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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,

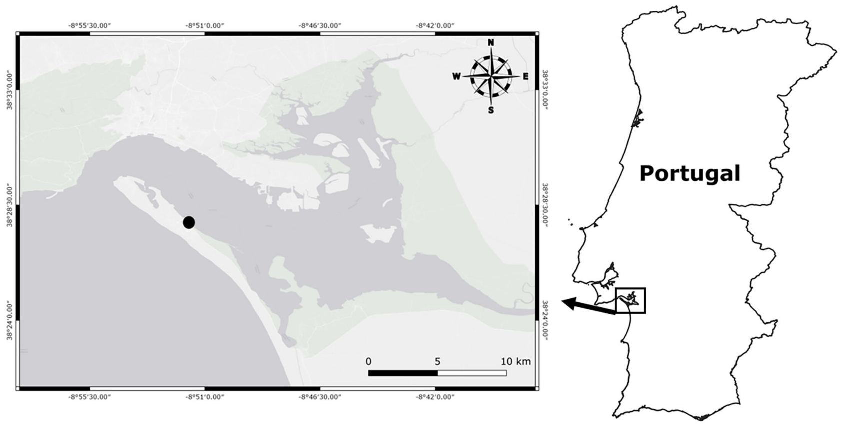

FIGURE 1
Field site location.

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).

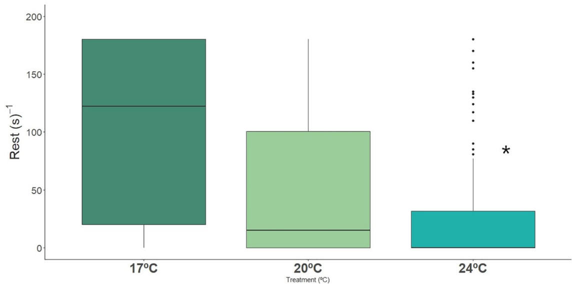

FIGURE 2
Time spent in stationary behavior (s^{-1}) by long-snouted seahorse exposed to different temperature treatments (17°C, 20°C and 24°C). Bold * represent significant differences from control treatment.

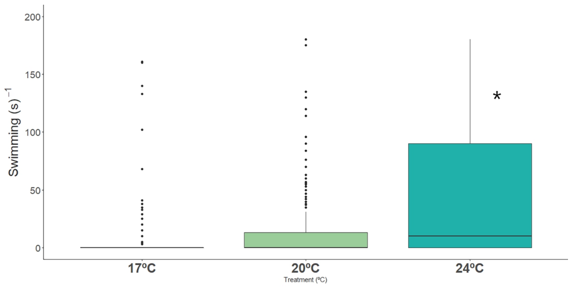

FIGURE 3
Time spent swimming (s^{-1}) by long-snouted seahorse exposed to different temperature treatments (17°C, 20°C and 24°C). Bold * represent significant differences from control treatment.

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.

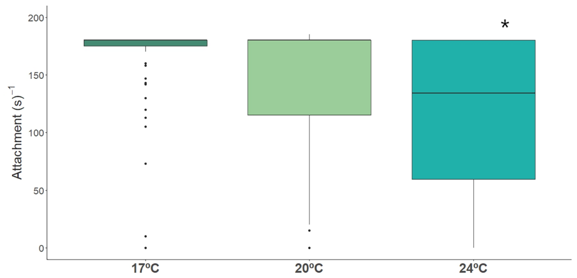

FIGURE 4
Time spent attached (s^{-1}) by long-snouted seahorse exposed to different temperature treatments (17°C, 20°C and 24°C). Bold * represent significant differences from control treatment.

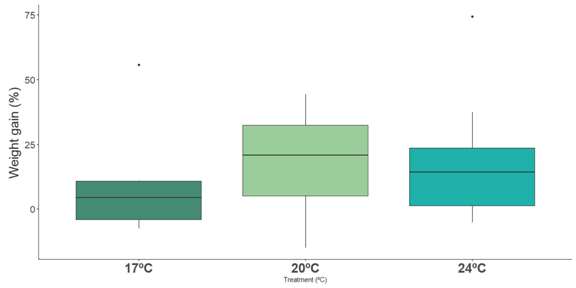

FIGURE 6
Impact of ocean warming on weight gain (%) of long-snouted seahorse exposed to different temperature treatments (17°C, 20°C and 24°C).

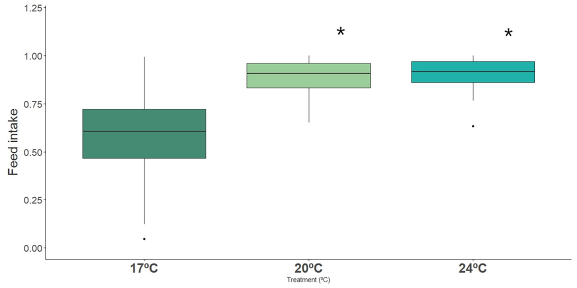

FIGURE 5
Impact of ocean warming on the feed intake of long-snouted seahorse exposed to different temperature treatments (17°C, 20°C and 24°C). Bold * represent significant differences from control treatment.

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

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