mouth by Albany Coast Water Board.* IECM Report No. C84, University of Port Elizabeth, Port Elizabeth.

Boukhdoud, L., Saliba, C., Parker, L. D., McInerney, N. R., Kahale, R., Saliba, I., et al. (2021). Using DNA metabarcoding to decipher the diet plant component of mammals from the Eastern Mediterranean region. *Metabarcoding Metagenom.* 5, 219–231. doi: 10.3897/mbmg.5.70107

Bovo, S., Ribani, A., Utzeri, V. J., Schiavo, G., Bertolini, F., and Fontanesi, L. (2018). Shotgun metagenomics of honey DNA: evaluation of a methodological approach to describe a multi-kingdom honey bee derived environmental DNA signature. *PloS One* 13, e0205575. doi: 10.1371/journal.pone.0205575

Chua, P. Y. S., Crampton-Platt, A., Lammers, Y., Alsos, I. G., Boessenkool, S., and Bohmann, K. (2021). Metagenomics: a viable tool for reconstructing herbivore diet. *Mol. Ecol. Resour.* 21, 2249–2263. doi: 10.1111/1755-0998.13425

Claassens, L., de Villiers, N. M., Seath, J., and Wasserman, J. (2022). Distribution and density of the Critically Endangered estuarine pipefish across its range – implications for conservation. *Aquat. Conserv.* 32, 28–41. doi: 10.1002/aqc.3742

Clarke, L. J., Beard, J. M., Swadling, K. M., and Deagle, B. E. (2017). Effect of marker choice and thermal cycling protocol on zooplankton DNA metabarcoding studies. *Ecol. Evol.* 7, 873–883. doi: 10.1002/ECE3.2667

Doyle, J. (1991). "DNA Protocols for plants", in *Molecular techniques in taxonomy* (Berlin Heidelberg: Springer), 283–293. doi: 10.1007/978-3-642-83962-7_18

Edgar, G. J., Barrett, N. S., Graddon, D. J., and Last, P. R. (2000). The conservation significance of estuaries: a classification of Tasmanian estuaries using ecological, physical and demographic attributes as a case study. *Biol. Conserv.* 92, 383–397. doi: 10.1016/S0006-3207(99)00111-1

Emami-Khoyi, A., Hartley, D. A., Paterson, A. M., Boren, L. J., Cruickshank, R. H., Ross, J. G., et al. (2016). Identifying prey items from New Zealand fur seal

(*Arctocephalus forsteri*) faeces using massive parallel sequencing. *Conservation Genet. Resour.* 8, 343–352. doi: 10.1007/s12686-016-0560-9

Ferravante, C., Memoli, D., Palumbo, D., Ciaramella, P., di Loria, A., D'Agostino, Y., et al. (2021). HOME-BIO (shotgun metagenomic analysis of biological entities): a specific and comprehensive pipeline for metagenomic shotgun sequencing data analysis. *BMC Bioinf.* 22, 106. doi: 10.1186/s12859-021-04004-y

Grange, N., Whitfield, A. K., De Villiers, C. J., and Allanson, B. R. (2000). The response of two South African east coast estuaries to altered river flow regimes. *Aquat. Conserv.* 10, 155–177. doi: 10.1002/1099-0755(200005/06)10:3<155::AID-AQC406>3.0.CO;2-Z

Gray, J. S., Poore, G. C. B., Ugland, K. I., Wilson, R. S., Olsgard, F., and Johannessen, O. (1997). Coastal and deep-sea benthic diversities compared. *Mar. Ecol. Prog. Ser.* 159, 97–103. doi: 10.3354/MEPS159097

Gumm, J. M., Snekser, J. L., Leese, J. M., Little, K. P., Leiser, J. K., Imhoff, V. E., et al. (2011). Management of interactions between endangered species using habitat restoration. *Biol. Conserv.* 144, 2171–2176. doi: 10.1016/J.biocon.2011.05.006

Gurevich, A., Saveliev, V., Vyahhi, N., and Tesler, G. (2013). QUAST: quality assessment tool for genome assemblies. *Bioinformatics* 29, 1072–1075. doi: 10.1093/bioinformatics/btt086

Harper, L. R., Watson, H., Donnelly, R., Hampshire, R., Sayer, C. D., Breithaupt, T., et al. (2020). Using DNA metabarcoding to investigate diet and niche partitioning in the native European otter (*Lutra lutra*) and invasive American mink (*Neovison vison*). *Metabarcoding Metagenom.* 4, 113–133. doi: 10.3897/mbmg.4.56087

Hawlitschek, O., Fernández-González, A., Balmori-de la Puente, A., and Castresana, J. (2018). A pipeline for metabarcoding and diet analysis from fecal samples developed for a small semi-aquatic mammal. *PloS One* 13, e0201763. doi: 10.1371/journal.pone.0201763

Hebert, P. D. N., Ratnasingham, S., and DeWaard, J. R. (2003). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proc. R. Soc. B* 270, S96–S99. doi: 10.1098/RSBL.2003.0025

Hilmer, T., and Bate, G. C. (1990). Covariance analysis of chlorophyll distribution in the Sundays River estuary, Eastern Cape. *S. Afr. J. Aquat. Sci.* 16, 37–59. doi: 10.1080/10183469.1990.10557366

Kahlke, T., and Ralph, P. J. (2019). BASTA – taxonomic classification of sequences and sequence bins using last common ancestor estimations. *Methods Ecol. Evol.* 10, 100–103. doi: 10.1111/2041-210X.13095

Kajee, M., Griffiths, C. L., and Lamberth, S. J. (2018). Long-term physico-chemical and faunal changes in a small, rural South African estuary. *Afr. Zool.* 53, 127–137. doi: 10.1080/15627020.2018.1552838

Kartzinel, T. R., Chen, P. A., Coverdale, T. C., Erickson, D. L., Kress, W. J., Kuzmina, M. L., et al. (2015). DNA metabarcoding illuminates dietary niche partitioning by African large herbivores. *Proc. Natl. Acad. Sci. U.S.A.* 112, 8019–8024. doi: 10.1073/pnas.1503283112

Kaselowski, T., and Adams, J. B. (2013). Not so pristine - characterising the physico-chemical conditions of an undescribed temporarily open/closed estuary. *Water SA.* 39, 627–636. doi: 10.4314/wsa.v39i5.6

Klare, U., Kamler, J. F., and Macdonald, D. (2011). A comparison and critique of different scat-analysis methods for determining carnivore diet. *Mamm. Rev.* 41, 294–312. doi: 10.1111/j.1365-2907.2011.00183.x

Krehenwinkel, H., Wolf, M., Lim, J. Y., Rominger, A. J., Simison, W. B., and Gillespie, R. G. (2017). Estimating and mitigating amplification bias in qualitative and quantitative arthropod metabarcoding. *Sci. Rep.* 7, 1–12. doi: 10.1038/s41598-017-17333-x

Kurtz, S. (2003). The *Vmatch* large scale sequence analysis software. Available at: http://www.vmatch.de 2003 [Accessed April 15, 2023].

Landa, A., Flagstad, Ø., Areskoug, V., Linnell, J. D. C., Strand, O., Ulvund, K. R., et al. (2017). The endangered Arctic fox in Norway – the failure and success of captive breeding and reintroduction. *Polar Res.* 36, 9. doi: 10.1080/17518369.2017.1325139

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. (2021). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10, R25. doi: 10.1186/gb-2009-10-3-r25

Leray, M., Yang, J. Y., Meyer, C. P., Mills, S. C., Agudelo, N., Ranwez, V., et al. (2013). A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. *Front. Zool.* 10, 34. doi: 10.1186/1742-9994-10-34

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The sequence Alignment/Map format and SAMtools. *Bioinformatics* 25, 2078–2079. doi: 10.1093/bioinformatics/btp352

Li, D., Liu, C.-M., Luo, R., Sadakane, K., and Lam, T.-W. (2015). MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct *de Bruijn* graph. *Bioinformatics* 31, 1674–1676. doi: 10.1093/bioinformatics/btv033

Lockyear, J., Hecht, T., Kaiser, H., and Teske, P. (2006). The distribution and abundance of the endangered Knysna seahorse *Hippocampus capensis* (Pisces: Syngnathidae) in South African estuaries. *Afr. J. Aquat. Sci.* 31, 275–283. doi: 10.2989/16085910609503897

Mata, V. A., Rebelo, H., Amorim, F., McCracken, G. F., Jarman, S., and Beja, P. (2019). How much is enough? effects of technical and biological replication on metabarcoding dietary analysis. *Mol. Ecol.* 28, 165–175. doi: 10.1111/mec.14779

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 17, 10–12. doi: 10.14806/ej.17.1.200

Mkare, T. K., van Vuuren, B. J., and Teske, P. R. (2017). Conservation implications of significant population differentiation in an endangered estuarine seahorse. *Biodivers. Conserv.* 26, 1275–1293. doi: 10.1007/s10531-017-1300-5

Morrill, A., Kaunisto, K. M., Mlynarek, J. J., Sippola, E., Vesterinen, E. J., and Forbes, M. R. (2021). Metabarcoding prey DNA from fecal samples of adult dragonflies shows no predicted sex differences, and substantial inter-individual variation, in diets. *PeerJ* 9, e12634. doi: 10.7717/peerj.12634

Mumma, M. A., Adams, J. R., Zieminski, C., Fuller, T. K., Mahoney, S. P., and Waits, L. P. (2016). A comparison of morphological and molecular diet analyses of predator scats. *J. Mammal.* 97, 112–120. doi: 10.1093/jmammal/gyv160

Ntuli, N. N., Nicastro, K. R., Zardi, G. I., Assis, J., McQuaid, C. D., and Teske, P. R. (2020). Rejection of the genetic implications of the "Abundant Centre Hypothesis" in marine mussels. *Sci. Rep.* 10, 604. doi: 10.1038/s41598-020-57474-0

Orth, R. J., Carruthers, T. J. B., Dennison, W. C., Duarte, C. M., Fourqurean, J. W., Heck, K. L., et al. (2006). A global crisis for seagrass ecosystems. *Bioscience* 56, 987–996. doi: 10.1641/0006-3568(2006)56[987:AGCFSE]2.0.CO;2

Payne, M. F., Rippingale, R. J., and Longmore, R. B. (1998). Growth and survival of juvenile pipefish *Stigmatopora argus* fed live copepods with high and low HUFA content. *Aquaculture* 167, 237–245. doi: 10.1016/S0044-8486(98)00318-4

Piñol, J. (2021). Genotype by sequencing: an alternative new method to amplicon metabarcoding and shotgun metagenomics for the assessment of eukaryote biodiversity. *Mol. Ecol. Resour.* 21, 1001–1004. doi: 10.1111/1755-0998.13320

Pompanon, F., Deagle, B. E., Symondson, W. O. C., Brown, D. S., Jarman, S. N., and Taberlet, P. (2012). Who is eating what: diet assessment using next generation sequencing. *Mol. Ecol.* 21, 1931–1950. doi: 10.1111/j.1365-294X.2011.05403.x

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 2016, e2584. doi: 10.7717/peerj.2584

Shehzad, W., Riaz, T., Nawaz, M. A., Miquel, C., Poillot, C., Shah, S. A., et al. (2012). Carnivore diet analysis based on next-generation sequencing: application to the leopard cat (*Prionailurus bengalensis*) in Pakistan. *Mol. Ecol.* 21, 1951–1965. doi: 10.1111/j.1365-294X.2011.05424.x

Sousa, L. L., Silva, S. M., and Xavier, R. (2019). DNA Metabarcoding in diet studies: unveiling ecological aspects in aquatic and terrestrial ecosystems. *Environ. DNA* 1, 199–214. doi: 10.1002/edn3.27

Srivathsan, A., Ang, A., Vogler, A. P., and Meier, R. (2016). Fecal metagenomics for the simultaneous assessment of diet, parasites, and population genetics of an understudied primate. *Front. Zool.* 13, 17. doi: 10.1186/s12983-016-0150-4

Srivathsan, A., Sha, J. C. M., Vogler, A. P., and Meier, R. (2015). Comparing the effectiveness of metagenomics and metabarcoding for diet analysis of a leaf-feeding monkey (*Pygathrix nemaeus*). *Mol. Ecol. Resour.* 15, 250–261. doi: 10.1111/1755-0998.12302

Strum, S. C. (2005). Measuring success in primate translocation: a baboon case study. *Am. J. Primatol.* 65, 117–140. doi: 10.1002/AJP.20103

Tedersoo, L., Anslan, S., Bahram, M., Põlme, S., Riit, T., Liiv, I., et al. (2015). Shotgun metagenomes and multiple primer pair-barcode combinations of amplicons reveal biases in metabarcoding analyses of fungi. *MycoKeys* 10, 1–43. doi: 10.3897/mycokeys.10.4852

Tran, B. T., Kim, K. Y., Heo, J. S., Park, S. J., Park, H. K., and Choi, Y. H. (2022). Determination of the pacific oyster *Magallana gigas* (*Crassostrea gigas*) diet composition in two aquaculture farms by fecal DNA metabarcoding. *Aquaculture* 552, 738042. doi: 10.1016/j.aquaculture.2022.738042

Turpie, J. K., Adams, J. B., Joubert, A., Harrison, T. D., Colloty, B. M., Maree, R. C., et al. (2002). Assessment of the conservation priority status of South African estuaries for use in management and water allocation. *Water SA.* 28, 191–206. doi: 10.4314/wsa.v28i2.4885

van Wassenbergh, S., Strother, J. A., Flammang, B. E., Ferry-Graham, L. A., and Aerts, P. (2008). Extremely fast prey capture in pipefish is powered by elastic recoil. *J. R. Soc. Interface* 5, 285–296. doi: 10.1098/rsif.2007.1124

Weiss, S.-E., Emami-Khoyi, A., Kaiser, H., Cowley, P. D., James, N. C., Jansen van Vuuren, B., et al. (2022). The last two remaining populations of the critically endangered estuarine pipefish are inbred and not genetically distinct. *Front. Mar. Sci.* 8, 756595. doi: 10.3389/fmars.2021.756595

Whitfield, A. K. (1995). Threatened fishes of the world: *Syngnathus watermeyeri* Smith, 1963 (Syngnathidae). *Environ. Biol. Fishes* 43, 1–2. doi: 10.1007/BF00002484

Whitfield, A. K., Mkare, T. K., Teske, P. R., James, N. C., and Cowley, P. D. (2017). Life-histories explain the conservation status of two estuary-associated pipefishes. *Biol. Conserv.* 212, 256–264. doi: 10.1016/j.biocon.2017.06.024

Wooldridge, T. H., and Callahan, R. (2000). The effects of a single freshwater release into the Kromme Estuary. 3: Estuarine zooplankton response. *Water SA.* 26, 311–318.

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The response of the brood pouch transcriptome to synthetic estrogen exposure in the Gulf pipefish (*Syngnathus scovelli*)

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Endocrine disruptors have devastating impacts on the reproductive physiology of aquatic organisms. The Gulf pipefish, *Syngnathus scovelli*, is a sexually dimorphic species, which demonstrates predictable morphological, physiological, behavioral, and genetic responses to synthetic estrogen exposure. It has a broad geographic range, spanning freshwater and marine environments, making it a potential sentinel species across a wide range of habitats. In this study, we investigated the effects of ecologically relevant levels of 17α-ethinylestradiol (EE2) exposure on gene expression patterns in the male pipefish's brood pouch. We also characterized the extent to which EE2-exposed males developed coloration patterns that are normally restricted to females. We identified differentially expressed genes in the brood pouches of pregnant and non-pregnant males when males were exposed to 5ng/L EE2 from the second to eighth day of pregnancy (which normally lasts about 2 weeks). Our result revealed several potential candidate genes that have a role in the brood pouch's response to environmental estrogens. We also identified genes that were differentially expressed between mid-gestation pregnant males and non-pregnant males. We found an overall greater effect of EE2 exposure in the transcriptomes of non-pregnant males, which may explain why estrogen-exposed males exhibited difficulty receiving eggs in previous studies. The offspring developed similarly in the control and estrogen treatments, highlighting a potential link between the timing of EE2 exposure and its effects on male pregnancy. These results provide insight into how breeding pipefish populations may still exist even though they are found in freshwater and coastal locations where they are periodically exposed to potentially high concentrations of endocrine-disrupting compounds. We also present examples of female-typical coloration development on males due to EE2 exposure and identify candidate brood pouch genes that can be utilized as biomarkers, contributing to the development of the Gulf pipefish as a sentinel model for ecotoxicology.

KEYWORDS

syngnathidae, synthetic estrogen, brood pouch, transcriptomics, male pregnancy

Introduction

In aquatic environments, endocrine-disrupting compounds, such as 17α-ethinylestradiol (EE2), have been shown to affect physiology, gene expression patterns, and reproductive behaviors (Porte et al., 2006; Orlando and Guillette, 2007; Saaristo et al., 2010). For example, EE2 contamination can cause severe physiological changes in exposed aquatic male organisms, resulting in male sterilization, partial feminization, and even complete sex reversal in some cases (Allen et al., 1999; Jobling et al., 2003). Historically, the model systems for ecotoxicology research have been freshwater species, such as zebrafish and fathead minnows (Porte et al., 2006). More recently, marine species have revealed valuable insights into intraspecific variation in responses to endocrine disruptor contamination in coastal ecosystems (Gouveia et al., 2019; DeCourten et al., 2020). Long-term exposure to low levels of synthetic estrogens can cause entire breeding populations of fish to collapse as a consequence of reproductive failure in both fresh and saltwater environments (Nash et al., 2004; Kidd et al., 2007; Sárria et al., 2013).

Syngnathid fishes (i.e., seahorses, pipefishes, and seadragons) have served as flagship species for coastal environments due to their site fidelity and reliance on these threatened habitats during their entire lifespans (Shokri et al., 2009). Recently, they have emerged as sentinel species for ecotoxicology, with an increase in research utilizing several pipefish species, such as *Syngnathus abaster* and *S. scovelli* (Ueda et al., 2005; Partridge et al., 2010; Sárria et al., 2011; Rose et al., 2013; Sárria et al., 2013; Rose et al., 2015), and seahorse species, including *Hippocampus erectus* and *H. guttulatus* (Qin et al., 2020; D'Alvise et al., 2020). The Gulf pipefish (*S. scovelli*) has many advantageous characteristics for a toxicology model system. For instance, it is an estuarine fish and carries out its entire lifecycle in shallow habitats throughout the Gulf of Mexico and along the Florida Atlantic coast near large, populated cities, where effluent from wastewater treatment facilities is released (Dawson, 1982). These characteristics suggest that the Gulf pipefish might be useful as a bioindicator of coastal contamination throughout its life stages. Additional benefits include that Gulf pipefish can be collected throughout the year in many locations (Flanagan et al., 2016; Flanagan et al., 2021), that all ages can be maintained in laboratory conditions (Anderson and Jones, 2019), and that the developmental stages of embryos can be assessed through the male's transparent brood pouch (Paczolt and Jones, 2010). This species exhibits sexual dimorphism, has a polyandrous mating system, and is sex-role-reversed (Jones and Avise, 1997; Jones et al., 2000). Female pipefish are deeper bodied, have larger dorsal fins, and possess iridescent bands on their abdomens at sexual maturity. These traits are likely targets of sexual selection (Flanagan et al., 2014). These female secondary sexual traits can also develop in male Gulf pipefish that are exposed to EE2, so changes in male morphology can signal estrogen contamination in surrounding waters (Partridge et al., 2010).

In a review of integrative behavioral ecotoxicology, Peterson et al. (2017) observed that only a few studies have assessed the role of behavioral changes caused by toxin exposure on an organism's overall fitness. The well-described mating system of the Gulf pipefish has provided the opportunity to establish a link between endocrine disruptors and reproductive success in a sex-role-reversed species (Jones and Avise, 1997; Jones et al., 2001; Rose et al., 2013). Gulf pipefish research over the past two decades has investigated the effects of endocrine disruptors on morphological changes, particularly secondary sexual traits (Ueda et al., 2005; Partridge et al., 2010), courtship behaviors, the strength of sexual selection, and fitness (Partridge et al., 2010; Rose et al., 2013). In addition, the effects of endocrine disruptors on the transcriptomes of the liver, skin, and now brood pouch have been documented (Rose et al., 2015; Anderson et al., 2020). A well-annotated, chromosome-scale genome has provided the necessary backdrop for the development of biomarkers emerging from RNA-seq studies (Small et al., 2016; Ramesh et al., 2023). Studies of the population genomics of Gulf pipefish across saltwater and freshwater habitats (Flanagan et al., 2016; Flanagan et al., 2021) provide insights into population structure that lay a groundwork for future studies of population-level effects of endocrine disruptors in nature (Martyniuk et al., 2020).

High concentrations of synthetic estrogen contamination alter the function of the brood pouch when males are exposed prior to mating, and females prefer to mate with control males over those exposed to estrogen (Partridge et al., 2010; Rose et al., 2013). However, little is known about the effects of estrogen exposure on brood pouches of Gulf pipefish during a male's gestation period. Given the diversity of processes that take place in the brood pouch during pregnancy, such as waste removal and osmoregulation (Ripley, 2009), vascularization of the growing epithelial tissues (Ripley et al., 2010), adaptive immune responses (Parker et al., 2021), and many other vital processes to ensure embryo development (Carcupino et al., 1997), there is a need to understand the effects of EE2 exposure during pregnancy (Whittington et al., 2015). The brood pouch in seahorses displays morphological changes when exposed to 5 ng/L of EE2, with modifications in the expression patterns of genes related to the collagen family and many other cell proliferation and differentiation processes (Qin et al., 2020), but no pipefish has yet been studied in this regard.

The first goal of this study was to determine the effects of low, ecologically relevant levels of 17α-ethinylestradiol (EE2) exposure during and prior to pregnancy in the Gulf pipefish by identifying changes in gene expression patterns in the male pipefish's brood pouch. Since the study aimed to identify the impacts of EE2 exposure in the brood pouch during the male's pregnancy and not alter the process of egg transfer or fertilization, we chose to expose the males to contamination 24 hours after copulation. The second goal was to identify the effects of EE2 exposure on the success of the pregnancy and the changes in morphological patterning on the male's abdomens. This required an identification of stages in the banding ornamentation on the abdomen of male Gulf pipefish exposed to varying concentrations of EE2, which were then used to assess the levels of feminization for the males. The stages of ornamentation identified in this study provides a standard that can be used for future studies of both laboratory-exposed and wild-caught Gulf pipefish. To determine if the pregnancy was successful at the midpoint, we allowed the males to gestate for approximately half their pregnancy and compared the offspring from each treatment after eight days of pregnancy (Scobell and MacKenzie, 2011). The chosen concentration of 5 ng/L EE2 exposure was selected because this level of contamination is ecologically relevant (Zuo et al., 2006; Martyniuk et al., 2020; Klaic and Jirsa, 2022), has been shown to affect the reproductive abilities of laboratory exposed male Gulf pipefish (Rose et al., 2013), and has been detected in wastewater effluent in Florida seagrass communities where wild Gulf pipefish reside (Cook, 2015).

Materials and methods

Pipefish collection, experimental design, and EE2 exposure

Male Gulf pipefish (*S. scovelli*) were collected from Redfish Bay, Texas (N 27 53 39.07, W 97 7 51.69) in July of 2013 under the Texas Parks and Wildlife permit number SPR-0808-307. Only sexually mature females and pregnant males, those that had visible and functional brood pouches with developing offspring, were collected and brought into the lab under IACUC approval (Animal Use Protocol # 2013-0020, Reference #001898). All fish were dipped in freshwater for 10 minutes to remove any external parasites and then housed in 26-ppt salinity tanks at Texas A&M University (College Station, Texas). All field-caught males gave birth in lab prior to being used in the experiment and were assigned to either the non-pregnant category or mated in the lab to serve as a pregnant male for the experiment.

Methods used for the EE2 exposure treatments were stated in Rose et al. (2015). Powdered 17α-ethinylestradiol, of 98% purity, was acquired from Sigma and dissolved in ethanol. Tanks, holding 7-liters of saltwater, were separated and treated as either experimental or control tanks. Experimental tanks were dosed with 50 µl of 7ng/10µl stock EE2 ethanol solution to obtain a final concentration of 5ng of EE2 per liter of saltwater (5ng/L of EE2). Control tanks were dosed with 50 µl of pure ethanol with no EE2. Ten percent water changes were completed daily to ensure the concentrations remained at constant 5ng/L as established by Partridge et al. (2010).

To generate the pregnant males and establish successful pregnancies prior to assigning the fish to either the control or EE2-exposure treatments, the non-pregnant males and females were paired together in clean, EE2-free saltwater tanks until the male became pregnant. Pregnant males were then assigned a non-pregnant male for each replication. The males used in the study ranged from 84-94 mm in body length, and the size distribution did not differ between the treatments (mean = 89 mm). On the second day of the male's pregnancy, the fish were photographed, measured, and randomly assigned to treatments in either experimental tanks containing 5ng/L EE2 or the EE2-free control tanks. After seven days of exposure, that is, on the eighth day of the male's pregnancy, fish were humanely euthanized in MS-222 (IACUC-2013-0020) and their brood pouches dissected, imaged, and coated in RNA-later solution for the removal of offspring. Photographs were taken of the offspring, both in the pouch to detect any brood reduction (Paczolt and Jones, 2010) and after being dissected out of the pouch to assess the development of the embryos.

RNA extraction and sequencing

RNA from brood pouch flap tissues was isolated from 8 control males and 8 EE2 exposed males using a TRIzol® Reagent (Life Technologies, Carlsbad, CA) extraction method modified from (Leung and Dowling, 2005) as described in (Rose et al., 2015). All RNA samples were confirmed to be of high quality using a Caliper GX instrument before library construction. Each sample had its library individually prepared using the TruSeq mRNA Library Prep Kit v2 at the RTSF Genomics Core at Michigan State University. Caliper GX and qPCR methods were used for quality control on prepared libraries. Libraries were sequenced on an Illumina HiSeq 2500. Illumina Real Time Analysis (RTA, v1.17.21.3) was used for base calling. The RTA output was demultiplexed and converted to FASTQ using Illumina Bcl2fastq (v1.8.4) resulting in 150bp paired-end reads. This resulted in the following RNA-sequencing (RNA-seq) read files for each of the sixteen samples: (1) EE2-exposed and not pregnant (E51NPP, E52NPP, E54NPP, E57NPP), (2) EE2-exposed and pregnant (E51PP, E52PP, E54PP, E57PP), (3) control (i.e., non-exposed) and pregnant (C4PP, C7PP, C8PP, C9PP), and (4) control and not pregnant (C4NPP, C7NPP, C88NPP , C9NPP). The number of reads per sample ranged from 7,038,156 to 24,648,065 read pairs.

Transcriptome assembly and quantification

Reads from each sample were trimmed to remove Illumina adapters and low quality bases using Trimmomatic (v0.39) (ILLUMINACLIP : TruSeq3-PE.fa:2:30:10:2:keepBothReads LEADING:3 TRAILING:3 MINLEN:75) (Bolger et al., 2014). Paired-end reads were aligned and mapped to the Gulf pipefish (*S. scovelli*) genome (obtained from the William Cresko Lab at the University of Oregon) using HISAT2 (v2.2.1) on default parameters (Kim et al., 2015; Kim et al., 2019). The overall alignment rate averaged across all samples was 88.61%, with the lowest sample alignment rate being 83.08%. Reads were then assembled and quantified using StringTie (v.2.1.3b) without a reference annotation file in transcript merge mode (Pertea et al., 2015; Pertea et al., 2016). We chose not to use a reference annotation file to prevent the relatively poor annotation quality of the Gulf pipefish genome from biasing resulting read counts. Transcripts were then merged from all samples into a non-redundant set of transcripts (n = 21,721). A gene count matrix was generated from the abundances of the merged transcripts using the prepDE.py script from StringTie. Read counts and TPM values for each gene are provided in Supplemental Table 3.

Differential expression analysis

From this gene count matrix, we used the R (R Core team, 2015) and Bioconductor package edgeR (v.3.1.3) to identify transcripts that were differentially expressed between the conditions of EE2-exposure and pregnancy status (Robinson et al., 2010). Our goal was to understand whether EE2-exposure had an overall effect on the male brood pouch regardless of pregnancy, and whether EE2-exposure influenced the brood pouches of males that were pregnant. This resulted in four pairwise comparisons based on the following conditions: (1) EE2-exposure regardless of pregnancy status, (2) pregnancy status regardless of EE2-exposure, (3) EE2-exposure for only pregnant fish, and (4) EE2-exposure for only non-pregnant fish. Only genes with a false discovery rate ≤ 0.05 for differential expression were used for downstream analyses. For each of the four comparisons, we were left with a list of differentially expressed genes (Figure 1). The nucleotide sequences for the corresponding transcripts were obtained from the Gulf pipefish genome using the program gffread (v12.7) and FastaRecordExtractor (v2) (Pertea and Pertea, 2020). Each resulting sequence was converted to its longest open reading frame via FastaToORF (v1). Both FastaRecordExtractor and FastaToORF are available via the Jones Lab Github (https://github.com/JonesLabIdaho/). Sequences from differentially expressed genes were compared against all *Syngnathus* species present in the NCBI database using BLASTX, and the best matching protein ID was retained (Supplemental File 1: DE_syngnathus.xlsx). Sequences from differentially expressed genes were also used in the downstream gene ontology analysis.

Analysis of abundant transcripts

Apart from the differential expression analysis, we were also interested in the nature of abundant transcripts within the brood pouches of the different experimental groups. We assessed the overall expression profile and clustering of samples with a heatmap and the variance between samples with a PCA. For these analyses, we used the TPM (transcripts per million) values calculated by StringTie. We only included transcripts that had a minimum expression level in at least one of the four experimental groups. Our minimum expression level was set by averaging the TPM values across transcripts between samples of the same condition (EE2-exposure and pregnancy status) and keeping transcripts that had an average TPM value ≥ 25 in at least one of these four groups (n = 4,892). We excluded transcripts that have very low expression across all samples because they do not provide enough information on the variance between the samples.

To identify the variance between and clustering of samples, we performed a principal component analysis using the R function prcomp() within the package stats (v.3.6.2) (Figure 2). For all samples, we used only the TPM-filtered transcripts, with the expression value for these transcripts calculated as the log(TPM +1). Samples are labelled and plotted by pregnancy and EE2-exposure status on the first two principal components (Figure 2A). The first two principal components were also plotted in comparison with the third (Figures 2B, C).

We then took fifty transcripts with the highest average TPM value in each of the four experimental groups and searched for their corresponding protein ID and name in NCBI's *S. scovelli*

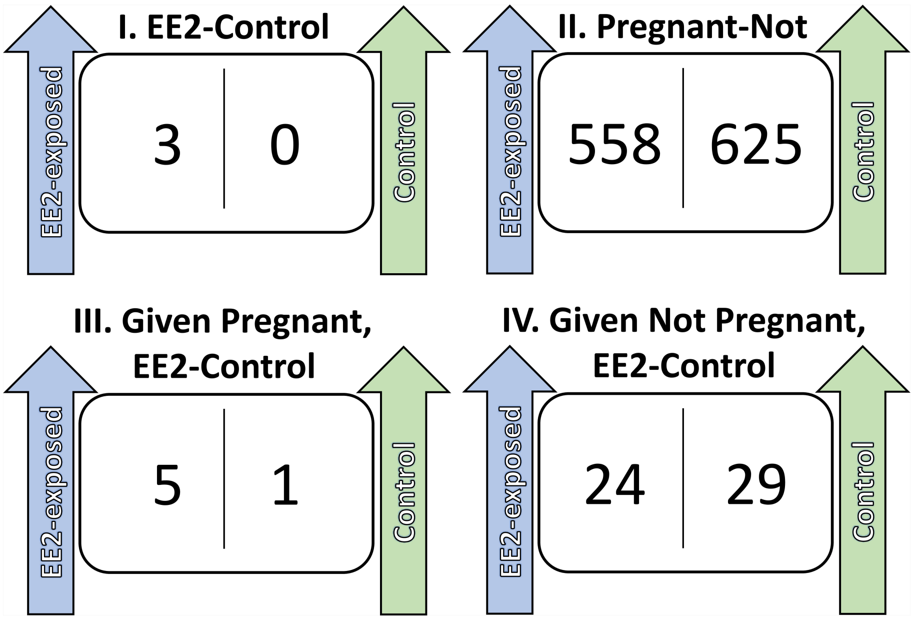


FIGURE 1
Differentially expressed genes from pairwise analyses. These are the number of significantly differentially expressed genes (FDR ≤ 0.05), that are up-regulated for their respective condition. There are four pairwise tests: (1) EE2-exposed versus control, including all males (regardless of pregnancy status), (2) pregnant versus not, including all males (regardless of EE2-exposure), (3) EE2-exposed versus control, for only pregnant males, and (4) EE2-exposed versus not, for only non-pregnant males.

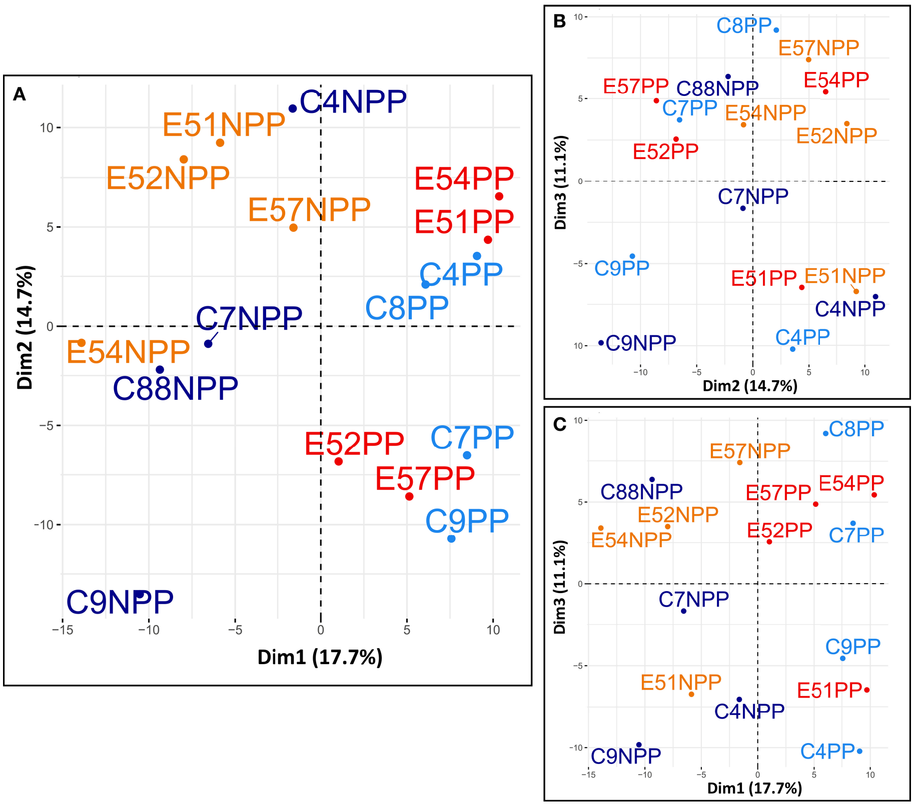


FIGURE 2

PCA between all individual male samples. Samples are labelled by their EE2-exposure status (E-EE2 exposed or C-control) and their pregnancy status (NPP-not pregnant, PP-pregnant). The first principal component separates males by pregnancy status **(A, C)**. The first two principal components explain 32.4% of the total variance between samples **(A–C)**. The third principal component explains an additional 11.1% of the total variance **(B, C)**.

database using BLASTX. The protein sequence ID with the best *E*-value score per transcript was retained (Supplemental File 2: top50_genes.xlsx).

Gene ontology analysis of differentially expressed genes

To explore the genes present within our differential expression comparison, we performed a gene ontology analysis through the PANTHER database using a common reference proteome. To begin, the zebrafish (*Danio rerio*) proteome (UP000000437_7955), was obtained from UniProt and used to construct a local NCBI BLAST database. The differentially expressed transcripts were searched against the zebrafish proteome using BLASTX to identify potential orthologs. When multiple differentially expressed transcripts within the pairwise comparison matched to the same zebrafish protein sequence ID, duplicates were removed to prevent potential inflation bias. If there was no match for a differentially expressed transcript, its sequence was not included for the PANTHER analysis. For these BLAST searches, matching protein sequence IDs with an *E*-value score of $1x10^{-20}$ or less were retained, and the protein sequence ID with the best *E*-value score per transcript was used.

For the gene ontology analysis, we used the PANTHER database (v16.0) (Thomas et al., 2003; Mi et al., 2013; Mi et al., 2019). The PANTHER database is a protein classification system that provides information on gene ontology and gene families. It also provides details on the molecular function, biological process, and pathways in which a gene is implicated. Use of PANTHER requires a reference organism for which PANTHER has an established database. For this reason, we used zebrafish, as it is one of the most heavily studied fish with respect to gene function. For the PANTHER analysis, we uploaded the list of protein sequence IDs for each of the differential expression comparisons. We performed a gene ontology analysis for biological processes, cellular components, and molecular function using the curated PANTHER GO-Slim database, and an analysis using the PANTHER Protein Class database. Results were recorded and assessed as percentages of the total hits in each database (Figure 3).

Assessment of male banding ornamentation stages

The morphological changes in the male abdomen were categorized using a scale of ornamental band intensity, similar to that used to quantify ornamentation in female *S. scovelli* by Jones and colleagues

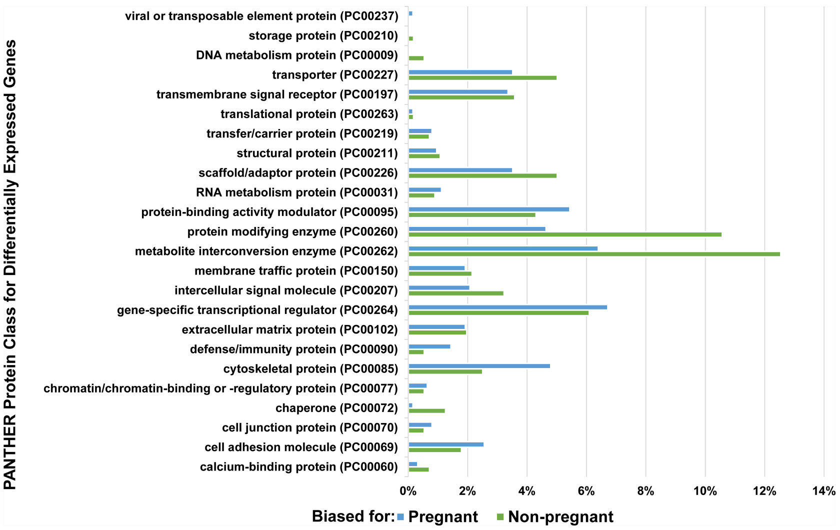


FIGURE 3
Protein classes from PANTHER for pregnancy-related differentially expressed genes. Differentially expressed genes from the second pairwise comparison (pregnant versus not, including all males, regardless of EE2-exposure) were BLAST searched for in the zebrafish (*Danio rerio*) proteome to find a corresponding protein ortholog. Resulting zebrafish protein IDs were used for a PANTHER protein class analysis. The percentage of proteins that fall into a specific protein class are report as the total number of proteins in a protein class divided by the total number of differentially expressed genes (pregnant males = 625 and non-pregnant males = 558). Differentially expressed genes that did not match to a zebrafish protein, and zebrafish proteins that did not have a PANTHER protein class are not displayed (pregnant males = 47%, and non-pregnant males = 34%).

(2001). To generate the ornamentation scale, we produced images representing a wide range of variation among exposed males. The requisite variation in male morphology was generated by exposing them to 4 combinations of EE2 concentration and exposure time. We used a 2ng/L exposure for seven days (n = 8 male fish) to replicate the concentrations from Rose et al. (2013). A 5ng/L exposure for seven days (n= 8 fish not used in the present study) represented the EE2 exposure of the present study. We also exposed males to 5ng/L for 18 days (n= 5) and 10ng/L for 18 days (n =5) to establish males that had full iridescence, approaching that observed in typical female specimens. Images were taken under a dissecting microscope to create the scale ranging from no banding pattern in control fish to female-like iridescent bands in the high exposure treatments (Figure 4). We used this newly established scale of ornamentation intensity to score the fish in the present study (which, as noted above, were not involved in the establishment of the scale). In a double-blind process, three reviewers were assigned the images of the male abdomens for the 16 fish included in the RNA-seq dataset for staging. The average score for each fish was taken across the three reviewers for statistical analysis. A Wilcoxon rank sum test was conducted due to the violation of normality for the data comparing the control males (n=8) with the EE2-exposed males (n=8) for band development.

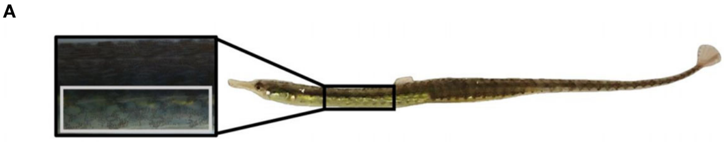
Results

Phenotypic response to EE2 exposure

During the seven days of 5ng/L of EE2 exposure, all males maintained their pregnancies with normal pouches and their offspring did not show any signs of brood reduction. The offspring developmental stages were assessed during the brood pouch dissection. The embryos for the control and EE2-exposed pregnant males were determined to be at the correct developmental stage for embryos at mid-gestation according to Sommer et al., 2012 and Schneider et al., 2023. Adult male fish in the EE2 treatment had begun demonstrating morphological signs of feminization during their seven days of exposure to estrogen, as seen in Partridge et al., 2010, whereas the control males did not show any phenotypic changes. Through an assessment of the images taken after seven days of EE2 contamination, all of the estrogen exposed males, including the pregnant and non-pregnant fish, had begun developing the female secondary sexual traits with banding patterns on their torsos. The staging of the band development was significantly higher in the EE2-exposed males with an average stage of 1.88 ± 0.37 compared to 0.17 ± 0.09 for the non-exposed males (Wilcoxon rank sum: W=1.5, p=0.0012).

Transcriptional response to EE2 exposure

The sequencing of the brood pouch RNA resulted in a total of 1,245 differentially expressed genes across pairwise comparisons between the four treatments (n=16, Figure 1). The brood pouch expression profiles were more greatly influenced by their pregnancy status than their exposure to EE2 (Figure 1). The largest number of differentially expressed genes were within the males in regard to their pregnancy status. There was 558 genes significantly upregulated in pregnant males and 625 genes upregulated in non-pregnant males (Figure 1, Supplemental Table 1A). There were only three genes that were statistically significantly different (p<0.05), as a result of EE2 exposure for all males, regardless of their pregnancy



B

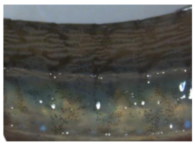
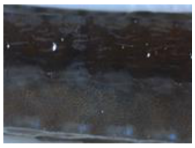
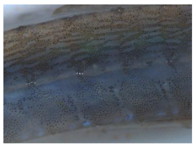
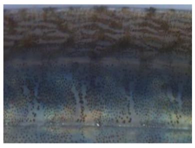
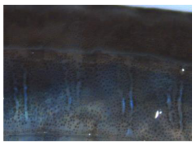
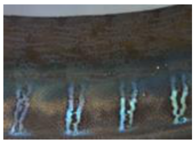
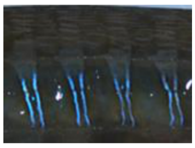
Male Banding Stage	Representative Image	Descriptive details of pigment for stages	EE2 Exposure Level
0		No line patterns of pigment cells	None
0		No line patterns of black pigment cells for bands, Reflective spots on lower abdomen	None
1		Reflective spots on lower abdomen expand to have wider sections of iridescence developing, no bands yet	2 ng/L for 7 days
1		Reflective spots on lower abdomen expand to have wider sections of iridescence developing, no bands yet	5 ng/L for 7 days
2		Black pigment is moving to create sections with thin single band-shaped cleared lines free of black pigment for bands to develop	2 ng/L for 7 days
2		Black pigment is moving to create sections with thin band-shaped cleared lines free of black pigment for paired bands to develop	5 ng/L for 7 days
3		Paired lines lacking black pigment are developed for the bands to form but lack iridescence within bands	5 ng/L for 7 days
4		Developed paired bands with black pigment outlines on edges, iridescence starting within the paired band sections lacking black piment	5 ng/L for 18 days
5		Blue iridescence on well-developed paired bands	10 ng/L for 18 days
Female		Blue iridescence on well-developed paired bands	None

FIGURE 4 (Continued)

FIGURE 4 (Continued)
Developmental stages of the male banding pattern resulting from exposure to estrogen. The torso section outlined in gray indicates the location of the banding pattern on the abdomen of the Gulf pipefish on this non-exposed male in **(A)**. The table for **(B)** contains images for the stages ranging from 0 with no band development through stage 5 with fully developed paired bands, similar to the last column displaying female bands. Descriptions with details of the pigmentation patterns and the EE2-exposure level of the fish is provided for each stage.

status, with all three genes being upregulated in the EE2-exposed males. These three estrogen-responsive genes included *coagulation factor XIII A chain-like, peptidyl-prolyl cis-trans isomerase FKBP11*, and *transcription factor Sp7 isoform X1* (Supplemental Table 1B). Gene expression patterns were less altered by EE2 exposure in pregnant males compared with their non-pregnant counterparts. There were only 6 genes differentially expressed when comparing the control pregnant males with the pregnant males in the EE2 treatment (Supplemental Table 1C) However, when comparing the non-pregnant males across treatments, there were 53 genes reported as differentially expressed due to EE2 exposure (Supplemental Table 1D).

The most highly expressed genes in all four categories of male brood pouches included overrepresentation of genes related to cell proliferation, including lectin, keratin, actin, and myosin proteins (Supplemental Table 2). Additional genes detected in the top 25 of all four male groups included *glyceraldehyde-3-phosphate dehydrogenase, creatine kinase muscle b, cytochrome c oxidase, cAMP-specific 3' 5'-cyclic phosphodiesterase 4C-like isoform X, aldolase a fructose-bisphosphate b*, and *parvalbumin beta-like*. The top expressed genes in this study for the brood pouch parallel the top expressed genes found in the Gulf pipefish skin and muscle transcriptome (Anderson et al., 2020). The top four genes for muscle and skin tissues included lectin proteins, *glyceraldehyde-3-phosphate dehydrogenase*, actin proteins, and *creatine kinase muscle b*, which were all found in the top 10 highest expressed genes in the brood pouches of pregnant males. The EE2-exposed males had similar highly expressed genes when compared with their control counterparts.

We identified genes regulating embryo development that were only highly expressed in the brood pouches for the control and EE2 exposed pregnant males. The reproductive gene *hatching enzyme 1.2-like isoform X1* was the third most highly expressed transcript for both pregnant groups. This gene is likely categorized as a patristacin-like gene (Small et al., 2016) and was significantly downregulated in expression for non-pregnant males compared to the pregnant males. Another reproductive gene that was differentially expressed in pregnant males compared with non-pregnant males was *low choriolytic enzyme-like isoform*, which is likely another *patristacin* or *patristacin*-like gene. Given that the pregnant males were dissected during their mid-gestation stage, the offspring were likely nearing the developmental stage where embryos begin hatching from their chorion (Sommer et al., 2012; Whittington et al., 2015; Schneider et al., 2023).

The PANTHER analyses identified protein classes for all the differentially expressed genes that could be assigned to the zebrafish proteome. The protein classes with the largest number of proteins assigned, particularly for the non-pregnant males, included metabolite interconversion enzyme, protein modifying enzyme, and gene-specific transcriptional regulator. The pregnant male genes were assigned to the cytoskeletal protein and protein binding activity modulator categories in higher percentages compared to their non-pregnant counterparts. Many of the protein classes were related to restructuring occurring in the pouches either during or in preparation for pregnancy, such as extracellular matrix proteins, cytoskeletal proteins, cell adhesion proteins and cell junction proteins (Qin et al., 2020). Although the results indicated that the defense/immunity protein group had greater representation for the pregnant males, lectin proteins were found to be one of the most highly expressed genes across all four male categories rather than being differentially expressed, thus not being reported in the PANTHER data set. Interestingly, C-type lectin transcripts, which have been shown to be highly expressed in seahorse brood pouches and suggested as an immune response gene in the brood pouch, were upregulated in EE2-exposed non-pregnant males compared to their control counterparts (Small et al., 2013; Whittington et al., 2015; Kawaguchi et al., 2017).

Discussion

This study's findings provide valuable insights into the effects of EE2 contamination on the brood pouch and stages of morphological changes in ornamentation of males, contributing to the development of the Gulf pipefish as an ecotoxicology model system. We also provide additional estrogen-responsive biomarkers for male brood pouches and a photographic scale for quantifying EE2-induced banding patterns in males that can be used in wild populations for predicting EE2-exposure. Additionally, we did not detect a substantial alteration in embryo morphology or developmental stage when comparing the offspring from the control and treatment males. We observed no direct evidence of brood reduction (Paczolt and Jones, 2010), and the staging of the embryos was similar to that of, *Syngnathus typhle* during mid prerelease developmental stages, as outlined by Schneider and colleagues (2023), when the eye is well developed and pigment has spread across the head and trunk. These results provide insight into why pipefish populations can remain reproductively active in locations of known endocrine-disruptor contamination. Gene expression data for the brood pouch also revealed that non-pregnant males show a greater transcriptional response to EE2 exposure when compared to pregnant males, providing support for previous studies where males were unable to receive eggs after exposure as non-pregnant or late-stage pregnant males when exposed to similar estrogen concentrations (Rose et al., 2013). Many of the top expressed transcripts were the same genes as the top expressed genes in the muscle and skin tissues studied by Anderson and colleagues (2020). The overall fold changes in gene expression patterns of the brood pouch paralleled those of the Gulf pipefish skin and muscle tissues (Anderson et al., 2020). The brood pouch transcriptome showed substantially fewer differentially expressed genes between control and estrogen treated fish (see Supplemental Table 1) compared to the dramatic effects of EE2 on the liver transcriptome (Rose et al., 2015), indicating variation in EE2 responsiveness across different organs.

The results from the current study indicate that timing of exposure to EE2 can affect the male pipefish's ability to successfully carry developing offspring in their brood pouches. Previous studies have shown that male pipefish and seahorses, when exposed prior to mating, experience reduced or receding pouch tissue, difficulties receiving eggs from females, or termination of the pregnancy with non-surviving offspring (Partridge et al., 2010; Rose et al., 2013; Sárria et al., 2013; Qin et al., 2020). However, in this study the males in the EE2 treatment were not exposed until 24 hours after mating, and all showed normal development of their offspring at the midpoint of the pregnancy. Previous studies conducted at 2ng/L allowed for Gulf pipefish males to become impregnated and an increase in female reproductive yields, yet there was complete reproductive failure documented for males at 5 ng/L (Rose et al., 2013). Partridge and colleagues (2010) reported that males exposed to 5 ng/L of EE2 had difficulty receiving eggs and continued to have reduced mating activity several days after being removed from exposure. Interestingly, the male pipefish in this study did display feminizing morphological changes as a result of the 5 ng/L EE2 contamination, similar to the males in other studies (Partridge et al., 2010; Rose et al., 2013) with the major difference in the studies being the timing of the exposure relative to the pregnancy.

Understanding how populations of pipefish can remain reproductively active despite the threats of contamination is an important aspect of learning about ecotoxicology model systems in wild populations (Matthiessen et al., 2018). One potential explanation is that estrogen or other contaminants can vary in their concentrations over time depending on the frequency and quantity of contaminated effluent being introduced into coastal environments (Zuo et al., 2006). For example, the concentrations of EE2 detected in water bodies can vary seasonally in the dry season compared to the wet season and the distance of the fishes from where the wastewater effluent is introduced (Klaic and Jirsa, 2022). The range in detected concentrations in natural bodies of water could be due in part to varying rates of photodegradation of EE2 contamination, as a result of variation in sun exposure and water depths within pipefish habitats, given that EE2 has a half-life of less than 1.5 days during days with maximum sun (Zuo et al., 2006). Although the pipefish in this study were exposed to 5 ng/L of EE2, which is higher than the concentrations for surface waters reported in marine locations further from the wastewater effluent sources reported in Klaic and Jirsa (2022), wildlife are often exposed to several endocrine disrupting compounds that have similar estrogenic effects on the physiology and behaviors of exposed organisms (Matthiessen et al., 2018). The combination of varying concentration at the site of introduction and potential movement patterns of the fish can lead to individuals being exposed at different stages of their reproductive cycle. Future directions for this model system include monitoring wild populations for contamination levels and long-term surveys of their reproduction in locations with evidence of estrogen contamination. Studies in Tampa Bay have identified locations where 5ng/L EE2 has been detected entering the coastal waters where pipefish reside, providing the opportunity for testing multibiomarkers (Cook, 2015). Implementing the tools developed in the Gulf pipefish through comparative RNA-sequencing conducted over the past several years and identification of biomarker candidates will enable the next stage in biomonitoring *in situ* (Gouveia et al., 2019; Martyniuk et al., 2020).

One of the primary goals of the present study was to identify gene expression patterns in the brood pouch prior to mating and during the male's pregnancy when success of developing offspring could be confirmed. The males in the present study were either non-pregnant after recently having given birth or halfway through the gestation of their offspring, representing a diverse array of metabolic and epithelial changes occurring in the male's reproductive structure during the two different stages of reproduction. For example, non-pregnant males are typically preparing their pouch for mating (Carcupino et al., 1997). Thus, this stage could require the differentiation of the epithelial cells and other changes to the structure of the pouch flaps. On the other hand, pregnant males would likely experience epithelial proliferation, growth of new blood vessels, and waste removal within the pouch as the offspring develop (Scobell and MacKenzie, 2011). Since males included in this study were dissected during the middle of their pregnancy, the data set does not include genes related to fertilization, early gestation or parturition. Nevertheless, a time-series of the brood pouch transcriptome of the big-bellied seahorse (*H. abdominalis*) by Whittington and colleagues (2015) indicated that the mid-pregnancy stage differed the most from non-pregnant males, with 966 genes up or down regulated. In contrast, early, late, and post-parturition stages showed 527, 535, and 325 genes, respectively, that were differentially expressed compared to non-pregnant males. Comparatively, the largest difference in gene expression patterns in the seahorse brood pouch transcriptome occurred between mid-stage pregnant males and post-parturition males, indicating that the pouch undergoes a remodeling after the offspring are born to prepare for the next pregnancy (Whittington et al., 2015). This pattern is also seen in the pipefish brooding structures for *Nerophis ophidion* and *Syngnathus rostellatus*, where transcriptomes from early-stage pregnant males showed greater numbers of differentially expressed genes when compared to the parturition males, whereas the late stage transcriptomes were not as different from the parturition males (Parker et al., 2021). The *serine/threonine-protein kinase* transcripts were significantly biased in non-pregnant males in the present study and also in the Gulf pipefish brood pouch study conducted by Small et al. (2016). Seahorse males during post parturition experience an increase in *estrogen receptor 2a* expression in the brood pouch (Whittington et al., 2015), consistent with our findings in the Gulf pipefish. Curiously, neither seahorses nor pipefish in the present study showed a significant change in estrogen receptor expression when exposed to EE2 contamination (Qin et al., 2020).

Our differential expression analyses identified three EE2 responsive genes to use as biomarkers for contamination in all male brood pouches. One of these genes is *coagulation factor XIII A* *chain-like*, which is also upregulated by EE2 contamination in Gulf pipefish male's skin tissues where the female-specific iridescent bands develop (Anderson et al., 2020). The exposure to EE2 resulted in a greater change in gene expression patterns for non-pregnant male brood pouches compared to pregnant males, which could lead to a decline in pouch function for males or their ability to receive eggs from females during future courtship events. EE2 exposure and pregnancy stage did not lead to differences in the representation of genes with the greatest expression levels, as seen by the similar breakdowns in the makeup of the top 25 most expressed genes across groups reported in Supplemental Table 2. The gene *cathepsin* was upregulated in the brood pouch of EE2 exposed pregnant males compare to their control counterparts and has also been identified as an estrogen biomarker in the skin and the liver of male Gulf pipefish (Rose et al., 2015; Anderson et al., 2020). The gene with the greatest upregulation in EE2 non-pregnant males, *C-type lectin domain family 4 member M-like*, which potentially regulates cell growth and immune responses, has also been shown to be over expressed in brood pouches of non-pregnant male seahorses compared to pregnant males (Whittington et al., 2015).

In conclusion, the gene expression changes identified in the brood pouch transcriptome and the documentation of male morphological changes in male banding patterns due to EE2 exposure contribute to the growing development of the Gulf pipefish as a model system for ecotoxicology. The EE2-responsive genes identified in the brood pouch for this study, along with the extreme gene expression patterns in the livers of EE2-exposed pipefish (Rose et al., 2015) and the genes previously identified that underlie the development of female secondary sexual traits in the skin of EE2-exposed males (Anderson et al., 2020), provide several excellent candidate genes for developing biomarkers to detect endocrine disruptor contamination in wild populations of pipefish. While previous studies indicated EE2-exposed pipefish males experienced a reduction in the function of their brood pouches prior to mating, males in this study successfully maintained their pregnancies when exposed 24 hours after copulation. Although the EE2 exposed males in this study displayed morphological feminization and began developing the female-specific sexually selected banding on their abdomen, the offspring displayed normal developmental patterns through the 7 days of exposure, indicating that the timing of exposure to EE2 is a critical factor affecting male pouches. Given that EE2 can alter the strength of sexual selection in the Gulf pipefish (Rose et al., 2013), affect the preferences of choosy females (Partridge et al., 2010), and has the potential to alter the operational sex ratios and effective population sizes due to non-receptive or sterile males (Lane et al., 2011), there is still a need to test the effects of contaminants on wild populations. The combination of the molecular tools and the well characterized morphological changes in EE2 exposed males provide an excellent arsenal of techniques for detecting estrogen contamination in wild populations across various ecosystems where the Gulf pipefish resides, particularly when quantification of contamination in water samples is difficult. The collection of previous laboratory research has now set the stage for the Gulf pipefish to become a well-established model system for investigating how endocrine disruptors, such as EE2, can alter the mating system of exposed pipefish populations in the natural environment.

Data availability statement

The RNA-seq raw reads generated in this study were deposited in NCBI's SRA database under BioProject PRJNA980712. The morphological data generated for this study is available https://zenodo.org/record/7996477.

Author contributions

Conceived and designed the experiments: ER and AJ. Performed the experiments: ER. Analyzed the data: ER, BJ, VA, AJ. Wrote the paper: ER, BJ, AJ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1138597/full#supplementary-material

SUPPLEMENTARY TABLE 1
Gene expression patterns for all comparative groups. Differentially expressed genes for all control and EE2-exposed males **(A)**, all pregnant and non-pregnant males **(B)**, pregnant control and EE2-exposed males **(C)**, and non-pregnant control and EE2-exposed males **(D)**.

SUPPLEMENTARY TABLE 2

Top 50 expressed transcripts for all four male groups. Average TPM is provided along with the NCBI protein match for each of the highest expressed transcripts for the four male groups.

SUPPLEMENTARY TABLE 3

Counts for all genes. TPM counts provided for all the transcripts generated from the brood pouches for all control and EE2-exposed males.

References

Allen, Y., Scott, A., Matthiessen, P., Haworth, S., Thain, J., and Feist, S. (1999). Survey of estrogenic activity in united kingdom estuarine and coastal waters and its effects on gonadal development of the flounder *Platichthys flesus*. *Environ. Toxicol. Chem.* 18, 1791–1800. doi: 10.1002/etc.5620180827

Anderson, A. P., and Jones, A. G. (2019). Choosy gulf pipefish males ignore age but prefer active females with deeply keeled bodies. *Anim. Behav.* 155, 37–44. doi: 10.1016/j.anbehav.2019.05.018

Anderson, A. P., Rose, E., Flanagan, S. P., and Jones, A. G. (2020). The estrogen-responsive transcriptome of female secondary sexual traits in the gulf pipefish. *J. Heredity* 111 (3), 294–306. doi: 10.1093/jhered/esaa008

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for illumina sequence data. *Bioinformatics* 30 (15), 2114–2120. doi: 10.1093/bioinformatics/btu170

Carcupino, M., Baldacci, A., Mazzini, M., and Franzoi, P. (1997). Morphological organization of the male brood pouch epithelium of *Syngnathus abaster* risso (Teleostea, syngnathidae) before, during, and after egg incubation. *Tissue Cell* 29 (1), pp.21–pp.30. doi: 10.1016/S0040-8166(97)80068-7

Cook, M. M. (2015). *Endocrine-disrupting compounds: measurement in Tampa bay, removal from sewage and development of an estrogen receptor model* (University of South Florida ProQuest Dissertations Publishing, 3688382).

D'Alvise, N. P., Richard, S., Aublanc, P., Bunet, R., and Bonnefont, J. L. (2020). When male seahorses take the female contraceptive pill. *Environ. Sci. pollut. Res.* 27 (14), 16528–16538. doi: 10.1007/s11356-020-08152-1

Dawson, C. E. (1982). *Fishes of the Western north Atlantic, part 8, order gasterosteiformes, suborder syngnathoidei* (New Haven, CT: Sears Foundation for Marine Research, Yale University).

DeCourten, B. M., Forbes, J. P., Roark, H. K., Burns, N. P., Major, K. M., White, J. W., et al. (2020). Multigenerational and transgenerational effects of environmentally relevant concentrations of endocrine disruptors in an estuarine fish model. *Environ. Sci. Technol.* 54 (21), 13849–13860. doi: 10.1021/acs.est.0c02892

Flanagan, S. P., Johnson, J. B., Rose, E., and Jones, A. G. (2014). Sexual selection on female ornaments in the sex-role-reversed gulf pipefish (*Syngnathus scovelli*). *J. Evolution. Biol.* 27 (11), 2457–2467. doi: 10.1111/jeb.12487

Flanagan, S. P., Rose, E., and Jones, A. G. (2016). Population genomics reveals multiple drivers of population differentiation in a sex-role-reversed pipefish. *Mol. Ecol.* 25 (20), 5043–5072. doi: 10.1111/mec.13794

Flanagan, S. P., Rose, E., and Jones, A. G. (2021). The population genomics of repeated freshwater colonizations by gulf pipefish. *Mol. Ecol.* 30 (7), 1672–1687. doi: 10.1111/mec.15841

Gouveia, D., Almunia, C., Cogne, Y., Pible, O., Degli-Esposti, D., Salvador, A., et al. (2019). Ecotoxicoproteomics: a decade of progress in our understanding of anthropogenic impact on the environment. *J. Proteomics* 198, 66–77. doi: 10.1016/j.jprot.2018.12.001

Jobling, S., Casey, D., Rodgers-Gray, T., Oehlmann, J., Schulte-Oehlmann, U., Pawlowski, S., et al. (2003). Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. *Aquat. Toxicol.* 65 (2), 205–220. doi: 10.1016/S0166-445X(03)00134-6

Jones, A. G., and Avise, J. C. (1997). Microsatellite analysis of maternity and the mating system in the gulf pipefish *Syngnathus scovelli*, a species with male pregnancy and sex-role reversal. *Mol. Ecol.* 6 (3), 203–213. doi: 10.1046/j.1365-294X.1997.00173.x

Jones, A. G., Rosenqvist, G., Berglund, A., Arnold, S. J., and Avise, J. C. (2000). The Bateman gradient and the cause of sexual selection in a sex–role–reversed pipefish. *Proc. R. Soc. London. Ser. B: Biol. Sci.* 267 (1444), 677–680. doi: 10.1098/rspb.2000.1055

Jones, A. G., Walker, D., and Avise, J. C. (2001). Genetic evidence for extreme polyandry and extraordinary sex-role reversal in a pipefish. *Proc. R. Soc. London. Ser. B: Biol. Sci.* 268 (1485), 2531–2535. doi: 10.1098/rspb.2001.1841

Kawaguchi, M., Okubo, R., Harada, A., Miyasaka, K., Takada, K., Hiroi, J., et al. (2017). Morphology of brood pouch formation in the pot-bellied seahorse hippocampus abdominalis. *Zool. Lett.* 3, 1–16. doi: 10.1186/s40851-017-0080-9

Kidd, K., Blanchfield, P., Mills, K., Palace, V., Evan, R., Lazorchak, J., et al. (2007). Collapse of a fish population after exposure to synthetic estrogen. *PNAS* 104, 8897–8901. doi: 10.1073/pnas.0609568104

Kim, D., Langmead, B., and Salzberg, S. L. (2015). HISAT: a fast spliced aligner with low memory requirements. *Nat. Methods* 12 (4), 357–360. doi: 10.1038/nmeth.3317

Kim, D., Paggi, J. M., Park, C., Bennett, C., and Salzberg, S. L. (2019). Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat. Biotechnol.* 37 (8), 907–915. doi: 10.1038/s41587-019-0201-4

Klaic, M., and Jirsa, F. (2022). 17α-ethinylestradiol (EE2): concentrations in the environment and methods for wastewater treatment–an update. *RSC Adv.* 12 (20), 12794–12805. doi: 10.1039/D2RA00915C

Lane, J. E., Forrest, M. N., and Willis, C. K. (2011). Anthropogenic influences on natural animal mating systems. *Anim. Behav.* 81 (5), 909–917. doi: 10.1016/j.anbehav.2011.02.003

Leung, Y. F., and Dowling, J. E. (2005). Gene expression profiling of zebrafish embryonic retina. *Zebrafish* 2 (4), 269–283. doi: 10.1089/zeb.2005.2.269

Martyniuk, C. J., Feswick, A., Munkittrick, K. R., Dreier, D. A., and Denslow, N. D. (2020). Twenty years of transcriptomics, 17alpha-ethinylestradiol, and fish. *Gen. Comp. Endocrinol.* 286, 113325. doi: 10.1016/j.ygcen.2019.113325

Matthiessen, P., Wheeler, J. R., and Weltje, L. (2018). A review of the evidence for endocrine disrupting effects of current-use chemicals on wildlife populations. *Crit. Rev. Toxicol.* 48 (3), 195–216. doi: 10.1080/10408444.2017.1397099

Mi, H., Muruganujan, A., Casagrande, J. T., and Thomas, P. D. (2013). Large-Scale gene function analysis with the PANTHER classification system. *Nat. Protoc.* 8 (8), 1551–1566. doi: 10.1038/nprot.2013.092

Mi, H., Muruganujan, A., Ebert, D., Huang, X., and Thomas, P. D. (2019). PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. *Nucleic Acids Res.* 47 (D1), D419–D426. doi: 10.1093/nar/gky1038

Nash, J. P., Kime, D. E., van der Ven, L. T., Wester, P. W., Brion, F., Maack, G., et al. (2004). Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environ. Health Perspect.* 112 (17), 1725–1733. doi: 10.1289/ehp.7209

Orlando, E. F. , and Guillette, L. J. Jr. (2007). Sexual dimorphic responses in wildlife exposed to endocrine disrupting chemicals. *Environ. Res.* 104 (1), 163–173. doi: 10.1016/j.envres.2006.06.002

Paczolt, K. A., and Jones, A. G. (2010). Post-copulatory sexual selection and sexual conflict in the evolution of male pregnancy. *Nature* 464 (7287), 401–404. doi: 10.1038/nature08861

Parker, J., Dubin, A., Schneider, R., Wagner, K. S., Jentoft, S., Böhne, A., et al. (2021). Immunological tolerance in the evolution of male pregnancy. *Mol. Ecol.* 32 (4), 819–840. doi: 10.1111/mec.16333

Partridge, C., Boettcher, A., and Jones, A. G. (2010). Short-term exposure to a synthetic estrogen disrupts mating dynamics in a pipefish. *Horm. Behav.* 58 (5), 800–807. doi: 10.1016/j.yhbeh.2010.08.002

Pertea, M., Kim, D., Pertea, G. M., Leek, J. T., and Salzberg, S. L. (2016). Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and ballgown. *Nat. Protoc.* 11 (9), 1650–1667. doi: 10.1038/nprot.2016.095

Pertea, G., and Pertea, M. (2020). GFF utilities: GffRead and GffCompare. *F1000Research* 9, 304. doi: 10.12688/f1000research.23297.1

Pertea, M., Pertea, G. M., Antonescu, C. M., Chang, T. C., Mendell, J. T., and Salzberg, S. L. (2015). StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat. Biotechnol.* 33 (3), 290–295. doi: 10.1038/nbt.3122

Peterson, E. K., Buchwalter, D. B., Kerby, J. L., LeFauve, M. K., Varian-Ramos, C. W., and Swaddle, J. P. (2017). Integrative behavioral ecotoxicology: bringing together fields to establish new insight to behavioral ecology, toxicology, and conservation. *Curr. Zool.* 63 (2), 185–194. doi: 10.1093/cz/zox010

Porte, C., Janer, G., Lorusso, L. C., Ortiz-Zarragoitia, M., Cajaraville, M. P., Fossi, M. C., et al. (2006). Endocrine disruptors in marine organisms: approaches and perspectives. *Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol.* 143 (3), 303–315. doi: 10.1016/j.cbpc.2006.03.004

Qin, G., Zhang, Y., Zhang, B., Zhang, Y., Liu, Y., and Lin, Q. (2020). Environmental estrogens and progestins disturb testis and brood pouch development with modifying transcriptomes in male-pregnancy lined seahorse. *Hippocampus erectus. Sci. Total Environ.* 715, 136840. doi: 10.1016/j.scitotenv.2020.136840

Ramesh, B., Small, C. M., Healey, H., Johnson, B., Barker, E., Currey, M., et al. (2023). Improvements to the gulf pipefish syngnathus scovelli genome. *Gigabyte*. doi: 10.46471/gigabyte.76

R Core Team (2015). *R: a language and environment for statistical computing* (Vienna, Austria: R Foundation for Statistical Computing). Available at: http://www.R-project.org/.

Ripley, J. L. (2009). Osmoregulatory role of the paternal brood pouch for two syngnathus species. *Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol.* 154 (1), 98–104. doi: 10.1016/j.cbpa.2009.05.003

Ripley, J. L., Williams, P. S., and Foran, C. M. (2010). Morphological and quantitative changes in paternal brood-pouch vasculature during embryonic development in two syngnathus pipefishes. *J. Fish Biol.* 77 (1), 67–79. doi: 10.1111/j.1095-8649.2010.02659.x

Robinson, M. D., McCarthy, D. J., and Smyth, G. K. (2010). edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *bioinformatics* 26 (1), 139–140. doi: 10.1093/bioinformatics/btp616

Rose, E., Flanagan, S. P., and Jones, A. G. (2015). The effects of synthetic estrogen exposure on the sexually dimorphic liver transcriptome of the sex-role-reversed gulf pipefish. *PloS One* 10 (10), e0139401. doi: 10.1371/journal.pone.0139401

Rose, E., Paczolt, K. A., and Jones, A. G. (2013). The effects of synthetic estrogen exposure on premating and postmating episodes of selection in sex-role-reversed gulf pipefish. *Evolution. Appl.* 6 (8), 1160–1170. doi: 10.1111/eva.12093

Saaristo, M., Craft, J. A., Lehtonen, K. K., and Lindström, K. (2010). An endocrine disrupting chemical changes courtship and parental care in the sand goby. *Aquat. Toxicol.* 97, 285–292. doi: 10.1016/j.aquatox.2009.12.015

Sárria, M. P., Santos, M. M., Castro, L. F. C., Vieira, N. M., and Monteiro, N. M. (2013). Estrogenic chemical effects are independent from the degree of sex role reversal in pipefish. *J. Hazard Mater.* 88 (1), 75–80. doi: 10.1016/j.jhazmat.2013.10.043

Sárria, M. P., Santos, M. M., Reis-Henriques, M. A., Vieira, N. M., and Monteiro, N. M. (2011). The unpredictable effects of mixtures of androgenic and estrogenic chemicals on fish early life. *Environ. Int.* 37 (2), 418–424. doi: 10.1016/j.envint.2010.11.004

Schneider, R. F., Woltering, J. M., Adriaens, D., and Roth, O. (2023). A comparative analysis of the ontogeny of syngnathids (pipefishes and seahorses) reveals how heterochrony contributed to their diversification. *Dev. Dyn.* 252 (5), 553–588. doi: 10.1002/dvdy.551

Scobell, S. K., and MacKenzie, D. S. (2011). Reproductive endocrinology of syngnathidae. *J. Fish Biol.* 78 (6), 1662–1680. doi: 10.1111/j.1095-8649.2011.02994.x

Shokri, M. R., Gladstone, W., and Jelbart, J. (2009). The effectiveness of seahorses and pipefish (Pisces: syngnathidae) as a flagship group to evaluate the conservation value of estuarine seagrass beds. *Aquat. Conserv: Mar. Freshw. Ecosyst.* 19, 588–595. doi: 10.1002/aqc.1009

Small, C. M., Bassham, S., Catchen, J., Amores, A., Fuiten, A. M., Brown, R. S., et al. (2016). The genome of the gulf pipefish enables understanding of evolutionary innovations. *Genome Biol.* 17 (1), 1–23. doi: 10.1186/s13059-016-1126-6

Small, C. M., Harlin-Cognato, A. D., and Jones, A. G. (2013). Functional similarity and molecular divergence of a novel reproductive transcriptome in two male-pregnant *Syngnathus* pipefish species. *Ecol. Evol.* 3 (12), 4092–4108. doi: 10.1002/ece3.763

Sommer, S., Whittington, C. M., and Wilson, A. B. (2012). Standardised classification of pre-release development in male-brooding pipefish, seahorses, and seadragons (Family syngnathidae). *BMC Dev. Biol.* 12 (1), 1–6. doi: 10.1186/1471-213X-12-39

Thomas, P. D., Campbell, M. J., Kejariwal, A., Mi, H., Karlak, B., Daverman, R., et al. (2003). PANTHER: a library of protein families and subfamilies indexed by function. *Genome Res.* 13 (9), 2129–2141. doi: 10.1101/gr.772403

Ueda, N., Partridge, C., Bolland, J., Hemming, J., Sherman, T., and Boettcher, A. (2005). Effects of an environmental estrogen on male gulf pipefish, *Syngnathus scovelli* (Evermann and Kendall), a male brooding teleost. *Bull. Environ. contam. Toxicol.* 74 (6), 1207–1212. doi: 10.1007/s00128-005-0709-9

Whittington, C. M., Griffith, O. W., Qi, W., Thompson, M. B., and Wilson, A. B. (2015). Seahorse brood pouch transcriptome reveals common genes associated with vertebrate pregnancy. *Mol. Biol. Evol.* 32 (12), 3114–3131. doi: 10.1093/molbev/msv177

Zuo, Y., Zhang, K., and Deng, Y. (2006). Occurrence and photochemical degradation of 17α-ethinylestradiol in acushnet river estuary. *Chemosphere* 63 (9), 1583–1590. doi: 10.1016/j.chemosphere.2005.08.063

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Different behavioral strategies of two sympatric seahorses: habitat availability and increased density of *Hippocampus guttulatus* alter the behavior of *Hippocampus hippocampus*

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In the light of future ocean warming scenarios and habitat fragmentation, coastal fishes must adjust their physiological and behavioral traits to face the combined effect of global warming and ecological interactions. Seahorses (*Hippocampus* spp.) are charismatic fishes with peculiar life cycle traits, which make them vulnerable to several anthropic pressures and natural disturbances. In this study, we investigated the behavior of two sympatric seahorse species, the long-snouted *Hippocampus guttulatus* (Cuvier, 1829) and the short-snouted *Hippocampus hippocampus* (Linnaeus, 1758). Specifically, we carried out two manipulative experiments to assess (i) the effect of temperature and habitat availability on both *H. hippocampus* and *H. guttulatus* behavior and (ii) the effect of temperature and an increased density of *H. guttulatus* (by 3×) on the activity level of congeneric species *H. hippocampus.* Our results showed that +3°C warming did not affect seahorse behavior in both experiments, suggesting greater behavioral tolerance to thermal variation. However, a significant reduction of the active behavior of *H. hippocampus* was observed when the artificial habitat was introduced in the tank, while *H. guttulatus* maintained its activity. Furthermore, a significant decrease of the *H. hippocampus* activity was observed with an increased relative dominance of *H. guttulatus.* Our results suggest that both increased density of *H. guttulatus* and habitat availability, but not ocean warming, will affect the behavior of *H. hippocampus*. Therefore, different interspecific behavioral strategies may occur, thus affecting the distribution of the two species among shallow habitats when they occur in sympatry.

KEYWORDS

behavioral plasticity, *Hippocampus guttulatus*, *Hippocampus hippocampus*, climate change, seahorse, coastal habitat

1 Introduction

The study of animal behavior is critically important in understanding the status and welfare of species and populations (Sih et al., 2004; Clements and Hunt, 2015) and can reveal the ecological processes structuring marine populations and communities (Hay, 2009; Schmidt et al., 2010; Nagelkerken and Munday, 2016). Species behavior is a crucial factor in shaping ecological interactions, thus representing a powerful tool to better understand the combined effects of species interactions, their response in a shifting environment, including in a climate change scenario (Harmon and Barton, 2013; Milazzo et al., 2013; Levine et al., 2017; Boulanger et al., 2022).

Warming has been shown to influence the distribution and behavior of many organisms (Abram et al., 2017; Freitas et al., 2021; Spatafora et al., 2021). Higher temperature may induce behavioral changes by altering activity rate (Biro et al., 2010), foraging (Biro et al., 2007; Hu et al., 2021), habitat use and selection (Freitas et al., 2016; Matis et al., 2018; Freitas et al., 2021), and reproduction (Hopkins et al., 2011; Miller et al., 2015; Spatafora et al., 2021). However, temperature-related changes in the organism's behavior may in turn affect species interactions and ecological processes (Harmon and Barton, 2013; Freitas et al., 2021; Zhang et al., 2022). For example, higher temperature can create novel interspecific interactions (e.g., invasive vs. native species; Galaiduk et al., 2013; Galasso et al., 2015) or alter the balance of pre-existing antagonistic ones (Nogués-Bravo & Rahbek, 2011; Milazzo et al., 2013; Wernberg et al., 2013; Alexander et al., 2016; Bonaviri et al., 2017; Yeruham et al., 2020). This may allow species coexistence through thermal resource partitioning, thus maintaining their functional roles in the ecosystem (Fey et al., 2019; Freitas et al., 2021). Moreover, organism behavior (e.g., habitat use and selection, foraging and anti-predator responses) can also be indirectly affected *via* temperature-dependent changes in the abundance or quality of resources (Ockendon et al., 2014; Nagelkerken and Munday, 2016; Matis et al., 2018; Nay et al., 2020; Freitas et al., 2021).

Seahorses are extremely vulnerable to natural and anthropogenic impacts (Foster and Vincent, 2004) due to their distinct life history, including sparse distribution, low mobility, small home ranges, low fecundity, lengthy parental care, and mate fidelity. Over the past few years, populations of seahorses showed a sudden and sharp decline mainly due to habitat loss, fragmentation, illegal trade, and incidental capture in fishing gears. Only recently, seahorse species were listed on Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES Convention) (Foster and Vincent, 2004) and in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Pollom, 2014; IUCN, 2020; Pollom et al., 2021). Given their extreme vulnerability to natural and anthropogenic impacts, climate changes may have a larger impact on seahorses compared with other fish species (Aurélio et al., 2013; Faleiro et al., 2015; Cohen et al., 2017; Pierri et al., 2021; Pollom et al., 2021; Costa et al., 2023). However, studies investigating seahorses' ability to cope with future ocean warming and other ecological factors (e.g., habitat availability and species interaction) are still scarce and limited to just a few species (Costa et al., 2023).

In the temperate coastal waters of the Europe, few studies have investigated the effects of ocean warming on the behavioral and physiological performance of *Hippocampus guttulatus* (Cuvier, 1829) (Aurélio et al., 2013; Faleiro et al., 2015; Costa et al., 2023). Although two of the three prementioned studies reported a normal increase in the metabolic rates (within their thermal sensitivity) and ventilation of adult *H. guttulatus* individuals with increasing temperature (18°C–26°C–30°C), the authors suggested great behavioral and physiological plasticity to warming (Aurélio et al., 2013; Faleiro et al., 2015). Additionally, Costa et al. (2023) found significant increases in the activity and food intake in the same species exposed to +7°C variation. While some information is present for *H. guttulatus*, no studies have been conducted to investigate the effect of temperature on behavior of the congeneric species *H. hippocampus* (Linnaeus, 1758). The two species inhabit lagoons, estuaries, and coastal marine waters (Pierri et al., 2022) where they generally share same habitat but with differences in niche partitioning (Curtis and Vincent, 2005). *H. hippocampus* has been mainly found in bare and sandy habitats, while *H. guttulatus* prefers more complex habitats such as seagrass beds (Curtis & Vincent, 2005; Caldwell and Vincent, 2012; Curtis et al., 2017; Correia et al., 2020; Correia, 2022). In most of the European study sites where the two species occurred sympatrically, the density of *H. guttulatus* was greater than that of *H. hippocampus* (Woodall et al., 2018; but not in Greece, see Correia et al., 2020). Although very low densities of *H. guttulatus* (0 to 0.51 ind. m^{-2}) and *H. hippocampus* (0.001–0.035 ind. m^{-2}*)* have been recorded in the wild, the two species tend to be patchily distributed and are occasionally found at very high density (e.g., 10 ind. *H. guttulatus* m^2, Foster and Vincent, 2004). Low densities are a recent characteristic of European seahorse populations mainly due to habitat loss, fragmentation, illegal trade, and incidental capture in fishing gears (Correia et al., 2018; Pierri et al., 2021). Only recently, European species were listed on Appendix II of CITES (Appendix II) and the IUCN Red List where they were classified as "Data Deficient" at a global level (Pollom, 2014; Pollom et al., 2021) and "Near Threatened" in the Mediterranean (Pollom, 2017). Indeed, IUCN Species Survival Commission (SSC) retain that the status of "Near Threatened" is not appropriate, and the two species should probably be included in the upper categories, as most of the known European populations faced declines of up to 90% in the past decade. Given the high vulnerability of these species and the predicted effects of the climate changes, many aspects of European seahorse biology remain unknown (especially for *H. hippocampus*), including wide-scale distribution data, abundance rates, and behavior. In addition, given seahorse tendency to be patchily distributed and positively correlated with the percentage of holdfasts' availability (Correia et al., 2015), the preferred habitat may become scarce, thus forcing these species (e.g., seagrasses) to occupy the same niche. Therefore, mechanisms underpinning the behavioral interaction between the two species need to be addressed to better understand the distribution of the two seahorse species in the European coastal ecosystems (Milazzo et al., 2013).

Here, we first examined the behavioral responses of *H. guttulatus* and *H. hippocampus* to increased temperature (+3°C, RCP 8.5, IPCC, 2014) in the presence/absence of an artificial habitat. As *H. guttulatus* generally prefers more shallow waters (with higher temperature variation) with complex habitats (e.g., seagrass beds) respect to the congeneric *H. hippocampus*, we predicted that the likely more thermal tolerant species *H. guttulatus* would maintain its activity levels regardless temperature conditions and habitat availability while *H. hippocampus* would reduce its activity under the same altered conditions. Second, as no information is available for *H. hippocampus* in relation to temperature variation and the species interaction with *H. guttulatus*, we explored the behavioral responses (i.e., activity level) of *H. hippocampus* to increased density (3×) of the congeneric species and elevated temperature conditions. We forecasted that *H. hippocampus* would reduce its activity under elevated temperatures and in presence of a higher density of the congeneric, thus, explaining their lower abundances when the two species share the same habitat.

Finally, given that sex-specific behavioral responses have been reported for *H. guttulatus* (Faleiro et al., 2008; Gristina et al., 2022) and other seahorses species (Freret-Meurer and Alves, 2018), sex effect, and its interaction with other experimental factors (temperature, habitat availability, and species) were also tested.

2 Materials and methods

2.1 Specimens rearing and stocking conditions

H. guttulatus (11 males and seven females) and *H. hippocampus* (24 males and 20 females), were selected in October 2021 from a captive broodstock held at the breeding structures of Ramalhete Research Station of the Centre of Marine Science—CCMAR (Faro, Portugal). Individuals of both species were randomly chosen from the broodstock and assigned to four holding tanks (100 cm × 70 cm × 50 cm) at two temperatures: two 22°C tanks (*H. hippocampus*, 11 males and 10 females; *H. guttulatus*, five males and three females) and two 25°C tanks (*H. hippocampus*, 13 males and 10 females; *H. guttulatus*, six males and four females). The mean temperature matched average summer temperature of 21.6°C (Newton and Mudge, 2003) experienced by these species where individuals were collected, while the higher temperature represented a mean temperature increase of +3°C forecasted by the end of the 21st century (RCP 8.5, IPCC, 2014). The elevated temperature (25°C) was reached by an increase of ±0.5°C per hour. The individuals of *H. hippocampus* (mean standard length ± SE: males = 12.26 ± 0.15 cm and female = 11.8 ± 0.18 cm) and *H. guttulatus* (mean standard length ± SE: males = 12.84 ± 0.17 cm and female = 12.81 ± 0.22 cm) (Supplementary Table S1) were acclimated to the different temperature conditions for two weeks prior to behavioral testing. Seawater temperature was set to 21.4 ± 0.5°C and 25.2 ± 0.5°C, while salinity and dissolved oxygen were 37.6 ± 0.1‰ and 6.9 ± 0.1 mg L^{-1} for both treatments. Holding tanks were illuminated from above with 2 × 36W fluorescent tubes with a light intensity of 900 ± 40 lx at the water surface and the photoperiod was set on 14h light/10h dark cycles without twilight periods. Artificial structures made of nautical rope (0.8 cm Ø) were provided as holdfasts. Seahorses were fed *ad libitum* once a day with a mix of live mysid shrimp (*Mesopodopsis slabberi* and *Leptomysis* sp.). Aquariums were daily cleaned by syphoning to remove debris and eventual uneaten food. Seawater parameters, such as ammonia, nitrates, and nitrites, were recorded biweekly and kept stable throughout the experiment.

2.2 Experimental setup

Four experimental tanks (60 cm × 50 cm × 40 cm) were kept in the same conditions (temperature, dissolved oxygen, and light intensity) as the holding tanks and used for two behavioral experiments. Seahorse behavior was observed and recorded using a set of GoPro Hero 8 video cameras each assembled in a PVC frame support placed in each aquarium at ≈15 cm above the water surface. A grid (mesh size: 20 cm × 17 cm) was superimposed on the tank bottom to help behavioral measurements. Fish were randomly assigned to different observation tanks. Behavioral observations were made daily in the morning (09.30h–13.30h.). During video recordings, temperature conditions were kept constant.

2.2.1 Experiment 1: Effect of habitat and temperature on H. guttulatus and H. hippocampus behavior

To determine the effects of elevated temperature and habitat availability on the behavior of *H. hippocampus* and *H. guttulatus*, a total of 21 and 18 individuals, respectively, at control temperature (22°C: *H. hippocampus*, five males and five females; *H. guttulatus*, five males and three females) and elevated temperature condition (25°C: *H. hippocampus*, seven males and four females; *H. guttulatus*, six males and four females) were used (see Supplementary Table S1 for details). One individual (same protocol for both species) was gently taken from the holding tank and introduced into one of the two experimental tanks in relation to the previous temperature condition of fish acclimation (22°C or 25°C). Recording began after 5 min of acclimation once the seahorse was introduced into the experimental tank. Behavioral observations consisted of recording seahorse responses before and after the introduction of an artificial habitat (Supplementary Figure S1). For each replicate, a 10-min pre-introduction period was followed by a 10-min post-introduction period during which each fish was exposed to their new habitat: three nautical rope (0.8 cm Ø) placed in the middle of the experimental tank mimicking the *Posidonia oceanica* (Delile, 1813) seagrass (Correia et al., 2013). The tested artificial structures used for this experiment were the same as those available inside the holding tanks and have been proven to be a suitable habitat for *H. hippocampus* and *H. guttulatus* (Faleiro et al., 2008; Correia et al., 2013).

2.2.2 Experiment 2: Effect of temperature and increased density of H. guttulatus (3×) on H. hippocampus behavior

To investigate the effects of elevated temperature and increased density of *H. guttulatus* on the behavior of *H. hippocampus*, a total of 24 individuals of the latter species (22°C: seven males and five females; 25°C: six males and six females) were used (see Supplementary Table S2 in Supplementary Material for details).

A protocol similar to experiment 1 was followed to evaluate the behavior of *H. hippocampus* with increased densities of *H. guttulatus* (3×) under two temperature conditions. A single individual of both *H. hippocampus* and *H. guttulatus* was carefully taken from the holding tanks (at the same temperature conditions: 22°C or 25°C) and introduced into one experimental tank. Recording began after 5 min of acclimation once the seahorse was introduced into the experimental tank. Behavioral observations consisted of recording *H. hippocampus* responses before (*H. hippocampus* and *H. guttulatus*, density 1:1) and after the introduction of two additional individuals of *H. guttulatus* (*H. hippocampus and H. guttulatus*, density 1:3) (Supplementary Figure S2). Ten-minute time frame was recorded for each introduction period (pre- and post-introduction) at different combinations of *H. hippocampus* to *H. guttulatus* density (1:1 and 1:3). The 3× density of *H. guttulatus* was selected to mimicking wild condition where the density of *H. guttulatus* was greater than that of *H. hippocampus* and to preserve the welfare of these species under captive conditions (Faleiro et al., 2008). The experiment was run at the two selected temperature conditions (22°C or 25°C) and the same artificial holdfasts (three artificial plastic plants) during the two introduction periods. For this experiment, *H. guttulatus* were randomly selected with a sex ratio of 2:1 (two males, one female). A total of 12 individuals (a total of four trials) of *H. guttulatus* were tested daily, and after each trial, the fish were placed back in a separate tank and other fish were used. After four trials, the fish were kept for 24h before being tested again.

2.3 Behavioral analyses

The behavior of seahorses in the two experiments was assessed by a standard continuous focal sampling procedure (Martin and Bateson, 1993) using the VLC software (Software – VLC media player vs 3.0.11: VideoLAN; https://www.videolan.org/vlc/index.it.html). After video analyses, the behavior of seahorses was described through a species ethogram (adapted from Faleiro et al., 2008), and the behaviors were assigned in two categories: Activity and Inactivity. In detail, for the first experiment, a total of five behaviors for both *H. hippocampus* and *H. guttulatus* were recorded and grouped into two categories: (i) Inactive: resting and swinging and (ii) Active: swimming, crawling, and body movement (see Table 1A for the detailed description of the behavior). For the second experiment, a total of eight behaviors for *H. hippocampus* were recorded and grouped into two categories: (i) Inactive: resting, swinging, interactions with *H. guttulatus*, grasping holdfast and (ii) Active: swimming, crawling, and searching and body movement (see Table 1B for the detailed description of the behavior). For each individual and in both experiments, we recorded the time spent (in seconds, expressed as percentage) by seahorses on all behaviors during the 20-min video observations. In both experiments, the sum of active behaviors (defined as "Active") was compared between the two introduction periods (pre- and post-) and the two temperature conditions (22°C or 25°C).

2.4 Statistical analysis

For both experiments, linear mixed models allowing to account for the correlation between observations coming from the same

TABLE 1 Behavioral ethograms reporting the list of the behaviors grouped in the two categories (Active and Inactive) described for *H. guttulatus* and *H. hippocampus* (A, first experiment) and for *H. hippocampus* (B, second experiment).

	Movements	A			B
		Behavior description		Movements	Behavior description
Inactive	Resting	The seahorse remains resting, without performing any kind of movement, while attached or unattached to the holdfast.	**Inactive**	Resting	The seahorse remains resting, without performing any kind of movement, while attached or unattached to the holdfast.
	Swinging	The seahorse remains attached to the holdfast, with slight movements of the head or body.		Swinging	The seahorse remains attached to the holdfast, with slight movements of the head or body.
Active	Swimming	Actively swimming in water column with dorsal and pectoral fins moving.		Interactions with *H. guttulatus*	*H. hippocampus* is attached (grasping with his tail) to another seahorse in the tank when at rest.
	Crawl	The seahorse crawl on the bottom tank.		Grasp holdfast	The seahorse is attached to the provided holdfat in the tank.
	Body movements	The seahorse remains in the same place and move the body rapidly due to the dorsal and/or pectoral fins movements.	**Active**	Swimming	Actively swimming in water column with dorsal and pectoral fins moving.
				Crawl	The seahorse crawl on the bottom tank.
				Body movements	The seahorse remains in the same place and move the body rapidly due to the dorsal and/or pectoral fins movements.
				Searching	The seahorse swim actively seeking for potential preys.

individual (before and after the introduction of the habitat in the first experiment; before and after the introduction of two individuals of *H. guttulatus* in the second experiment) were used. In particular, for the first experiment, the differences in the time spent on active behaviors (*Active*) between the two species (*Species*) and sex (*Sex*) at different temperatures (*Temperature*) and before and after the introduction of the habitat (*Habitat*) were formulated as follows:

$$Log(Active_{ij} + 1) = \alpha + \beta \times Species_{ij} \times Habitat_{ij} \times Temperature_{ij} \times Sex_{ij} + \beta_2 + \alpha_i + \epsilon_{ij}$$

For the second experiment, the differences in the Active behavior of *H. hippocampus* before and after the introduction of the two *H. guttulatus* individuals (*Density*) and between the two sexes (*Sex*) at the two temperature conditions were modeled as follows:

$$Log(HH_{Active\ ij} + 1) = \alpha + \beta \times Density_{ij} \times Temperature_{ij} \times Sex_{ij} + \beta_2 + Length + \alpha_i + \epsilon_{ij}$$

For both model formulations, the response variable was log +1 transformed to meet the model assumptions of homoscedasticity and normality of the residuals. Specifically, for the first experiment, the log +1-transformed *Active* of the two species was modeled as an intercept α plus a linear combination of the interaction between *Species* [fixed factor with two levels: *H. hippocampus* (HH) and *H. guttulatus* (HG)], *Sex* (fixed factor with two levels: male and female), *Habitat* (fixed factor with two levels: *pre-* and *post-* introduction of the habitat) and *Temperature* (fixed factor with two levels: 22°C and 25°C). While for the second experiment the log+1-transformed *Activity of H. hippocampus* was modeled as an intercept α plus a linear combination of the interaction between *Density* (fixed factor with two levels: *pre-* and *post-* introduction of two individuals of *H. guttulatus*), and *Temperature* (fixed factor with two levels). In both formulations, the response was modeled by adding also a random factor α_i (allowing us to account for the correlation of the observations coming from the same individual for the first (*H. Hippocampus*: $n = 21$; *H. guttulatus*: $n = 18$) and the second experiment (*H. hippocampus*: $n = 24$). assumed to be normally distributed with mean 0 and variance σ_α^2 plus the model errors (ϵ_{ij}) assumed to be normally distributed with mean 0 and variance σ^2. The index *i* refers to a specific individual, while the index *j* to the observations coming from an individual. The analyses were performed using lme4 library (Bates et al., 2014) in R statistical Software (R Core Team, 2019).

3 Results

3.1 Experiment 1: Effect of habitat and temperature on *H. guttulatus* and *H. hippocampus* behavior

Obtained data are presented in Supplementary Table S3. A significant difference in the active behavior between the two species in two introduction periods (Supplementary Table S3; Figure 1A; *Habitat* x *Species*: $F_{1,78} = 14.84$, $p < 0.001$) was observed, but neither temperature nor sex or their interaction significantly affected the behavior (Supplementary Table S3). A *post hoc* test performed on the interaction terms showed that *H. hippocampus* active behavior (78.4 s ± 26.3 SE) was significantly lower than of *H. guttulatus* (414.6 s ± 54.5 SE) during the post-introduction period when the artificial habitat was provided (Supplementary Table S3; Figure 1A; $t = 3.507$, $p < 0.01$). In addition, *H. hippocampus* active behavior significantly decreased during the post-introduction period (78.4 s ± 26.3 SE) compared with the pre-introduction period (294.24 s ±

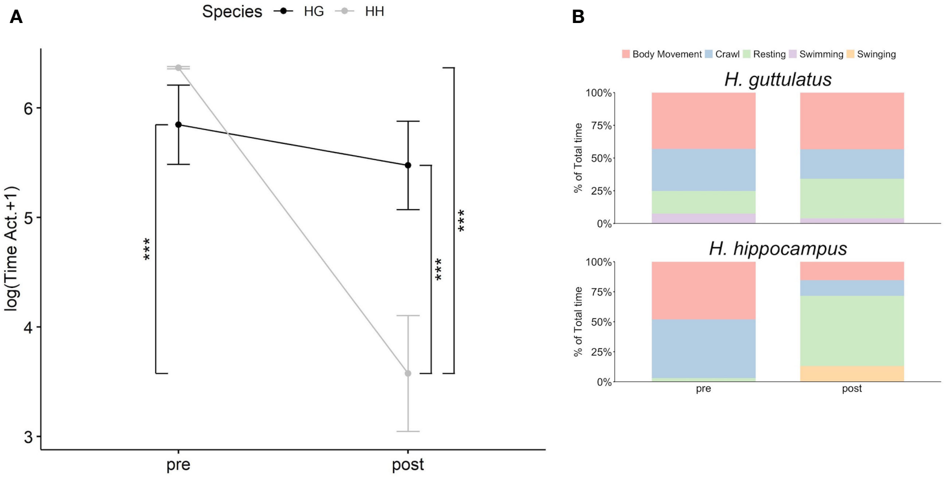

FIGURE 1
Relationship between the introduction periods (pre- and post-introduction of the habitat) and the sum of the time spent on active behaviors (in log +1) by the two seahorse species: *H. hippocampus* (gray line; $n = 21$) and *H. guttulatus* (black line; $n = 18$) **(A)**. Solid dots represent the median, top, and bottom vertical whiskers represent quartiles. Asterisk (*) indicates significant differences (p<.05) between the two seahorse species and the two introduction periods for *H. hippocampus* and *H. guttulatus*. Stacked bar charts represent the percentage of time spent on the different behaviors (body movement, crawl, resting, swimming, and swinging) by the two seahorse species between the two introduction periods **(B)**.

32.7 SE) (Supplementary Table S3; Figure 1A; $t = -6.649$, $p< 0.001$). The time spent by *H. guttulatus* on active behaviors was similar between the two introduction periods (Supplementary Table S3; Figure 1A; t = -0.887; p = 1). Descriptive results of the single behaviors showed that, in the absence of habitat, *H. guttulatus* spent approximately 81% of the total time performing body movement (36%), crawling (~33%), and swimming (~12%), while *H. hippocampus* spent 97% of the total time for body movement (~48%) and crawling (~49%). However, when artificial plants were added to the tank, the percentage of the activity of *H. hippocampus* was reduced to 28% (body movement = ~15%; crawling = ~13%) in favor to the resting behaviors. Finally, the time spent by *H. guttulatus* (in %) performing active behaviors during the post-introduction period was approximately 70% of the total activity (body movement = 43%, crawling = ~23% and swimming = ~4%) (Figure 1B). In addition, seahorse length did not affect, or change, their behavior (Supplementary Table S3).

3.2 Experiment 2: Effect of temperature and increased density of *H. guttulatus* (3×) on *H. hippocampus* behavior

The time spent by *H. hippocampus* on active behavior was significant different (*Density*: $F_{1,46} = 16.91$, $p< 0.001$) between the two introduction periods (pre- and post-) (Figure 2A; Supplementary Table S4) regardless temperature or sex (Supplementary Table S4). Specifically, a twofold decrease of *H. hippocampus* active behavior was observed during the postintroduction (164.7 s ± 41.9 SE) compared with the pre-introduction period (362.5 s ± 39.8 SE) (Supplementary Table S4, Figure 2A). Descriptive results of the single behaviors showed that, before the introduction of two individuals of *H. guttulatus*, the percentage of time spent by *H. hippocampus* on active behavior was approximately 67% of the total time distributed between crawling (~41%), body movement (~25%), and searching (~1%) (Figure 2B). During the post-introduction period, *H. hippocampus* spent approximately 30% of the total time for body movement (~18%), crawling (9%), and searching (~3%) (Figure 2B). No swimming activity was recorded in both introduction periods. As previously observed in Experiment 1, seahorse length did not affect, or change, their behavior (Supplementary Table S4).

4 Discussion

The results of the present study indicated that the two seahorse species reacted differently when a habitat was provided but were not affected by +3°C warming. *H. hippocampus* significantly reduced its activity in presence of a habitat, while *H. guttulatus* maintained its activity; additionally, *H. hippocampus* significantly reduced its activity when exposed to an increased density of the congeneric species regardless temperature.

Higher temperatures can increase fish activity as can raise metabolic rates (Biro et al., 2010; White and Kearney, 2014; Abram et al., 2017) of fish species (Ojanguren and Brañta, 2000; Biro et al., 2010; Laubenstein et al., 2018; Culumber, 2020). However, in our study, behavior of the two seahorse species did not change significantly with an increase in temperature. Wide thermal tolerance of *H. guttulatus* has been reported in a previous study where the species showed unaltered activity pattern under different temperature conditions (18°C, 26°C, 28°C, and 30°C) (Aurélio et al., 2013; Faleiro et al., 2015). Our findings were in accordance with previous studies conducted on *H. guttulatus* (Costa et al., 2023) and other seahorses (Qin et al., 2018) where seahorse activity became affected by extreme temperature increase (+7°C and +8°C, respectively) but not by a lower temperature increase (+3°C and +4°C, respectively). Such a result for *H. guttulatus* was expected as the species inhabits shallow inshore waters (inlets and coastal lagoons) where there are large daily and seasonal temperature fluctuations (see Lourie et al., 2004; Woodall et al., 2018; Pierri et al.,

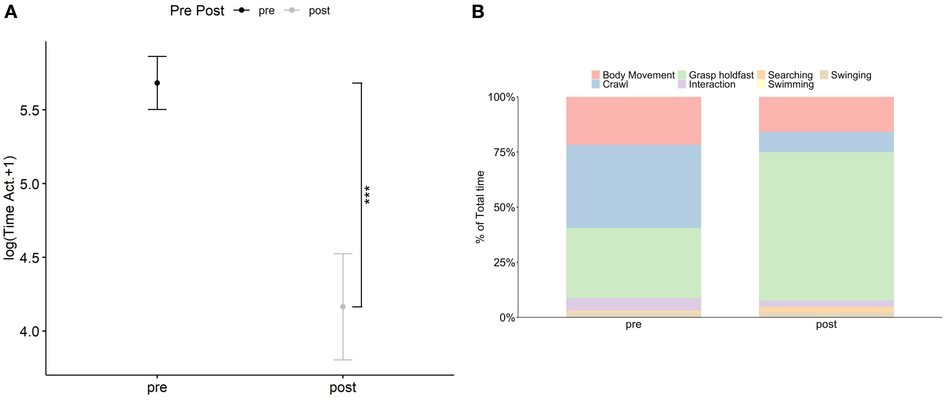

FIGURE 2
Relationship between the introduction periods (pre- and post-introduction of two ind. of *H. guttulatus*) and the sum of the time spent on active behaviors (in log +1) by *H. hippocampus* (n = 24) **(A)**. Solid dots represent the median; top and bottom-vertical whiskers represent quartiles. Asterisk (*) indicates significant differences (p<.05) between the two introduction periods for *H. hippocampus* Stacked bar chart represent the percentage of time spent on the different behaviors (body movement, crawl, resting, swimming, and swinging) by *H. hippocampus* between the two introduction periods **(B)**.

2021); however, the lack of same response for *H. hippocampus* was somewhat surprising as the species usually lives in deeper and cooler waters (Lourie et al., 2004). Indeed, unaltered activity displayed by the two seahorse species under increased temperature conditions suggests tolerance to warming. Given their poor swimming abilities, seahorse tolerance to +3°C warming may allow them to cope with future increases in water temperature. However, considering limited thermal range used in the present experiment and the captive conditions, further laboratory and field investigations are needed to exclude other negative impacts of long-term warming (Faleiro et al., 2015).

Behavioral differences between the two species were observed in relation to habitat availability. In the absence of habitat, both species had similar activity levels and spent a similar amount of time in body movement, crawling, and swimming. This contrasts with previous evidence of higher activity levels of *H. hippocampus* compared with *H. guttulatus* in areas with sparse vegetation (Curtis and Vincent, 2005; Curtis et al., 2007). When artificial habitat was introduced, *H. hippocampus* was less active than *H. guttulatus*. This may be related to different aspects of their lifestyle (e.g., locomotor activity or foraging strategy) (James and Heck, 1994; Curtis and Vincent, 2005). The reduction of active behaviors (i.e., lower crawling and higher resting, see Figure 1B) observed in *H. hippocampus* may reflect the species preference for less vegetated habitat as supported by previous study conducted on the same and other seahorse species (James and Heck, 1994; Curtis and Vincent, 2005). *H. guttulatus* prefers dense-vegetated habitats (Curtis and Vincent, 2005; Caldwell and Vincent, 2012; Correia, 2022) and reductions in species abundance have been associated with declines in these habitats (Ribeiro et al., 2006; Correia et al., 2015). However, no changes in the behavior of *H. guttulatus* were observed in presence and absence of the artificial holdfast. Although there were some differences in the experimental design (e.g., the use of different number of holdfast availability), this study findings are in line with previous evidences that failed to detect any effect of habitat availability on *H. guttulatus* activity (Faleiro et al., 2008). Our results suggest that *H. guttulatus* have a higher behavioral plasticity compared with congeneric species when a suitable holdfast is provided. This may support the density pattern trend observed in most of the European study sites where *H. guttulatus* tends to have higher densities than *H. hippocampus* when co-occuring (Woodall et al., 2018). The capacity of *H. guttulatus* to remain active in different habitats may allow this species to increase the chance to find prey or reproductive mates thus increasing its competitive ability as reported for other seahorse species (Freret-Meurer and Alves, 2018). Indeed, *H. guttulatus* can modulate its foraging strategy to different habitats including bare substrates when no potential holdfasts are available (Ape et al., 2019). Evidence of trophic flexibility in this species have been reported in other studies where *H. guttulatus* was able to switch from a *"sit and wait"* foraging strategy in vegetated habitat to active feeding while swimming or crawling in uncovered areas (James and Heck, 1994; Felício et al., 2006). On the other hand, a higher activity of *H. guttulatus* may reduce camouflage and increase predation susceptibility (Foster and Vincent, 2004) with this potentially favoring the less active species. Seahorses used for this study came from a captive broodstock and were maintained under controlled conditions with limited holdfast diversity compared with the wild populations. However, the two seahorse species were kept isolated in different tanks, and the same artificial structures were provided to both species. Therefore, as they were previously adapted to these artificial structures, the difference in the activity level between the two seahorses may be related only to species-specific behavioral responses.

A significant reduction in the active behavior of *H. hippocampus* was observed when its ecological congeneric species was on patrol at 3× relative dominance. Despite the limited thermal range used in this study, this result was observed independently of the temperature condition thus ulteriorly supporting thermal plasticity of *H. hippocampus* behavior. The highest density of *H. guttulatus* along with the reduced activity of *H. hippocampus* seemed mostly related to the increased time spent, by the latter, grasping artificial substrata. This may be due to the higher risk to lose their holdfasts thus maximizing substrate occupation when more individuals compete for the same habitat in line with the "The competitive exclusion principle" (Gause, 1934). Indeed, the benefit of remaining still enough while grasping the holdfast for camouflage reduce seahorse visibility to both predator and prey (Foster and Vincent, 2004; Webster et al., 2009). Although not tested in this study, the reduced activity may however limit other crucial behavioral activities such as foraging, especially because *H. hippocampus* is considered an active forager of both planktonic and epibenthic prey (Curtis and Vincent, 2005; Kitsos et al., 2008). Overall, and despite the limited number of information available in literature, our results pointed out that both less-preferred habitat and increased density of the congeneric species *H. guttulatus* cause changes in the behavior of *H. hippocampus*. To date, only few information is present in literature regarding similar interspecific interactions among sympatric fish species (e.g., labrids) showing evident behavioral alterations with a reduction of the activity of subordinate species (Milazzo et al., 2013). However, further investigation is needed to determine whether behavioral changes observed in this study affected the intraspecific interactions of the two seahorses.

Broadly, this study emphasized the behavior as a primary mechanism in understanding the effects of warming and other ecological factors (i.e., habitat availability) on *H. hippocampus* and *H. guttulatus*. In addition, our results provided preliminary insight into ocean warming and species interaction that may affect patterns of distribution of the two seahorses. The paucity of behavioral and ecological information on seahorses is hampered by their naturally low densities (Foster and Vincent, 2004), low catchability (Curtis et al., 2007) and challenges associated with differentiating species (Lourie and Vincent, 1999; Curtis et al., 2007).

However, given that one of the main threats to seahorse populations on a global scale is represented by degradation of coastal habitats (Olden et al., 2007), this study helps to understand the behavioral traits that drive distribution of these sensitive species and could be therefore useful for future management and conservation projects. Additional factors, which were not explored in this study, could also have some influence on behavioral responses in the wild, including other alternative habitats, predator presence, and abundance (Shelton and Mangel, 2011; Harasti et al., 2014), as well as food availability (Felício et al., 2006; Correia et al., 2015). We believe that future studies should account for these additional factors that could affect the distribution and abundance of seahorse populations.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The use of the animals was approved by the ethics committee from the Veterinary Medicines Directorate, the Portuguese competent authority for the protection of animals, Ministry of Agriculture, Rural Development and Fisheries, Portugal. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

DS and MG: Conceptualization (Lead); data curation (lead); formal analysis (lead); investigation (lead); methodology (lead); writing— original draft (lead). FQ: Data curation (Supporting); formal analysis (support- ing); investigation (supporting); methodology (supporting). CP: Conceptualization (Equal); investigation (equal); methodology (equal). TL: Conceptualization (Equal); investigation (equal); methodology (equal). JP: Conceptualization (Equal); data curation (equal); writing— original draft (lead); investigation (equal); methodology (equal). All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1138296/full#supplementary-material

References

Abram, P. K., Boivin, G., Moiroux, J., and Brodeur, J. (2017). Behavioural effects of temperature on ectothermic animals: unifying thermal physiology and behavioural plasticity. *Biol. Rev.* 92, 1859–1876. doi: 10.1111/brv.12312

Alexander, J. M., Diez, J. M., Hart, S. P., and Levine, J. M. (2016). When climate reshuffles competitors: A call for experimental macroecology. *Trends Ecol. Evol.* 31 (11), 831–841. doi: 10.1016/j.tree.2016.08.003

Ape, F., Corriero, G., Mirto, S., Pierri, C., Lazic, T., and Gristina, M. (2019). Trophic flexibility and prey selection of the wild long-snouted seahorse Hippocampus guttulatus Cuvier 1829 in three coastal habitats. *Estuarine Coast. Shelf Sci.* 224, 1–10. doi: 10.1016/j.ecss.2019.04.034

Aurélio, M., Faleiro, F., Lopes, V. M., Pires, V., Lopes, A. R., Pimentel, M. S., et al. (2013). Physiological and behavioral responses of temperate seahorses (*Hippocampus guttulatus*) to environmental warming. *Mar. Biol.* 160, 2663–2670. doi: 10.1007/s00227-013-2259-8

Bates, D., Mächler, M., Bolker, B., and Walker, S. (2014). Fitting Linear Mixed-Effects Models using lme4. *J. Stat. Softw.* 67, 1–48. doi: 10.48550/arXiv.1406.5823

Biro, P. A., Beckmann, C., and Stamps, J. A. (2010). Small within-day increases in temperature affects boldness and alters personality in coral reef fish. *Proc. R. Soc. B.: Biol. Sci.* 277, 71–77. doi: 10.1098/rspb.2009.1346

Biro, P. A., Post, J. R., and Booth, D. J. (2007). Mechanisms for climate-induced mortality of fish populations in whole-lake experiments. *PNAS* 104, 9715–9719. doi: 10.1073/pnas.0701638104

Bonaviri, C., Graham, M., Gianguzza, P., and Shears, N. T. (2017). Warmer temperatures reduce the influence of an important keystone predator. *J. Anim. Ecol.* 86, 490–500. doi: 10.1111/1365-2656.12634

Boulanger, E., Benestan, L., Guerin, P.-E., Dalongeville, A., Mouillot, D., and Manel, S. (2022). Climate differently influences the genomic patterns of two sympatric marine fish species. *J. Anim. Ecol.* 91, 1180–1195. doi: 10.1111/1365-2656.13623

Caldwell, R., and Vincent, A. C. J. (2012). Revisiting two sympatric European seahorse species: Apparent decline in the absence of exploitation. *Aquat. Conserv. Mar. Freshw. Ecosyst.* 22, 427–435. doi: 10.1002/aqc.2238

Clements, J., and Hunt, H. (2015). Marine animal behaviour in a high CO2 ocean. *Mar. Ecol. Prog. Ser.* 536, 259–279. doi: 10.3354/meps11426

Cohen, F. P. A., Valenti, W. C., Planas, M., and Calado, R. (2017). Seahorse aquaculture, biology and conservation: knowledge gaps and research opportunities. *Rev. Fish. Sci. Aquac.* 25, 100–111. doi: 10.1080/23308249.2016.1237469

Core Team, R. (2019). *R: A Language and Environment for Statistical Computing* (Vienna, Austria: R Foundation for Statistical Computing). Available at: https://www.R-project.org/.

Correia, M. (2022). Monitoring of seahorse populations, in the Ria Formosa Lagoon (Portugal), reveals steep fluctuations: potential causes and future mitigations. *Proc. Zool. Soc.* 75, 190–199. doi: 10.1007/s12595-021-00394-2

Correia, M., Caldwell, I. R., Koldewey, H. J., Andrade, J. P., and Palma, J. (2015). Seahorse (Hippocampinae) population fluctuations in the Ria Formosa Lagoon, south Portugal. *J. Fish. Biol.* 87, 679–690. doi: 10.1111/jfb.12748

Correia, M., Koldewey, H. J., Andrade, J. P., Esteves, E., and Palma, J. (2018). Identifying key environmental variables of two seahorse species (*Hippocampus guttulatus* and *Hippocampus hippocampus*) in the Ria Formosa lagoon. South Portugal. *Environm. Biol. Fish.* 101, 1357–1368. doi: 10.1007/s10641-018-0782-7

Correia, M., Palma, J., Koldewey, H., and Andrade, J. P. (2013). Can artificial holdfast units work as a habitat restoration tool for long-snouted seahorse (*Hippocampus guttulatus* Cuvier). *J. Exp. Mar. Biol. Ecol.* 448, 258–264. doi: 10.1016/j.jembe.2013.08.001

Correia, M., Paulo, D., Samara, E., Koulouri, P., Mentogiannis, V., and Dounas, C. (2020). Field studies of seahorse population density, structure and habitat use in a semi-closed north-eastern Mediterranean marine area (Stratoni, North Aegean Sea). *J. Fish. Biol.* 97, 314–317. doi: 10.1111/jfb.14371

Costa, A. B., Correia, M., Silva, G., Lopes, A. F., and Faria, A. M. (2023). Performance of the long-snouted seahorse, Hippocampus guttulatus, under warming conditions. *Front. Mar. Science.* 10. doi: 10.3389/fmars.2023

Culumber, Z. W. (2020). Thermal stress increases activity and risk-taking behavior but not anxiety in a livebearing fish. *Environm. Biol. Fish.* 103, 313–317. doi: 10.1007/s10641-020-00966-9

Curtis, J. M. R., Ribeiro, J., Erzini, K., and Vincent, A. C. J. (2007). A conservation trade-off? Interspecific differences in seahorse responses to experimental changes in fishing effort. *Aquat. Conservation: Mar. Freshw. Ecosyst.* 17, 468–484. doi: 10.1002/aqc.798

Curtis, J. M. R., Santos, S. V., Nadeau, J. L., Gunn, B., Bigney Wilner, K., Balasubramanian, H., et al. (2017). Life history and ecology of the elusive European short-snouted seahorse Hippocampus hippocampus. *J. Fish. Biol.* 91, 1603–1622. doi: 10.1111/jfb.13473

Curtis, J. M., and Vincent, A. C. (2005). Distribution of sympatric seahorse species along a gradient of habitat complexity in a seagrass dominated community. *Mar. Ecol. Prog. Ser.* 291, 81–91. doi: 10.3354/meps291081

Faleiro, F., Baptista, M., Santos, C., Aurélio, M. L., Pimentel, M., Pegado, M. R., et al. (2015). Seahorses under a changing ocean: the impact of warming and acidification on the behaviour and physiology of a poor-swimming bony-armoured fish. *Conserv. Physiol.* 3, 1–77. doi: 10.1093/conphys/cov009

Faleiro, F., Narciso, L., and Vicente, L. (2008). Seahorse behaviour and aquaculture: How to improve Hippocampus guttulatus husbandry and reproduction? *Aquaculture* 282, 33–40. doi: 10.1016/j.aquaculture.2008.05.038

Felício, A. K. C., Rosa, I. L., Souto, A., Renato, H. A., and Freitas, R. H. A. (2006). Feeding behavior of the long-snout seahorse Hippocampus reidi Ginsbur. *J. Ethol* 24, 219–225. doi: 10.1007/s10164-005-0189-8

Fey, S. B., Vasseur, D. A., Alujević, K., Kroeker, K. J., Logan, M. L., O'Connor, M. I., et al. (2019). Opportunities for behavioral rescue under rapid environmental change. *Global Change Biol.* 25, 3110–3120. doi: 10.1111/gcb.14712

Foster, S. J., and Vincent, A. C. J. (2004). Life history and ecology of seahorses: implications for conservation and management. *J. Fish. Biol.* 65, 1–61. doi: 10.1111/j.0022-1112.2004.00429.x

Freitas, C., Olsen, E. M., Knutsen, H., Albretsen, J., and Moland, E. (2016). Temperature-associated habitat selection in a cold-water marine fish. *J. Anim. Ecol.* 85, 628–637. doi: 10.1111/1365-2656.12458

Freitas, C., Villegas-Ríos, D., Moland, E., and Olsen, E. M. (2021). Sea temperature effects on depth use and habitat selection in a marine fish community. *J. Anim. Ecol.* 90, 1787–1800. doi: 10.1111/1365-2656.13497

Freret-Meurer, N. V., and Alves, M. A. S. (2018). Personality in the longsnout seahorse, Hippocampus reidi Ginsburg 1933: are males shyer than females? *Behav. Processes* 157, 106–110. doi: 10.1016/j.beproc.2018.09.006

Galaiduk, R., Figueira, W., Kingsford, M., and Curley, B. (2013). Factors driving the biogeographic distribution of two temperate Australian damselfishes and ramifications for range shifts. *Mar. Ecol. Progr. Ser.* 484, 189–202. doi: 10.3354/meps10300

Galasso, N. M., Bonaviri, C., Trapani, F. D., Picciotto, M., Gianguzza, P., Agnetta, D., et al. (2015). Fish-seastar facilitation leads to algal forest restoration on protected rocky reefs. *Sci. Rep.* 5, 12409. doi: 10.1038/srep12409

Gause, G. F. (1934). *The struggle for existence* (Baltimore: Williams & Wilkins).

Gristina, M., Pierri, C., Lazic, T., and Palma, J. (2022). "Behavioral traits of captive short-snouted seahorse Hippocampus hippocampus, Linnaeus 1758," in *2022 IEEE International Workshop on Metrology for the Sea* (Learning to Measure Sea Health Parameters (MetroSea), 545–548. doi: 10.1109/MetroSea55331.2022.9950976

Harasti, D., Martin-Smith, K., and Gladstone, W. (2014). Ontogenetic and sex-based differences in habitat preferences and site fidelity of White's seahorse. *Hippocampus Whitei. J. Fish. Biol.* 85, 1413–1428. doi: 10.1111/jfb.12492

Harmon, J. P., and Barton, B. T. (2013). On their best behavior: how animal behavior can help determine the combined effects of species interactions and climate change. *Ann. N. Y. Acad. Sci.* 1297, 139–147. doi: 10.1111/nyas.12192

Hay, M. E. (2009). Marine chemical ecology: chemical signals and cues structure marine populations, communities, and ecosystems. *Ann. Rev. Mar. Sci.* 1, 193–212. doi: 10.1146/annurev.marine.010908.163708

Hopkins, K., Moss, B. R., and Gill, A. B. (2011). Increased ambient temperature alters the parental care behaviour and reproductive success of the three-spined stickleback (*Gasterosteus aculeatus*). *Environ. Biol. Fish.* 90, 121–129. doi: 10.1007/s10641-010-9724-8

Hu, N., Yu, Z., Huang, Y., Liu, D. A., Wang., F., and Zhang, T. (2021). Elevated temperatures increase growth and enhance foraging performances of a marine gastropod. *Aquacult. Environ. Interact.* 13, 177–188. doi: 10.3354/aei00398

IPCC. (2014). Climate change 2014: synthesis report. Contribution of Working Groups I, II and III to the fifth assessment report of the Intergovernmental Panel on Climate Change. IPCC, Geneva.

IUCN. (2020). 2020 IUCN Red List of Threatened Species (Online). Retrieved from http://www.iucnredlist.org/.

James, P. L., and Heck, K. L. J. (1994). The effects of habitat complexity and light intensity on ambush predation within a simulated seagrass habitat. *J. Exp. Mar. Biol.* 176, 187–200. doi: 10.1016/0022-0981(94)90184-8

Kitsos, M.-S., Tzomos, T. H., Anagnostopoulou, L., and Koukouras, A. (2008). Diet composition of the seahorses, *Hippocampus guttulatus* Cuvier 1829 and *Hippocampus hippocampus* (L. 1758) (Teleostei, Syngnathidae) in the Aegean Sea. *J. Fish. Biol.* 72, 1259–1267. doi: 10.1111/j.1095-8649.2007.01789.x

Laubenstein, T. D., Rummer, J. L., Nicol, S., Parsons, D. M., Pether, S. M. J., Pope, S., et al. (2018). Correlated effects of ocean acidification and warming on behavioral and metabolic traits of a large Pelagic fish. *Diversity* 10, 35. doi: 10.3390/d10020035

Levine, J. M., Bascompte, J., Adler, P. B., and Allesina, S. (2017). Beyond pairwise mechanisms of species coexistence in complex communities. *Nature* 546, 56–64. doi: 10.1038/nature22898

Lourie, S. A., Foster, S. J., Cooper, E. W. T., and Vincent, A. C. J. (2004). *A Guide to the Identification of Seahorses* (Washington: University of British Columbia and WorldWildlife Fund), 1–114.

Lourie, S. A., and Vincent, A. C. J. (1999). *Hall, H. Seahorses: An Identification Guide to the World's Species and Their Conservation* (Project Seahorse: London, UK), 186.

Martin, P., and Bateson, P. (1993). *Measuring behaviour—an introductory guide. 2nd edition* (Cambridge University Press, Cambridge, UK).

Matis, P. A., Donelson, J. M., Bush, S., Fox, R. J., and Booth, D. J. (2018). Temperature influences habitat preference of coral reef fishes: Will generalists become more specialised in a warming ocean? *Global Change Biol.* 24, 3158–3169. doi: 10.1111/gcb.14166

Milazzo, M., Mirto, S., Domenici, P., and Gristina, M. (2013). Climate change exacerbates interspecific interactions in sympatric coastal fishes. *J. Anim. Ecol.* 82, 468–477. doi: 10.1111/j.1365-2656.2012.02034.x

Miller, G. M., Kroon, F. J., Metcalfe, S., and Munday, P. L. (2015). Temperature is the evil twin: effects of increased temperature and ocean acidification on reproduction in a reef fish. *Ecol. Applicat.* 25, 603–620. doi: 10.1890/14-0559.1

Nagelkerken, I., and Munday, P. L. (2016). Animal behaviour shapes the ecological effects of ocean acidification and warming: moving from individual to community-level responses. *Global Change Biol.* 22, 974–989. doi: 10.1111/gcb.13167

Nay, T. J., Johansen, J. L., Rummer, J. L., Steffensen, J. F., Pratchett, M. S., and Hoey, A. S. (2020). Habitat complexity influences selection of thermal environment in a common coral reef fish. *Conserv. Physiol.* 8, coaa070. doi: 10.1093/conphys/coaa070

Newton, A., and Mudge, S. M. (2003). Temperature and salinity regimes in a shallow, mesotidal lagoon, the Ria Formosa, Portugal. Estuarine. *Coast. Shelf Sci.* 57, 73–85. doi: 10.1016/S0272-7714(02)00332-3

Nogués-Bravo, D., and Rahbek, C. (2011). Communities under climate change. *Science* 334, 1070–1071. doi: 10.1126/science.1214833

Ockendon, N., Baker, D. J., Carr, J. A., White, E. C., Almond, R. E. A., Amano, T., et al. (2014). Mechanisms underpinning climatic impacts on natural populations: altered species interactions are more important than direct effects. *Global Change Biol.* 20, 2221–2229. doi: 10.1111/gcb.12559

Ojanguren, A. F., and Brañta, F. (2000). Thermal dependence of swimming endurance in juvenile brown trout. *J. Fish. Biol.* 56, 1342–1347. doi: 10.1111/j.1095-8649.2000.tb02147.x

Olden, J. D., Hogan, Z. S., and Vander Zanden, M. J. (2007). Small fish, big fish, red fish, blue fish: size biased extinction risk of the world's freshwater and marine fishes. *Global Ecol. Biogeography* 16, 694–701. doi: 10.1111/j.1466-8238.2007.00337.x

Pierri, C., Cardone, F., Corriero, G., Lazic, T., Quattrocchi, F., Alabiso, G., et al. (2021). Density decline in a Mediterranean seahorse population: natural fluctuations or new emerging threats? *Front. Mar. Sci.* 8. doi: 10.3389/fmars.2021.692068

Pierri, C., Lazic, T., Gristina, M., Corriero, G., and Sinopoli, M. (2022). Large-scale distribution of the European Seahorses (Hippocampus Rafinesque 1810): A systematic review. *Biology* 11, 325. doi: 10.3390/biology11020325

Pollom, R. (2014) *Hippocampus Hippocampus. The IUCN Red List of Threatened Species. 2014*. Available at: https://www.iucnredlist.org/species/10069/54904826 (Accessed 10 September 2021).

Pollom, R. (2017)Hippocampus Guttulatus. In: *The IUCN Red List of Threatened Species*. Available at: https://www.iucnredlist.org/species/41006/67617766 (Accessed 10 September 2021).

Pollom, R. A., Ralph, G. M., Pollock, C. M., and Vincent, A. C. J. (2021). Global extinction risk for seahorses, pipefishes and their near relatives (*Syngnathiformes*). *Oryx* 55, 497–506. doi: 10.1017/S0030605320000782

Qin, G., Johnson, C., Zhang, Y., Zhang, H., Yin, J., Miller, G., et al. (2018). Temperature-induced physiological stress and reproductive characteristics of the migratory seahorse *Hippocampus erectus* during a thermal stress simulation. *Biol. Open* 7, bio032888. doi: 10.1242/bio.032888

Ribeiro, J., Bentes, L., Coelho, R., Gonçalves, J. M. S., Lino, P. G., Monteiro, P., et al. (2006). Seasonal, tidal and diurnal changes in fish assemblages in the Ria Formosa lagoon (Portugal). *Estuar. Coast. Shelf Sci.* 67, 461–474. doi: 10.1016/j.ecss.2005.11.036

Schmidt, K. A., Dall, S. R. X., and Van Gils, J. A. (2010). The ecology of information: an overview on the ecological significance of making informed decisions. *Oikos* 119, 304–316. doi: 10.1111/j.1600-0706.2009.17573.x

Shelton, A. O., and Mangel, M. (2011). Fluctuations of fish populations and the magnifying effects of fishing. *PNAS* 108, 17. doi: 10.1073/pnas.1100334108

Sih, A., Bell, A., and Johnson, J. C. (2004). Behavioral syndromes: an ecological and evolutionary overview. *Trends Ecol. Evol.* 19, 372–378. doi: 10.1016/j.tree.2004.04.009

Spatafora, D., Massamba N'Siala, G., Quattrocchi, F., Milazzo, M., and Calosi, P. (2021). Plastic adjustments of biparental care behavior across embryonic development under elevated temperature in a marine ectotherm. *Ecol. Evol.* 11, 11155–11167. doi: 10.1002/ece3.7902

Webster, M. M., Ward, A. J. W., and Hart, P. J. B. (2009). Individual boldness affects interspecific interactions in sticklebacks. *Behav. Ecol. Sociobiol.* 63, 511–520. doi: 10.1007/s00265-008-0685-2

Wernberg, T., Smale, D. A., Tuya, F., Thomsen, M. S., Langlois, T. J., de Bettignies, T., et al. (2013). An extreme climatic event alters marine ecosystem structure in a global biodiversity hotspot. *Nat. Climate Change* 3 (1), 78–82. doi: 10.1038/nclimate1627

White, C. R., and Kearney, M. R. (2014). Metabolic scaling in animals: methods, empirical results, and theoretical explanations. *Compr. Physiol.* 4 (1), 231–256. doi: 10.1002/cphy.c110049

Woodall, L. C., Otero-Ferrer, F., Correia, M., Curtis, J. M., Garrick-Maidment, N., Shaw, P. W., et al. (2018). A synthesis of European seahorse taxonomy, population structure, and habitat use as a basis for assessment, monitoring and conservation. *Mar. Biol.* 165, 1–19. doi: 10.1007/s00227-017-3274-y

Yeruham, E., Shpigel, M., Abelson, A., and Rilov, G. (2020). Ocean warming and tropical invaders erode the performance of a key herbivore. *Ecology* 101, e02925. doi: 10.1002/ecy.2925

Zhang, Y., Zhang, C., Xu, B., Ji, Y., Ren, Y., and Xue, Y. (2022). Impacts of trophic interactions on the prediction of spatio-temporal distribution of mid-trophic level fishes. *Ecol. Indic.* 138, 108826. doi: 10.1016/j.ecolind.2022.108826

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Magnitude of bycatch of *Hippocampus patagonicus*, an endangered species, in trawl fisheries in Southeast and South Brazil

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One of the biggest threats to the world's fish stocks is trawling with indiscriminate capturing of non-target organisms, typically referred to as bycatch. Some species of seahorses are globally threatened and are often targets caught as bycatch. The aim of this work is to describe the magnitude of bycatch of the Patagonian seahorse *Hippocampus patagonicus* (Sygnathidae) in trawling fisheries in the Southeast and South Brazil. Between 2016 and 2018 we monitored the catch of five trawling vessels off the coast of Rio de Janeiro and Sao Paulo. A total of 2,041 individuals of *H. patagonicus* were captured, 1,183 males and 858 females. They were evaluated as for injuries suffered during dragging. The average rate of body damage was 33%. Based on the individuals captured and the analysis of on-board maps, an average CPUE of 3.36 with a standard deviation of 5.95/ind./day/vessel was estimated for the study area, extrapolating an incidental removal of 2,282,515 individuals per year, or 9,427 metric tons of seahorses, along the South and Southeast Brazil, where 3700 trawlers operate, an area recognized as the geographic distribution of *H. patagonicus* in Brazil

KEYWORDS

incidental capture, fish stock, bycatch, unsustainability, seahorse, impact, syngnathidae

1 Introduction

Negative impacts of trawling are known all over the world. These effects indiscriminately mow down the fauna and flora of the ocean floor, damaging habitat and leading to bycatch of vulnerable species. Bycatch fauna, captured in large numbers and usually discarded before the boats dock in ports, includes animals of varying sizes or species considered of no commercial interest (Haimovici and Mendonça, 1996; Soetaert et al., 2015). Discarding small fish affects the composition of natural stocks and has a direct effect on the decrease in the volume caught in the next harvest, making the activity less productive and more costly each year (Polet et al., 2005). It also changes the distribution of sizes of affected populations and species composition in fishing areas and causes loss of diversity (Viana, 2020).

One of the biggest threats to the world's fish stocks is commercial trawling, especially the indiscriminate capturing of non-target organisms, typically referred to as bycatch (Foster and Arreguin-Sánchez, 2014). Just as the bycatch can be sold, it can also be unused, being therefore called discard. According to this definition, global marine fishery data indicate that 9.1 million tons of discards are produced annually and that 45% of this volume is due to bottom trawling (Pérez Roda et al., 2019). In Brazil, the discard rate in fisheries of shrimp, fish, and other organisms varies from 24% to 65,2% (Davies et al., 2009; Perez et al., 2013; Cardoso et al., 2021).

Global fish production (marine + inland waters) was estimated at 96.4 million tons in 2018, a 5.4% increase over the past three years (FAO, 2020). However, after the global problems of coping with COVID-19, with direct effects on marine extractive production, which showed a temporary decrease (FAO, 2021), it has again regained strength.

In Brazil, marine extractive production was estimated at 489,000 tons during 2017-2018 (FAO, 2020). However, the lack of Brazilian management of these resources, whose monitoring ceased in 2009, opened gaps in the data of more than one decade (Dias et al., 2020).

Although monitoring at a national level has ceased, in the State of Rio de Janeiro the Instituto de Pesca Foundation carried out a fishing monitoring program between the regions of Paraty and Cabo Frio. The results for 2018 show a discharge of 27,187.3 tons of fish, with industrial fishing accounting for 70.8% and artisanal fishing for 29.2% of this amount (PMAP-RJ, 2019). The four main ports in the State of Rio de Janeiro are Niterói, São Gonçalo, Angra dos Reis, and Cabo Frio. Niterói and São Gonçalo together account for 58.2% of all fishing production, 69.4% of industrial fishing, and 30.9% of artisanal fishing. Angra dos Reis ranks third in production port, accounting for 21.3% of the State production, and Cabo Frio accounts for 10.6%.

Among fishing gears, the Siege trawler accounted for 80.5% of industrial catches and 50.9% of artisanal catches. In second place, the double trawl accounted for 10.6% of industrial catches, and in fourth position was artisanal fishing catches (6.6%). The industrial double trawl fleet was the second most important in number of vessels (71) and unloaded production: 10.6%, i.e., 2,045.3 tons (Dias et al., 2020).

The impacts of fishing on target populations result in overfishing of many species (Dias et al., 2020). Fishing removed from the sea, as bycatch fauna, non-target populations of endangered species, such as seahorses (Foster and Vincent, 2004; Choo and Liew, 2005; Foster and Vincent, 2010; Silveira, 2011; Filiz and Taşkavak, 2012; Foster and Arreguin-Sánchez, 2014; Foster et al., 2017; Silveira et al., 2018). The three species of seahorses in Brazil (*Hippocampus reidi, H. erectus, and H. patagonicus*) are listed on the official Brazilian list of endangered species as Vulnerable (MMA, 2022b). They are protected by the National Action Plan for the Conservation of Coral Environments – PAN Corais (ICMBio, 2022). Internationally, *H. reidi* is considered "Near Threatened" (NT, A2d+4d)), while *H. erectus* and *H. patagonicus* are listed as "Vulnerable" (VU, A2cd) (IUCN, 2017). The three species are also included in the Appendix II of the International Convention on Trade in Endangered Fauna and Flora (CITES, 2022).

Considering the need for long-term monitoring of bycatch to obtain information that supports conservation actions, since more than 95% of seahorses traded worldwide are bycatches mainly from trawling (Foster and Vincent, 2004), the objective of this work is to describe the incidental capture of the seahorse *Hippocampus patagonicus* in trawl fisheries in Southeastern and South Brazil and estimate the number of metric tons and individuals captured per year throughout its geographic distribution area in Brazil.

2 Material and methods

2.1 Study area

The State of Rio de Janeiro is one of the main fishing landing ports in Brazil (Figure 1A). It has a Coastal Zone with 640 km in length and 156 docking sites distributed over 25 coastal municipalities (Begot and Vianna, 2014). The climate is tropical and the summers have more rainfalls than the winters. The annual average is 1,252 mm. The climate classification is Aw, according to Köppen and Geiger, and the average temperature is 23.6°C (Climate Data, 2022). The continental shelf in this region is classified as a "platform of tropical and equatorial climates under the action of river inputs," whose morphological type occurs in the regions located between the parallels 30° South and North (Corrêa, 2021).

2.2 Data collection and analyses

Data collection took place from December 2016 to August 2018. The data collected consisted of completing an on-board spreadsheet containing general information about fishing and the number of seahorses captured. It was adapted from Silveira et al. (2018). Fishing was carried out with four double trawl boats and one single trawler (all of them in industrial fishing) that landed products in five different ports: Angra dos Reis, Niterói, São Gonçalo, Cabo Frio, and Macaé, the latter using simple trawl. The fishing sites of the monitored vessels ranged from Maranduba, in the State of São Paulo, to Campos dos Goytacazes, in the State of Rio de Janeiro (Figure 1B). Fishing focused on marine shrimp (*Farfantepenaeus* spp.) and demersal fish, such as *Micropogonias furnieri*, *Umbrina canosai*, *Cynoscion striatus*, *Merluccius hubbsi*, *Lophius gastrophysus*, *Percophis brasiliensis*, *Scomberomorus* spp, *Paralichthys brasiliensis*, in addition to the mollusks *Sepioteuthis sepioidea* and *Octopus vulgaris*. The mesh used varied between 12 and 30 mm between opposite nodes. The anglers used a Global Positioning System (GPS) device attached to the boat to record the initial and final coordinates of the net in each haul, as well as capture depths, and recorded the number of seahorses caught. The trawls worked at a depth of up to 100 m.

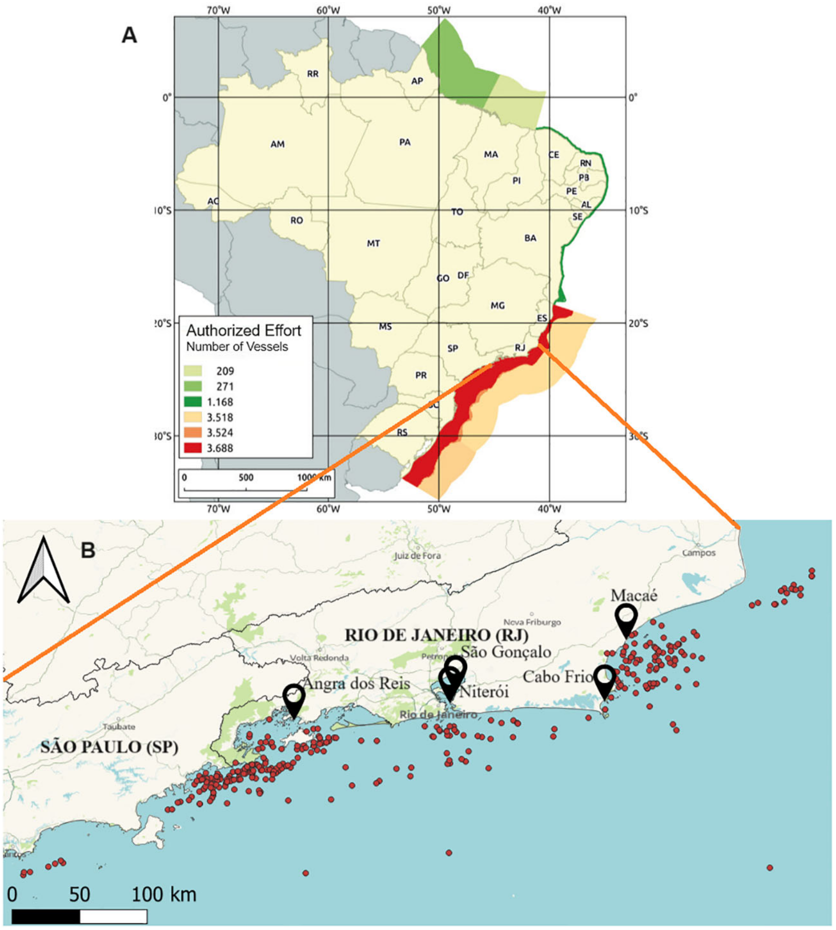

FIGURE 1
(A) Brazilian coastline showing the marine zones with their respective number of vessels authorized for trawling (Dias et al., 2020). **(B)** Study area covered by this work (red dots), represented by the numerous fishing trips and the fishing landing ports of this study (black markers).

The captured seahorses were deposited in containers with 90% alcohol provided by the research team. Once a month, researchers collected the containers with seahorses and on-board spreadsheets filled out by the fishermen themselves. All fishermen who participated in the activities received in return monthly food for the family until the end of the study.

The captured animals were transported to the Hippocampus Project laboratory in the State of Pernambuco. They were identified according to Silveira et al. (2014) and Piacentino and Luzzatto (2004). Height (linear measurement from the top of the head to the tip of the stretched out tail) was measured with a digital caliper (0.1 mm). The analysis of injuries resulting from trawl nets was performed in pregnant males (PM), non-pregnant males (NPM), and females (F) (data in percentage). The most common injuries found in fish expected to occur are crushing, abrasion (Davis, 2002), and organ eversion (Silveira et al., 2018).

Catch per effort unit (CPUE) was estimated by dividing the number of seahorses captured by the number of days, hauls, and total hours worked. The calculation of the total number of hours was performed by multiplying the number of hours of each throw by the total number of throws. Fishing effort focused on seahorses is presented in individuals per day (ind./day), per throw (ind./throw), and per hour (ind./hour) for all ports. Based on the CPUE of each port, the annual average CPUE was also estimated for the Southeast and South Brazil, where strong fishing pressure has already been recorded for seahorses that are removed as bycatch (Silveira et al., 2018; Silveira et al., 2020). The annual CPUE estimate for the South/Southeast Brazil is presented in individuals per year (ind./year) and metric tons per year (metric tons/year). Information on the size of the trawl fishing fleet (3,700 vessels), to calculate the annual CPUE, was obtained from Dias et al. (2020). To estimate metric tons/year, 557 individuals from the samples were weighed, and the average weight was multiplied by the estimated amount of ind./year. All seahorses were preserved in 70°gl alcohol after fishing and none of the individuals that were weighed were dry or missing any part of the body.

The reproductive period of seahorses was determined by the relative frequency of pregnant males over months.

2.3 Statistical analysis

Spearman's correlation was used to assess the relationship between the number of seahorses captured, the number of hours trawled, and the size of the net mesh. To test the relationship between sex (males, pregnant males, and females) and bodily injuries, an Analysis of Variance (ANOVA) was conducted. Seahorse catches per unit effort were compared between ports using ANOVA and Tukey's *post-hoc* test. The Shapiro-Wilk test and the Levene test were used to verify the assumptions of normality and constant variance.

A scatterplot shows the relationship between the number of seahorses captured and the depth of capture. The estimates that represent capture depth were made using Kriging with a linear variogram, in which the blue scale represents the capture depth estimate and the red scale represents the number of captured animals. The contours of the territorial limits of the States were obtained through shapefiles made available by the Brazilian Institute of Geography and Statistics (IBGE, 2022). All analyses were performed using the R software (R Core Team, 2022), version 4.2.0, at a significance level of 5%.

3 Results

During the study period, 2,041 individuals of *H. patagonicus* were captured as bycatch, 1,183 males and 858 females, with no difference in sex ratio (p=0.298). The height of individuals ranged from 6.5 to 15.9 cm (10.47 ± 1.38 cm) and the weight varied between 1.0 and 12.1 g (4.13 ± 1.54 g). Six individuals of *H. reidi* were collected. They were not representative in the sample. No individual of *H. erectus* was identified. The ports that received the highest number of seahorses (relative frequency) were Niterói, São Gonçalo, Angra dos Reis, Macaé, and Cabo Frio (Figure 2). All seahorses captured in the State of São Paulo (n = 25) were landed to the port of Angra do Reis, in Rio de Janeiro.

There was no correlation between the hours of trawling and the number of seahorses caught overall (p=0.565). When we separated the data by depth range (18 to 48 m and 50 to 100 m), the number of hours of trawling did not interfere with the capture at the lowest depths (p=0.414), but interfered with capture at greater depths (p=0.007). There was also a weak and positive correlation between the number of seahorses captured and the net mesh size (p=0.043).

Shrimp trawling was responsible for removing 38% of seahorses in the samples at capturing depths that varied between 18 and 48 m, while trawling for demersal fish captured 62% of seahorses at depths between 50 and 100 m. Of the five vessels monitored, three fished only shrimp, one fished shrimp and fish, and one vessel fished only fish.

The main injuries observed on the bodies of seahorses were everted digestive tract (65,6%), everted cloaca (32%), and broken tails (0,3%), broken heads (0,6%), and broken rings (0,87%). There was no significant difference in the percentage of injuries between males, pregnant males, or females (p=0.648). There were more animals without injuries than animals with apparent injuries (p=0.001). Table 1 shows the number of injuries/sex in the study period and the average percentage of injuries by port and total injuries.

The highest percentages of injuries to animals occurred with specimens landed in the ports of São Gonçalo and Niterói (Table 1). Of the 2,041 seahorses captured as bycatch, 1,183 were males and 44.5% were pregnant males. Among pregnant males, 39.73% suffered some type of injury. On the other hand, of the 858 females captured, 30.65% suffered injuries. Non-pregnant males (657 specimens) suffered injuries at a percentage of 30.90%. There was no significant difference between the percentage of injuries to males, pregnant males, or females (p=0.648). Regarding injuries and body size, there was no significant difference in height of males with or without injuries (p=0.1273), but for females there was a difference (p<0.001). The average height of females with injuries was 10.23 ± 1.10, while for females without injuries it was 9.63 ± 1.27. The reproductive period of *H. patagonicus* occurs throughout the year (Figure 3).

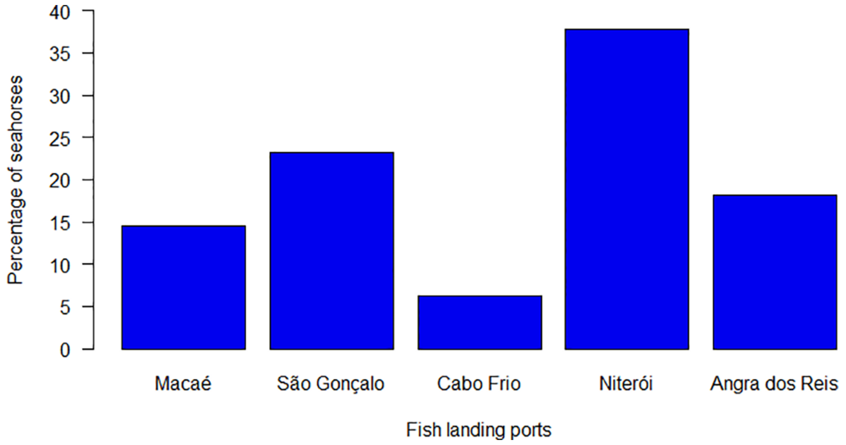

FIGURE 2
Percentage of seahorses captured as bycatch landed in monitored ports.

TABLE 1 Number of injuries by sex and percentage of injuries to animals by landing port.

Port	PM	NPM	Total M	F	Seahorse Total	INJ				
						PM	NPM	F	Total	%
CABO FRIO	21	55	76	52	128	1	1	12	14	10.9
SÃO GONÇALO	162	140	302	172	474	68	47	112	227	47.9
MACAÉ	59	88	147	149	296	8	4	7	19	6.4
NITEROI	170	264	434	337	771	90	126	103	319	41.4
ANGRA DOS REIS	114	110	224	148	372	42	25	29	96	25.8
TOTAL	526	657	1183	858	2041	209	203	263	675	33.1

PM, pregnant males; NPM, non-pregnant males; F, females; INJ, injury; MID%INJ, mean percentage of injury.

3.1 CPUE

Considering the five vessels monitored, 839 days of fishing were recorded, with 2,251 sets of nets thrown totaling 8,604 hours of fishing. Of the 2,251 sets of nets thrown, 1,783 were for shrimp fishing and 468 for demersal fish. These activities resulted in 2,041 seahorses caught as bycatch, of which 773 in shrimp fishing and 1268 in fish fishing. Also, 161 seahorses were captured (26 in Cabo Frio and 135 in Niterói), but they could not be considered in CPUE calculations because spreadsheets were lost.

Although the Port of Niterói recorded the highest absolute frequency of seahorses as bycatch (Table 1), the CPUE calculation shows that the rates of incidental capture and landing occurred (in order of importance) in the ports of São Gonçalo, Niterói, Cabo Frio, Angra dos Reis, and Macaé (Table 2). There was a significant difference between the number of seahorses landed in ports by trawl (p=0.0084), and São Gonçalo was the main landing port. When we compare the different fisheries, the CPUE calculation in ind/throw shows a significant difference with 0.75 ± 2 ind/throw for the marine shrimp fishery and 2.3 ± 3.4 ind/throw for the demersal fishery (p=0.001).

The CPUE calculation for the study region estimated 3.36 animals captured per day/boat considering an average of 15.3 (± 5.95) fishing days/boat/month, resulting in a bycatch of 2,282,515 individuals per year (3.36 seahorses/day x 3,700 boats x 183.6 days), or 9427 Kg year^{-1} of seahorses, along the South and Southeast Brazil, an area recognized as the main geographic distribution of *H. patagonicus*. Although fishing effort was standardized for the analyses here, Table 2 shows that the two ports with the highest rates of seahorse bycatch contributed the least in the sampled months, which suggests that our results may be underestimated.

Analyzing capture depth data allows establishing that *H. patagonicus* occurs along the coast of Rio de Janeiro at depths ranging from 25 m to 90 m, with a preference for the depth range between 40 and 80 m (Figures 4, 5).

4 Discussion

Considering that the data for the present study were collected entirely by the trawler fishermen and that, although they received a counterpart for collaborating, there is no way to be sure that all the material collected was delivered for research, since we detected a local trade in seahorses for curiosities and folk medicine. In addition, there may simply not have been enough effort to collect seahorses from the large amount of demersal fish or shrimp. Whatever the case, the data

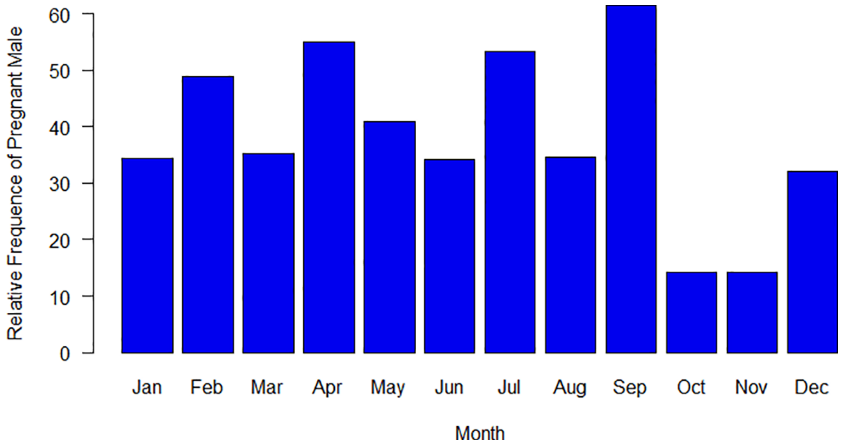

FIGURE 3
Reproductive period of *Hippocampus patagonicus* in the State of Rio de Janeiro (n = 1183).

TABLE 2 Capture per effort unit of seahorses as bycatch in trawl fisheries in the State of Rio de Janeiro between December 2016 and August 2018 (Same letters represent statistically similar averages).

Months	Port	CPUE/day	CPUE/throw	CPUE/hour
5	NITERÓI	2.59 ± 3.24^{B}	1.37 ± 1.71^{AB}	0.31 ± 0.37^{AB}
2	SÃO GONÇALO	8.62 ± 15.21^{A}	2.91 ± 5.05^{A}	0.72 ± 1.27^{A}
11	ANGRA DOS REIS	1.44 ± 5.23^{B}	0.60 ± 2.21^{B}	0.13 ± 0.45^{B}
6	CABO FRIO	2.52 ± 4.41^{B}	1.53 ± 2.19^{AB}	0.71 ± 1.01^{A}
9	MACAÉ	1.31 ± 1.66^{B}	0.31 ± 0.40^{B}	0.08 ± 0.10^{B}
33	Average	3.36 ± 5.95	1.34 ± 2.31	0.39 ± 0.64
	p-value (ANOVA)	0.0034	0.0084	<0.0001

would be underestimated and, unfortunately, the situation would be even more critical. However, the fact that we collected seahorses and fishing information (on-board spreadsheets) on a monthly basis allowed for verification and control between what was declared on the on-board map and the quantity delivered for the survey. The applied methodology was an excellent alternative for this type of work, since scientific observers on board are extremely expensive, especially in these cases where the permanence at sea is, on average, 20 days. However, very important information from these data is discussed here.

The reproductive period of *H. patagonicus* in Rio de Janeiro occurs in all seasons of the year and without interruptions, similar as *H. reidi* in tropical and temperate regions of Brazil (Silveira, 2005; Mai and Velasco, 2011; Silveira et al., 2022a) and other species around the world (Lourie et al., 2004). Although the samples of males had a good size (n=1183), they were collected according to the calendar of target fishing (shrimp and demersal fish), and showed a greater reproductive activity of *H. patagonicus* between February and September. However, it was not possible to establish a reproductive peak and perhaps it does not exist. Taking into account the fertility of *H. patagonicus* of 156.38 ± 66 embryos per pregnancy (Silveira et al., 2020) and that in the current survey 44.5% of males were pregnant, 81,588 animals still in formation were lost only in this sample.

In relation to seahorses that have suffered bodily injuries, 33% of captured seahorses had their body damaged to the point of death, but the remaining 77% (1,339 individuals) appeared to be in good condition. Perhaps, were they returned to the sea, they could have a chance of surviving and, eventually, find a reproductive partner. However, we have to consider the difference in atmospheric pressure to which *H. patagonicus* lives in Brazilian waters. These animals live in a preferred range around 40 and 80 m deep; having been captured at 90 m deep, this means that a pressure of five to ten atmospheres routinely act on their physiology. Suddenly, after hundreds of meters of trawling, the animals are brought to sea level after being carried by a net. In this case, not only pressure, but also temperature, which can quickly vary, cause thermal shock in fish and may lead to death before reaching the boat deck (Davis and Olla, 2001). For fish, mortality during trawling is related, among others, to trawling time, total catch weight, trawling depth, water temperature, time the fish stays on deck exposed to air, and atmospheric temperature (Morfin et al., 2017).

In this study, we could not assess the survival rate of seahorses that did not show apparent injuries, not even quantify death from

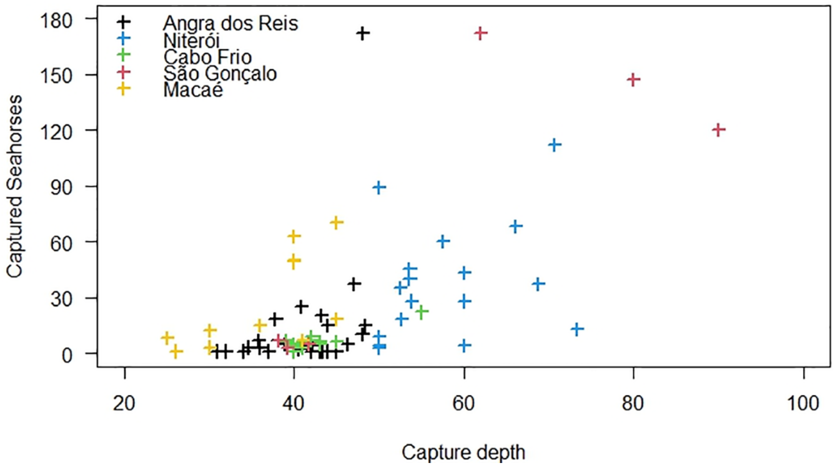

FIGURE 4
Frequency of occurrence of *Hippocampus patagonicus* captured as bycatch in relation to depth (m) and landing ports in Southeast Brazil.

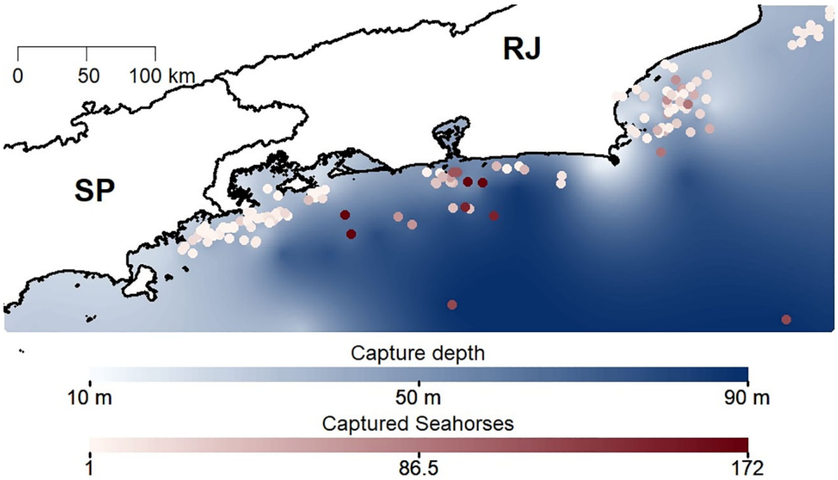

FIGURE 5
Spatial distribution map of *Hippocampus patagonicus* in relation to depth.

fatigue. A project for such a study would be very important in order to know whether there is a possibility of recovery and return of specimens, including pregnant males. According to Morfin et al. (2017), survival rates for plaice in trawl fisheries range between 45.2% and 66.6%. Among the fish that survive trawls and are exposed on deck for handling, most suffocate within the first 20 minutes out of the water and die. Animals that are still alive and thrown back into the sea are weakened individuals that are vulnerable to predation by birds or other fish (Davis and Olla, 2002; Macbeth et al., 2006).

The most frequent injuries found in this study were the eversion of the digestive tube and the cloaca (65.6 and 32%, respectively) where the meshes of the capture nets varied between 12-20 mm (shrimp) and 30 mm (fish). In general, the most common injuries in fish are crushing, abrasion, loss of scales, and fatigue from excessive swimming, which is aggravated by net overcrowding (Davis, 2002; Uhlmann et al., 2016; Morfin et al., 2017). Baum et al. (2003) observed that less than 1% of seahorses were killed in trawls where the mesh size of the net was 2.54 to 3.18 cm, and it was recorded as the main injury that 4.7% of the animals lost their tail rings (about 61% of tail size). The low trawl depth (1.8-6.4 m) and time per drag (40-50 min) contributed to results of less damage when compared to the results of the present study (33.1% of injuries), in depths between 20 and 100m and dragging time between one and six hours. The correlation between injuries and depth of capture was not verified, but it is likely that it exists, not only because of the greater stress to which the animal is exposed when removed from deeper places, but also because the number of seahorses increases with depth, between 20 and 60 m (increasing the sample number), and after that it starts to decrease (Figure 4).

The geographic area covered by the study was largely sampled by trawl nets that traversed the seabed, and the boats often overlapped fishing areas. In this study, we found a curious relationship between larger net mesh sizes and a larger number of seahorses captured. This possibly reflects the environment of the target fishery. Were sampled 2,251 net trawl and the most seahorses (62%) were captured by trawling aiming demersal fish; this type of fishing requires the largest meshes and the greatest depths (from 50 to 100 m). Although seahorses could escape due to the mesh size of this net, they are trapped by the accumulation of fish in the net and by anchoring their prehensile tail to the net itself or objects that have been dragged along. We would expect that smaller net meshes would capture more seahorses, as well as a longer trawl time, but these correlations were not seen, probably because the depth of occurrence of the species was the preponderant factor in this case. That is, regardless of the mesh size and dragging time, the capture takes place along the vertical distribution of *H. patagonicus*, although important, the other factors become secondary. The highest number of seahorses captured beyond 50 m of depth, even with the lowest number of bids for trawling for fish (compared to shrimp fishing), emphasizes the vertical distribution of the species. As for the horizontal distribution of *H. patagonicus*, there seem to be no gaps along the coast of Rio de Janeiro, but we must consider, in general, the skills/dedication of each fisherman in collecting seahorses from nets and the number of months sampled for each boat, influencing the different capture rates in the ports.

Our annual catch estimates for the Southeast and South Brazil (more than two million seahorses) place Brazil in a catch level similar to the Philippines (1.7 million seahorses/year), which are a known center of capture and trade of seahorses. However, what is the fate of seahorses captured as bycatch in Brazil? We do not know yet. Unfortunately, in Brazil there is no effective control over the seahorse trade. Only a few records in CITES exist, where an export of 16,669 individuals of *H. reidi* between 2010 and 2020 was reported (www.cites.org). There are no export records for *H. patagonicus*. There are records of Brazilian exports of 350 specimens of *H. erectus* in this period. Between 2000 and 2009, CITES recorded an export declaration of 5,588 specimens of *H. erectus* (tradeview.cites.org), which possibly means a misidentification of species or a strategy to disguise an over-quota in the export of *H. reidi* (Rosa et al., 2011). The Brazilian legislation that protects seahorses is satisfactory; however, once again, we fail to comply with and enforce laws. In the absence of government records and oversight of exports of live and dried seahorses (including correct species identification), alternative work by researchers may help to explain the problem (Rosa et al., 2011; Silveira et al., 2018), but how to solve it will require great government efforts.

In Brazil, seahorse bycatch data are scarce (Rosa et al., 2005; Vianna and Almeida, 2005; Rosa et al., 2011; Silveira, 2011; Silveira et al., 2018). Silveira et al. (2018) estimated 8,342 animals removed annually only by dragging pairs in southern Brazil (28 boats). However, according to Dias et al. (2020), the trawl fleet in the South and Southeast Brazil consists of boats proper for pair trawling, double trawling, and single trawling. The total is 3,700 vessels that operate in these areas. Many have additional authorizations that allow them to operate with other types of trawl or gillnet in the Territorial Sea and Exclusive Economic Zone - EEZ areas. All types of trawling and even gill fishing, at some point, captured seahorses in the South and Southeast Brazil (Pereira, 2016; Abilhoa et al., 2018; Silveira et al., 2018).

The trawling that occurs in South and Southeast Brazil is certainly the main source of dried seahorses that supplies the clandestine internal and external trade of seahorses (Rosa et al., 2011; Silveira et al., 2018) and removes a large number of *H. patagonicus* (Silveira et al., 2018; Silveira et al., 2022b). The clandestine trade of seahorses in Brazil also occurs through undeclared import. In response to a demand from the Chico Mendes Institute for Biodiversity Conservation - ICMBio (Taiamã Ecological Station, Cáceres), in 2016 the Hippocampus Project identified the species *H. ingens*. It was seized by the Federal Police while being smuggled from Peru, passing through Bolivia, and entering the State of Mato Grosso to be sold in the State of São Paulo, Brazil. Along with 30 kg of dried seahorses, sea cucumbers and shark claspers (copulatory organ) were also seized; they are used as an aphrodisiac delicacy in restaurants in São Paulo. The dried animals were being moved in cardboard boxes and plastic bags transported by line buses. Dried *H. patagonicus* were sold in the public market in Recife, PE, Northeast Brazil (Silveira, 2005). However, as we have seen, their distribution is restricted to the South and Southeast Brazil, which indicates a smuggling route of these animals.

The trade of dried seahorses (*H. patagonicus*) from the South and Southeast Brazil may occur illegally thanks to trawl fisheries. Therefore, what can we do? The zoning of fishing by fleet, further rules for trawling, further inspection, reduction of target stocks and bycatch species threatened with extinction or not, banning of trawling, and creation of marine protected areas are measures that scholars have claimed over the years (Haimovici and Mendonça, 1996; Perez et al., 2001; Haimovici et al., 2006; Hilborn et al., 2006; Lessa and Vooren, 2007; Rosso, 2015; Ricardo-Pezzuto and Mastella-Benincà, 2017; Dias et al., 2020). However, governments have done little. This absence places Brazil in the 26th position in world fishery management ranking; there are no positive outcomes for this performance (Melnychuk et al., 2017). Furthermore, the vertical distribution of *H. patagonicus* does not allow the protection of this species by PAN Corais (Silveira et al., 2020), the current protection instrument for seahorses in Brazil. In the absence of fishery management, the banning of trawl fishing within 12 miles of the Brazilian territorial sea promoted by the State of Rio Grande do Sul (RS) on its coast is certainly an effective action for the conservation of many species, including seahorses, and which could be replicated in other Brazilian States. However, the document banning trawling in RS was replaced for the Ordinance MAPA/SAP no. 634 (MAPA/SAP, 2022), which authorizes additional practices for sustainable fishing of motorized shrimp trawling in the maritime strip of the coastal zone adjacent to the RS, extending it from three nautical miles to 12 nautical miles. A few months after publication, the Ordinance no. 634 was suspended by the Federal Regional Court of the 4th Region (TRF4), as well as traction fishing. This measure benefits not only the thousands of species that are dragged for kilometers during motorized fishing, but also artisanal anglers, who feel impaired by the visibly unfair competition. In view of areas recognized as priority for conservation on the Brazilian coast (MMA, 2022a), the creation of new marine protected areas and their effective implementation in the Southeast and South Brazil may promote protection for *H. patagonicus* in its area of occurrence in Brazil. Strategies for the conservation of this and many other species caught in incidental trawl fisheries must be developed in partnership with environmental authorities, researchers, and the fishing industry.

Trawling is the world's "villain of the seas," claiming life in the oceans and providing 95% of seahorses for the global dry trade (Foster and Vincent, 2004). The global catch data for seahorses is alarming. According to data of 22 countries where capture and trade are known, it is estimated that around 76 million animals are removed from the sea annually (Lawson et al., 2017). Countries such as India, with estimates between 4.98 and 13.64 million seahorses/year, the Philippines, with 1.7 million, Thailand, with 29 million, and Vietnam, with 16.7 million seahorses, are known centers of capture and trade of these animals (Foster et al., 2017; Foster et al., 2019; Vaidyanathan et al., 2021). Unfortunately, Brazil appears in this scenario with alarming estimates and a complete lack of knowledge and control of the situation (Silveira et al., 2018; Silveira et al., 2020; this work). Based on these data, we need to build plans for monitoring and researching the possible and probable routes for the clandestine seahorse trade, providing people with knowledge and environmental education and ensuring the viability of seahorse populations in Brazilian waters.

5 Conclusion

Hippocampus patagonicus is a threatened species listed as "Vulnerable" in the international and Brazilian lists. However, this threat assumes even greater proportions when its only area of occupation in Brazil (Southeast and South) shows no subsidies for protection and conservation. Although there are many marine protected areas in the Southeast region, in the South region, such as in the State of Rio Grande do Sul, they are nonexistent. As we could see in this work, even in the Southeast region, with the presence of several marine protected areas, trawl fishing showed can further "vulnerability" and extrapolate it to a more serious condition if a government intervention is not carried out that can ensure sustainability to the natural stocks of this population or of populations of *H. patagonicus*. Unfortunately, the current seahorse protection laws in Brazil do not address this issue because seahorses are in the list of species protected by the PAN Corais. Furthermore, these reef environments are not preferred habitats of *H. patagonicus*, which continues to occupy preferential niches between 40 and 80 meters deep, thus risking being caught by the next trawl.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors upon request, without undue reservation.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because the seahorses used were caught incidentally, independently of our will.

Author contributions

RS contributed to the conceptualization, methodology, data analysis, writing – original draft, writing –, and review/editing. MV contributed to the data analysis, writing – review/editing. JS contributed to methodology, statistical analysis, preparation of figures, and review/editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Publisher's note

References

Abilhoa, V., Silveira, R., and Pereira, L. (2018). Feeding habits of the seahorse Hippocampus patagonicus (Actinopterygii: Syngnathiformes: Syngnathidae) on the southern coast of Brazil. *Acta Ichthyol. Piscat.* 48, 267–271. doi: 10.3750/AIEP/02379

Baum, J. K., Meeuwig, J. J., and Vincent, A. C. J. (2003). Bycatch of lined seahorses (Hippocampus erectus) in a Gulf of Mexico shrimp trawl fishery. *Fishery Bulletin* 101, 721–731.

Begot, L. H., and Vianna, M. (2014). A frota pesqueira costeira do estado do Rio de Janeiro. *Bol. do Inst. Pesca* 40, 79–94.

Cardoso, L. G., Monteiro, D. S., and Haimovici, M. (2021). Na assesment of discarded catches from the bottom pair trawling fishery in Southern Brazil. *Mar. Freshery Sci.* 34 (2), 197–210. doi: 10.47193/mafis.3422021010609

Choo, C. K., and Liew, H. C. (2005). Exploitation and trade in seahorses in peninsular Malaysia. *Malayan Nat. J.* 57, 57–66.

CITES (2022) *Convention on International Trade in Endangered Species of Wild Fauna and Flora*. Available at: https://cites.org/eng (Accessed May 16, 2022).

Climate Data (2022) *Dados climáticos para cidades mundiais*. Available at: www.climate-data.org (Accessed May 12, 2022).

Corrêa, I. C. S. (2021). *Morfologia do Ambiente Marinho* (Porto Alegre: CECO/PGGM/IGEO/UFRGS).

Davies, R. W. D., Cripps, S. J., Nickson, A., and Porter, G. (2009). Defining and estimating global marine fisheries bycatch. *Mar. Policy* 33, 661–672. doi: 10.1016/j.marpol.2009.01.003

Davis, M. W. (2002). Key principles for understanding fish bycatch discard mortality. *Can. J. Fish. Aquat. Sci.* 59, 1834–1843. doi: 10.1139/f02-139

Davis, M. W., and Olla, B. L. (2001). Stress and delayed mortality induced in pacific halibut by exposure to hooking, net towing, elevated seawater temperature and air: implications for management of Bycatch. *North Am. J. Fish. Manage.* 21, 725–732. doi: 10.1577/1548-8675(2001)021<0725:SADMII>2.0.CO;2

Davis, M. W., and Olla, B. L. (2002). Mortality of lingcod towed in a net as related to fish length, seawater temperature, and air exposure: ALaboratory Bycatch study. *North Am. J. Fish. Manage.* 22, 1095–1104. doi: 10.1577/1548-8675(2002)022<1095:MOLTIA>2.0.CO;2

Dias, M., Iwanicki, L., and Zamboni, A. (2020). *Auditoria da pesca: Brasil 2020: uma avaliação integrada da governança, da situação dos estoques e das pescarias* (Brasília:: Oceana Brasil).

FAO (2020). *The State of World Fisheries and Aquaculture 2020* (Rome: Food and Agriculture Organization of the United Nations). doi: 10.4060/ca9229en

FAO (2021). *The impact of COVID-19 on fisheries and aquaculture - A global assessment from the perspective of regional fishery bodies* (Rome: Food and Agriculture Organization of the United Nations). doi: 10.4060/cb5269en

Filiz, H., and Taşkavak, E. (2012). Field surveys on recent situation of seahorses in Turkey. *Biharean Biol.* 6, 55–60.

Foster, S. J., and Arreguin-Sánchez, F. (2014). Using distribution patterns of small fishes to assess small fish by-catch in tropical shrimp trawl fisheries. *Anim. Conserv.* 17, 217–224. doi: 10.1111/acv.12078

Foster, S. J., Aylesworth, L., Do, H. H., Bat, N. K., and Vincent, A. C. J. (2017). Seahorse exploitation and trade in Viet Nam. *Fish. Cent. Res. Rep.* 25, 50. doi: 10.14288/1.0389790

Foster, S. J., Kuo, T.-C., Wan, A. K. Y., and Vincent, A. C. J. (2019). Global seahorse trade defies export bans under CITES action and national legislation. *Mar. Policy* 103, 33–41. doi: 10.1016/j.marpol.2019.01.014

Foster, S. J., and Vincent, A. C. J. (2004). Life history and ecology of seahorses: implications for conservation and management. *J. Fish Biol.* 65, 1–61. doi: 10.1111/j.0022-1112.2004.00429.x

Foster, S. J., and Vincent, A. C. J. (2010). Using life-history information to assess potential effects of shrimp trawling on small fishes. *J. Fish Biol.* 76, 2434–2454. doi: 10.1111/j.1095-8649.2010.02631.x

Haimovici, M., and Mendonça, J. T. (1996). Descartes da fauna acompanhante na pesca de arrasto e tangones dirigida a linguados e camarões na plataforma continental do sul do Brasil. *Atlântica Rio Gd.* 18, 161–177.

Haimovici, M., Rossi-Wongtschowski, C. L. D. B., Cergole, M. C., Madureira, L. S.-P., and Bernardes, R.Á. (2006). “Recursos pesqueiros da região Sudeste e Sul,” in *Avaliação do potencial sustentável de recursos vivos na Zona Econômica do Brasil* (Brasília: Ministério do Meio Ambiente / Secretaria de QUlidade Ambiental ns Assentamentos Humanos), 39.

Hilborn, R., Micheli, F., and De Leo, G. A. (2006). Integrating marine protected areas with catch regulation. *Can. J. Fish. Aquat. Sci.* 63, 642–649. doi: 10.1139/f05-243

IBGE (2022) *Portal de Mapas do IBGE*. Available at: https://portaldemapas.ibge.gov.br/ (Accessed September 5, 2022).

ICMBio (2022). *Plano de Ação Nacional para Conservação de Ambientes Coralíneos* (Brasília: Instituto Chico Mendes de Conservação da Biodiversidade).

IUCN (2017) *IUCN Red List of Threatened Species. Version 2017.3. Int. Union Conserv. Nat.* Available at: http://www.iucnredlist.org (Accessed May 3, 2017).

Lawson, J. M., Foster, S. J., and Vincent, A. C. J. (2017). Low Bycatch rates add up to big numbers for a genus of small fishes. *Fisheries* 42, 19–33. doi: 10.1080/03632415.2017.1259944

Lessa, R. P., and Vooren, C. M. (2007). *Rhinobatos horkelii. The IUCN red list of threatened species 2007* (International Union for Conservation of Nature and Natural Resources: IUCN). doi: 10.2305/IUCN.UK.2007.RLTS.T41064A10396152.en

Lourie, S. A., Foster, S. J., Cooper, E. W. T., and Vincent, A. C. J. (2004). *A guide to the identification of seahorses* (Washington: Project seahorses & TRAFFIC North America - University of British Columbia and World Wildlife Fund).

Macbeth, W. G., Broadhurst, M. K., Paterson, B. D., and Wooden, M. E. L. (2006). Reducing the short-term mortality of juvenile school prawns (*Metapenaeus macleayi*) discarded during trawling. *ICES J. Mar. Sci.* 63, 831–839. doi: 10.1016/j.icesjms.2006.03.008

Mai, A. C. G., and Velasco, G. (2011). Population dynamics and reproduction of wild longsnout seahorse *Hippocampus* reidi. *J. Mar. Biol. Assoc. United Kingdom* 92, 421–427. doi: 10.1017/S0025315411001494

MAPA/SAP (2022). *Portaria no 634, de 21 de março de 2022* (Brasília: Diário Oficial da União / Ministério da Agricultura, Pecuária e Abastecimento / Secretaria de Aquicultura e Pesca).

Melnychuk, M. C., Peterson, E., Elliott, M., and Hilborn, R. (2017). Fisheries management impacts on target species status. *Proc. Natl. Acad. Sci.* 114, 178–183. doi: 10.1073/pnas.1609915114

MMA (2022a) *Ministério do Meio Ambiente*. Available at: www.gov.br/mma (Accessed August 10, 2022).

MMA (2022b). *Portaria no 148, de 7 de junho de 2022* (Brasília: Diário Oficial da União / Ministério do Meio Ambiente).

Morfin, M., Kopp, D., Benoît, H. P., Méhault, S., Randall, P., Foster, R., et al. (2017). Survival of European plaice discarded from coastal otter trawl fisheries in the English Channel. *J. Environ. Manage.* 204, 404–412. doi: 10.1016/j.jenvman.2017.08.046

Pereira, L. F., Silveira, R. B., and Abilhoa, V. (2016). New records of *Hippocampus patagonicus* Piacentino & Luzzatto, 2004 (Teleostei: Syngnathidae) from the coast of Paraná, southern Brazil. Check List. *Ecologia alimentar de Hippocampus patagonicus Piacentino & Luzzatto 2004 e a conservação de cavalos-marinhos (Teleostei : Syngnathidae) no Sul do Brasil* 12 (1), 1822. doi: 10.15560/12.1.1822

Perez, J. A. A., Pereira, B. N., Pereira, D. A., and Schroeder, R. (2013). Composition and diversity patterns of megafauna discards in the deep-water fishery off Brazil. *J. Fish Biol.* 83 (4), 804–825. doi: 10.1111/jfb.12141

Perez, J. A. A., Pezzuto, P. R., Rodrigues, L. F., Valentini, H., and Vooren, C. M. (2001). Relatório da reunião técnica de ordenamento de pesca de arrasto das regiões sudeste e sul do Brasil. *Notas Técnicas Facimar* 5, 1–34.

Pérez Roda, M. A., Gilman, E., Huntington, T., Kennelly, S. J., Suuronen, P., Chaloupka, M., et al. (2019). *A third assessment of global marine fisheries discards* (Rome: Food and Agriculture Organization of the United Nations).

Piacentino, G., and Luzzatto, D. (2004) *Hippocampus patagonicus sp. nov., nuevo caballito de mar para la Argentina (Pisces, Syngnathiformes). Rev. del Mus. Argentino Ciencias Nat.* Available at: http://revista.macn.gob.ar/ojs/index.php/RevMus/article/view/93 (Accessed March 20, 2017).

PMAP-RJ (2019). *Projeto Monitoramento da Atividade Pesqueira no Estado do Rio de Janeiro* (Rio de Janeiro: PETROBRAS/FUNDEPAG/FIPERJ).

Polet, H., Delanghe, F., and Verschoore, R. (2005). On electrical fishing for brown shrimp (*Crangon crangon*). *Fish. Res.* 72, 13–27. doi: 10.1016/j.fishres.2004.10.015

R Core Team (2022). *R: A language and environment for statistical computing* (Vienna, Austria: R Foundation for Statistical Computing). Available at: https://www.R-project.org/.

Ricardo-Pezzuto, P., and Mastella-Benincà, E. (2017). Challenges in licensing the industrial double-rig trawl fisheries in Brazil. *Lat. Am. J. Aquat. Res.* 43, 495–513. doi: 10.3856/vol43-issue3-fulltext-11

Rosa, I. M. L., Alves, R. R. N., Bonifácio, K. M., Mourão, J. S., Osório, F. M., Oliveira, T. P. R., et al. (2005). Fishers' knowledge and seahorse conservation in Brazil. *J. Ethnobiol. Ethnomed.* 1, 12. doi: 10.1186/1746-4269-1-12

Rosa, I. L., Oliveira, T. P. R., Osório, F. M., Moraes, L. E., Castro, A. L. C., Barros, G. M. L., et al. (2011). Fisheries and trade of seahorses in Brazil: historical perspective, current trends, and future directions. *Biodivers. Conserv.* 20, 1951–1971. doi: 10.1007/s10531-011-0068-2

Rosso, A. P. (2015). *Análise das relações entre frotas pesqueiras, recursos demersais e características do ecossistema: subsídios para identificação de Unidades Geográficas de Gestão para a pesca industrial do Sudeste-Sul do Brasil* (Itajaí: Universidade do Vale do Itajaí).

Silveira, R. B. (2005). *Dinâmica populacional do cavalo-marinho Hippocampus reidi (Syngnathidae) no manguezal de Maracaípe, Ipojuca, PE. Tese.* (Porto Alegre: Pontifícia Universidade Católica do Rio Grande do Sul).

Silveira, R. B. (2011). Registros de cavalos-marinhos (Syngnathidae: *Hippocampus*) ao longo da costa Brasileira. *Oecologia Aust.* 15, 316–325. doi: 10.4257/OECO.2011.1502.9

Silveira, R. B., Barcelos, B. T., MaChado, R., Oliveira, L., and Silva, J. R. S. (2018). Records of bycatch of *Hippocampus patagonicus* (Pisces: Syngnathidae) in commercial fishing in Southern Brazil. *Lat. Am. J. Aquat. Res.* 46(4), 744-755. doi: 10.3856/vol46-issue4-fulltext-12

Silveira, R. B., Da Silva, J. C., Benício, L., and Silva, J. R. S. (2020). Biology of *Hippocampus patagonicus* (Syngnathidae) in Brazilian waters. A species threatened with extinction, with suggestions for the conservation of seahorses in Brazil. *Lat. Am. J. Aquat. Res.* 48, 47–57. doi: 10.3856/vol48-issue1-fulltext-2307

Silveira, R. B., Siccha-Ramirez, R., Silva, J. R. S., and Oliveira, C. (2014). Morphological and molecular evidence for the occurrence of three *Hippocampus* species (Teleostei: Syngnathidae) in Brazil. *Zootaxa* 3861, 317–332. doi: 10.11646/zootaxa.3861.4.2

Silveira, R. B., Silva, J. R. S., Santos, L., Martins, J. C., Garcia, A. L., Montes., M. A., et al. (2022a). Evaluation of population parameters of seahorses in areas with and without tourism in federal marine protected areas in Northeast Brazil. *Biodiversidade Bras.* 12 (4), 1–18. doi: 10.37002/biobrasil.v12i4.2278

Silveira, R. B., Souza, F. B., Santos, E. P., and de Alcântara Santos, A. C. (2022b). Feeding ecology of seahorses (Syngnathidae: Hippocampus) on the coast of Rio de Janeiro state, Brazil. *Regional Stud. Mar. Sci.* 56 (2022), 744–755. doi: 10.1016/j.rsma.2022.102692

Soetaert, M., Chiers, K., Duchateau, L., Polet, H., Verschueren, B., and Decostere, A. (2015). Determining the safety range of electrical pulses for two benthic invertebrates: brown shrimp (*Crangon crangon* L.) and ragworm (*Alitta virens* S.). *ICES J. Mar. Sci.* 72, 973–980. doi: 10.1093/icesjms/fsu176

Uhlmann, S. S., Theunynck, R., Ampe, B., Desender, M., Soetaert, M., and Depestele, J. (2016). Injury, reflex impairment, and survival of beam-trawled flatfish. *ICES J. Mar. Sci.* 73, 1244–1254. doi: 10.1093/icesjms/fsv252

Vaidyanathan, T., Zhang, X., Balakrishnan, R., and Vincent, A. (2021). Catch and trade bans for seahorses can be negated by non-selective fisheries. *Aquat. Conserv. Mar. Freshw. Ecosyst.* 31, 43–59. doi: 10.1002/aqc.3419

Viana, D. (2020). *Efeitos subletais, mortalidade e sustentabilidade da captura incidental de peixes na pesca de camarão no litoral do Paraná* (Curitiba: Universidade Federal do Paraná).

Vianna, M., and Almeida, T. (2005). Bony fish bycatch in the southern Brazil pink shrimp (*Farfantepenaeus brasiliensis* and *F. paulensis*) fishery. *Braz. Arch. Biol. Technol.* 48, 611–623. doi: 10.1590/S1516-89132005000500014

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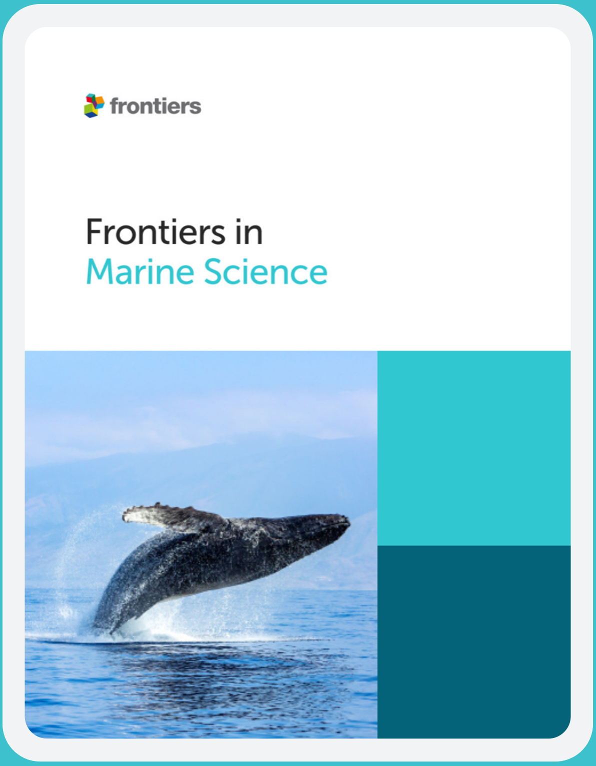

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