UNDERSTANDING THE IMPACT AND INVASION SUCCESS OF AQUATIC NON-NATIVE SPECIES: HOW THEY INTERACT WITH NOVEL ENVIRONMENTS AND NATIVE BIOTA

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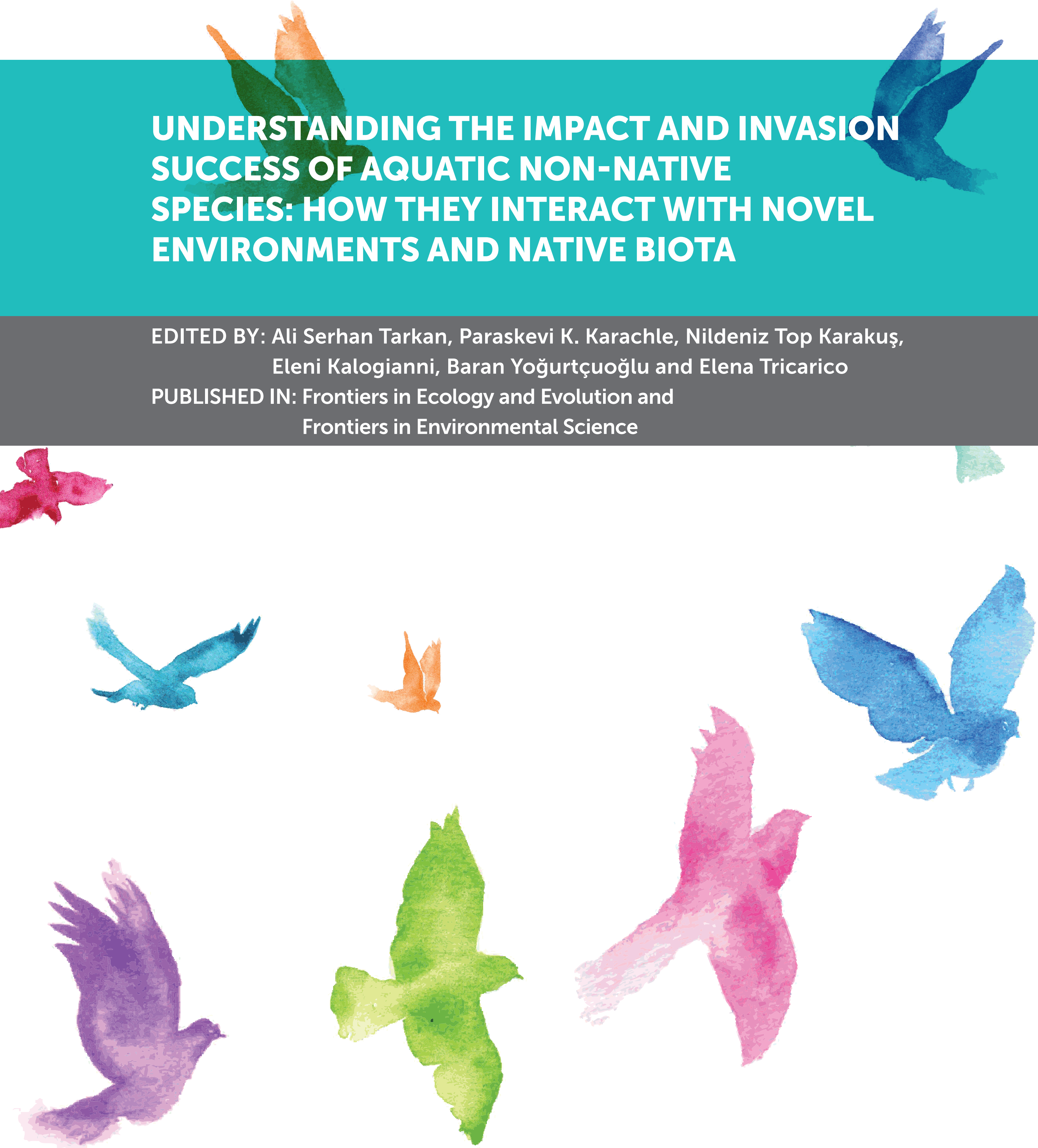

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EDITORIAL
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Editorial: Understanding the Impact and Invasion Success of Aquatic Non-native Species: How They Interact With Novel Environments and Native Biota

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Editorial on the Research Topic

Understanding the Impact and Invasion Success of Aquatic Non-native Species: How They Interact With Novel Environments and Native Biota

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INTRODUCTION

The introduction of non-native aquatic species through a wide range of human-induced pathways (e.g., fisheries, fish stocking, aquaculture, sport fishing, shipping, ornamental and aquarium fish trade, opening new inter-sea channels) is considered as a main driver of biodiversity loss and ecosystem services downgrading in aquatic ecosystems all over the world (García-Berthou et al., 2005; Katsanevakis et al., 2014; Havel et al., 2015). The current extent, frequency, and the rate of introduction of invasive species have dramatically increased in the recent years due to the higher mobilization and expanding demands in world-wide trade activities. While the spread of a species beyond its native range could be a natural process, proliferated intensity of human intervention in exploiting new ecosystems along with the effects of global climate change has long been thought to cause the increasing frequency of global invasions and range expansions of non-native species (Simberloff et al., 2013; Vilizzi et al., 2021).

It is a generally disregarded fact that not all non-native species are a threat to their hosting ecosystems. However, we should note those that may become invasive. The term "invasive" has been described in various ways but should refer to the non-native species that have proved to cause detrimental impacts on biodiversity and ecosystem services (Rejmánek, 2011). To understand whether a non-native species exhibits invasive characteristics, a wider set of detailed information on its specific impacts through field and experimental studies is needed. This is even more demanding for some invasive aquatic species that should be assessed on multiple scales. The other species present in the invaded environment, regardless of being native or non-native, are also expected to play a key role in the magnitude of the impacts of a new invader. Therefore, one of the most complex issues is to predict the ways in which introduced species can influence native communities under the diverse and stochastic nature of interactions within the environment that is novel to the invader

(Blossey, 2011), and how they can interact with other non-native species originating from different areas. Hence, standardized methods to assess impacts are rather hard to be developed as data on specific effects of invasive species are barely available and the nature of these impacts is variable. In this regard, it is essential to take up evidence-based science to drive better-informed policy and economic models so as to provide a more sustainable balance between ecosystem services and the conservation/protection of native/endemic species and fragile ecosystems.

To address these issues, our Research Topic titled "Understanding the Impact and Invasion Success of Aquatic Non-native Species: How They Interact with Novel Environments and Native Biota," compiled a series of research studies providing new data and approaches in assessing the invasion success and the impacts of various non-native species in the different regions of the world belonging to various groups of organisms and from various environments. A range of invasion ecology specialists endeavored to provide up-to-date information on the multifaceted issues of non-native species introductions and to map conservation priorities in terms of biological invasion. A total of 10 articles, including original pieces of research, a review, a brief research report and a hypothesis and theory, are included as part of this Research Topic. Below, an overview of these articles is provided.

RESEARCH TOPIC OVERVIEW

While the articles in this Research Topic can be roughly grouped based on their primary focus on the type of the organisms or environments, we follow an organization of the showcase with topics ranging from studies on single species to communities and, finally, to theoretical and modeling ones.

Atalah et al. quantified the ecological impacts of the Mediterranean fanworm (*Sabella spallanzanii*), an undesired invasive species in New Zealand, by investigating the diversity and abundance of benthic communities using a manipulative field experiment. Their research revealed compositional differences of benthic communities when exposed to *S. spallanzanii*. They concluded that the negative impacts have the potential to reach up to functioning of soft-sediment habitats through alterations to nutrient cycling, bioturbation, and benthic-pelagic coupling. Rolla et al. (1) assessed the extent of spatiotemporal variation in the growth and settlement rates of zebra mussel (*Dreissenapolymorpha*) in a recently colonized artificial lake area in the UK, aiming to better understand its establishment to propose more efficient management options. For control measures, they suggested that removal of mussels in deep waters might be beneficial during the summer and early autumn but depending on local conditions due to the existence of contrasting density-dependent mechanisms. Rolla et al. (2) investigated the variation in the trophic ecology of top mouth gudgeon (*Pseudorasbora parva*), the most invasive freshwater fish in Europe, in four contrasting freshwater habitats in South Wales, using stable isotope data and stomach content data as a complementary tool. Their study revealed great variation in diet and trophic position between neighboring waters even only a few kilometers apart. They also found higher diversity in the diet and a more generalist pattern in ponds containing fewer competitors, on the other hand, a poor condition and low-trophic position in the ponds with other cyprinids. Hudson et al. reviewed the genetic, genomic, and phenotypic pieces of research on three-spine stickleback (*Gasterosteus aculeatus*) in Central Europe, focusing on Switzerland and the invasion of the Lake Constance region. In the Lake Constance region, they found rapid phenotypic and genetic divergence between a lake population and some stream populations; and, also, considerable phenotypic variation within the lake population itself, alongside their attempt to document and discuss the complex colonization history, and trace the invasion pathways of stickleback. Haubrock et al. investigated the trophic interactions of the invasive alien species in the Arno River (Tuscany, Italy) using stable isotopes and dietary analyses. Their results suggested that species sharing close geographic origin (or shared histories of co-evolution) would exhibit a lower potential to compete than species from different origins, which affect their potential impact on native species. Jurlina et al. addressed the potential interactions between the native and alien trout groups (*Salmo* spp.) within the western part of the Balkans. Their study focused on the alternative life histories (e.g., occurrence of migratory behavior), cross-breeding, and introgression between the alien and native populations. Their results showed the occurrence of cross-breeding and introgression of genes between some of the alien and native populations. They concluded that migratory behavior might have two contrary consequences; i.e., it provides native trout stocks an alternative way to cope with the alien strains and/or species introduced into their home streams but also enables non-native brown trout to intrude into the recipient streams and introgress into their resident trout stocks. McCarthy et al. dealt with one of the poorly studied impacts of non-native species, i.e., their roles in changing biogeochemical processes, such as the emission of greenhouse gases (GHGs). They compared emissions of nitrous oxide (N_2O), methane (CH_4), and carbon dioxide (CO_2) produced by a native (*Crassostrea virginica*) and non-native (*Ostrea edulis*) oyster species. They briefly reported that the non-native oyster is a lower GHG emitter than the native one and emphasize that, at least in terms of GHG emissions, this non-native species introduction may not be detrimental to the environment. Their results also showed that GHG fluxes and chlorophyll-a consumption rates were not driven by a common set of environmental parameters, nor did fluxes vary consistently with oyster characteristics. South et al. evaluated the competitive interaction indirectly by comparing the closing force of two invasive crayfish species (*Cheraxquadricarinatus* and *Procambarusclarkii*) with a native analogous freshwater crab (*Potamonautesperlatus*). Their results suggested that the native crab had the capacity to hold a competitive mechanical advantage, a kind of biotic resistance toward both invaders, but that this varies with sex.

Finally, two articles contributed by their modeling approaches. Copp and Fox took up a critical perspective and reviewed their own model developed in 2007. The model is applied to non-native populations of pumpkinseed (*Lepomisgibbosus*) in nine countries of Europe and western

Asia by aiming to estimate the efficiency of the model for predicting the potential invasiveness of non-native freshwater fish populations. Their model was not strongly supported in their tests with the pumpkinseed likely due to the shortcomings in the dataset they used, as well as the uncertainty of the source populations. Yet, they concluded that, as long as the size and the quality of the life-history database are sufficient, such life-history models can be useful for predicting invasiveness status in non-native freshwater fishes. Yogurtçuoglu et al. addressed the establishment and invasiveness potential of the rainbow trout (*Oncorhynchus mykiss*) in Turkey, where it is the most cultured non-native fish species. They proposed an integrated approach in which habitat suitability and invasiveness risk assessment are used together to identify its geographic overlap probability with the native salmonid species of conservation concern. They further proposed a metric by blending the benefited risk assessment approach with the spatial analysis of the native trout species to prioritize and categorize the sensitivity of native salmonids against the overlap and interaction (including hybridization) with rainbow trout. Their results suggested that the northern region is the most suitable area for the rainbow trout, and *Salmo abanticus*, one of the endangered native trout, had the highest vulnerability and priority in conservation sense.

CONCLUSIONS

Conservationists are expected to elucidate the relations between the level and nature of propagule pressure from non-native species introductions and its potential ecological impacts on biodiversity. However, demonstrating ecological impact resulting from the introduction of non-native species is inherently difficult, as the impact can also be indirect through, for instance, the introduction of infectious agents, sexual pheromone pollution, or social network. This is also true since the long (lag) phase may pass before these impacts become apparent. Consequently, it may be highly difficult to foresee ecological impacts before they are eventually identified. This represents one of the most challenging aspects of invasion biology, which requires experimental design procedures to undertake large-scale replicated experiments. Such experiments should be set up using the latest innovative tools within a population level or, preferably, a community/ecosystem-level approach (e.g., involving dynamic food web modeling) to discriminate yet unseen non-native ecological impacts. The collection in this Research Topic is believed to serve this goal, providing state-of-the art experimental designs and risk assessment tools and models to predict impact of non-native species.

AUTHOR CONTRIBUTIONS

All the authors co-edited the Research Topic and approved the final version of editorial.

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Shared Histories of Co-evolution May Affect Trophic Interactions in a Freshwater Community Dominated by Alien Species

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Interactions occurring between species in multiple invaded freshwater ecosystems are often difficult to observe and study. Studies on invasive alien species typically focus on single species, and, when the species community is exclusively composed of alien species, their interactions, eventual facilitation and hindering processes are very scarcely assessed. To investigate such a community, the species community in the Arno River (Tuscany, Italy) was examined as a model system using the combined approach of stable isotopes and dietary analyses. Established alien species have formed a pyramid shaped community with the European catfish *Silurus glanis* and the North American channel catfish *Ictalurus punctatus* in the apex position, followed by opportunistic predatory (*Lepomis gibbosus*) and omnivore (*Alburnus alburnus*, *Cyprinus carpio*, *Barbus barbus, Pseudorasbora parva*, *Padogobius* sp., *Tinca tinca*) species. These species were observed to feed on a variety of primary producers (*Myriophyllum* sp., *Potamogeton* sp., *Phragmites australis*) and consumers (aquatic insects, molluscs, *Dikerogammarus villosus*, *Procambarus clarkii*, *Palaemon antennarius*, tadpoles). Remarkably, the channel catfish *Ictalurus punctatus* showed an ontogenetic niche shift, with juveniles occupying a slightly higher trophic position than mature individuals. *Pseudorasbora parva* and *A. alburnus* showed a strong niche overlap, with the former having a wider niche. Such wide niches were also found for the invasive crustaceans *D. villosus* and *P. clarkii.* Outgoing from our findings, we suggest that life-history and geographic origin play a role in determining competition and interaction type among alien species, with species from the same geographic area showing a lower potential to compete than species from different areas, hence affecting their potential impact on native species.

Keywords: stable isotopes, stomach contents, community structure, mixing models, feeding ecology, interactions

INTRODUCTION

The invasive potential as well as distribution of an alien species depend on various abiotic and biotic factors such as its life-history (Kolar and Lodge, 2001; Keller et al., 2007; Strayer, 2010; Blackburn et al., 2011), predator-prey dynamics, adaptability as well as interactions with other species (Ricciardi, 2003; Strayer et al., 2006; Ricciardi et al., 2013). Subsequent, the introduction and successive dispersal of alien species change the identity of entire species communities (Seebens et al., 2017). When multiple invasive alien species are dominating one freshwater ecosystem, they tend to occupy different trophic levels (Gamradt and Kats, 1996; Kiesecker and Blaustein, 1998), but the presence of invasive species interacting across trophic levels increases the already-existing complex threat through direct and indirect effects (Peckarsky and McIntosh, 1998; Huxel et al., 2002; Strayer, 2010), as the prevalence of omnivores (Pringle and Hamazaki, 1998), and interactions among predators (Sih et al., 1998) are unforeseeable. However, such complex interactions among invasive species are not well understood (Parker et al., 1999; Huxel et al., 2002; Strayer, 2010) but, as described in the "invasional meltdown theory," can enhance the chance of a species to successfully establish itself and increase or add further impacts on the recipient environment (Simberloff and Von Holle, 1999; Simberloff, 2006). Otherwise, these interactions can lower the impact of predatory invasive species on native species in certain cases (Soluk and Collins, 1988a,b; Soluk, 1993; Rosenheim, 1998; Bissattini et al., 2018). Despite the utmost importance of such studies on multiple invaded ecosystems for practical management and control efforts (Polis and Strong, 1996; Rosenheim, 1998), they are still scarce (but see Johnson et al., 2009; Gherardi et al., 2011; Haubrock et al., 2019a).

The use of Stable Isotopes Analyses (SIA) enables the study of invasive species and their interactions with native biota (Vander Zanden et al., 1999; Balzani et al., 2016). In particular, SIA proved to be a useful tool in investigating the impacts of alien fish (Cucherousset et al., 2012; Britton et al., 2018). Using SIA for carbon (C) and nitrogen (N), long-term and time-mediated information of a community trophic structure can be obtained (Boecklen et al., 2011; Layman et al., 2012; Middelburg, 2014), trophic levels can be estimated (Post, 2002), and feeding ecological niches finely quantified (Newsome et al., 2007). While C signatures identify the major energy sources, N signatures relate to the trophic position of a consumer within a food web (Fry, 2006; Layman et al., 2012). This relationship relies on predictable changes in the isotopic signal from prey to consumer, being enriched by 1‰ for C and by 2.5-5‰ for N between consecutive trophic levels (Post, 2002; Vanderklift and Ponsard, 2003). Combined with the analysis of dietary contents (DA), which provide a direct but short temporal insight into the feeding habit of a species, relationships among species can be investigated with a considerably higher resolution.

Aquatic ecosystems, especially in the Mediterranean areas, have been subjected to successive invasions (Dumont et al., 2004; Marr et al., 2010). For instance, Italian freshwaters, with an ichthyofauna composed of 48 native and 41 established introduced and further 15 introduced but not yet established species, have been imperiled by invasions (Nocita et al., 2017). Many of these species are notoriously invasive, like the European catfish *Silurus glanis* (Copp et al., 2007, 2009; Boulêtreau et al., 2018), which has become the object of various management efforts (Gualtieri and Mecatti, 2005; Cucherousset et al., 2018), while others like the channel catfish *Ictalurus punctatus* have received minor attention, with only few recent studies addressing its potential impacts (Haubrock et al., 2018a,b). In the Arno River in Tuscany, species introductions occurred repeatedly, as in the case of *Blicca bjoerkna*, *Rhodeus sericeus*, *I. punctatus*, and *S. glanis* (Nocita, 2002, 2007), that are now established (Nocita and Zerunian, 2007). Moreover, anthropogenic effects combined with introductions have caused the local extinction of native predators (e.g., *Esox lucius*; Nocita and Zerunian, 2007). Therefore, the Arno River is a peculiar ecosystem, because almost no native fish species is currently present (Nocita, 2002), and it is thus characterized by unique species composition, with so far unknown interactions and diverse introduction histories. Moreover, several of the species present in the Arno river (e.g., *P. clarkii*, *S. glanis*, *D. villosus*, *G. chinensis*, *P. antipodarum*, *P. parva*, *G. holbrooki*; Gherardi et al., 2008; Nocita and Lenuzza, 2016) are listed among the worst invasive species and other comparable lists of high-profile invasive species (Vilà et al., 2009; Nentwig et al., 2018). It can be considered as a unique melting pot for alien species from Asia and North America, mainly voluntarily introduced for sport fishing and commercial fishing purposes (Nocita, 2002; Vejrík et al., 2019). Consequently, manifold interactions that can only be observed in such an invaded range are of great importance from both a theoretic and management point of view.

We, thus, hypothesize that species interactions among and across trophic levels and, especially top-down impacts, are affected not only by differing life-histories but also a shared history of co-evolution of the invading species, with invasive alien species coming from different areas being more likely to compete due to the lack of the latter.

Hence, the aims of this work were to: (i) combine SIA and DA to investigate feeding preferences and impacts of alien species in the investigated community mainly composed by alien species; (ii) compare species' trophic niches and niche overlap to infer the potential degree of feeding competition among species; (iii) unravel important functional roles of organisms (as pointed out by (Bissattini and Vignoli, 2017) for *P. clarkii*, linking terrestrial and aquatic energy sources), and (iv) discuss results in regard to invasive species' origins as a potential factor determining the outcome of species interactions.

MATERIALS AND METHODS

Study Site and Sampling

The Arno River is with a length of 241 km and a drainage of more than 8.200 km² as well as an effluent flow of 100 m²/s the second most important river in Central Italy (Nocita and Zerunian, 2007). Its discharge regime is typical of Mediterranean rivers showing extreme flow reductions during summer. It flows through the major cities of Florence and Pisa and is especially within Florence divided by various weirs and characterized by a high density of non-native aquatic vegetation during spring and early summer. During summer, the water of the Arno River in the inner-city section of Florence can reach mean temperatures of 29.6°C (min: 27.2, max: 32.4; data from 2016 to 2018 http://www.arpat.toscana.it). The historic use and cultural value have led to high anthropogenic stress on communities. Indeed, the aquatic community consists of various alien species while only a minority of them is considered native (Nocita, 2002). However, a detailed list of alien species has not been published so far (pers. comm. Gianna Innocenti).

Sampling was conducted from April to June 2018, in a very anthropogenically disturbed part of the inner-city (Florence) section of the Arno River which is highly used for recreational angling (43.765606, 11.268234; DATUM WGS84). The specific period was chosen, as these months are the ideal activity period for several fish species (samples were taken approximately 2 month after the inactive winter period, thus after species had resumed their feeding activity). Moreover, the effect of angling as a stressor that could interfere with the species trophic positioning through the input of "unusual food sources" is limited due to low angling activity (pers. Comm, Thomas Bussatto). To collect the several species, a combined approach of several methods was applied. Fish were caught with standard fishing rods and the help of local fishermen using a variety of baits and fishing techniques. Macroinvertebrates and frog tadpoles were collected using funnel traps and hand nets. Sampled species were collected under the consideration of an even spatial distribution across the sampled stretch of the river ecosystem without bias toward either section, end or distance from riverbank (Willson et al., 2010). Samples of the local aquatic vegetation were also taken as they represent the baseline of the ecosystem. Sampled specimens were immediately euthanized (fish: gills cutting; macroinvertebrates: freezing), stored on ice during transport and then preserved in the freezer at −20°C until further processing. Overall, 232 specimens belonging to nine fish species, 71 invertebrates from at least six species, four green frog tadpoles, and thirteen samples from three different plant species (two hydrophytes: *Potamogeton* sp. and *Myriophyllum* sp., and one helophyte: *Phragmites australis*) were collected. Due to the rarity of both *Tinca tinca* and *Barbus barbus* in the study site, only one sample of each was caught and included in the dataset to roughly indicate these species positioning in the community. However, these samples were not used for any further statistical analysis. The sampling was authorized by the Tuscan Region ("Autorizzazione alla pesca scientifica, Regione Toscana").

Sample Preparation and Analyses

For all fish species, total length (TL) was measured to infer age, as fish growth is generally continuous, presenting a link between size and age (Hopkins, 1992). Resultant, over TL; the age can be inferred and used to identify fish maturity, which can in turn be used to investigate behavioral differences (Haubrock et al., 2019b). Additionally, weight (W), and, if distinguishable, also sex were recorded. TL of fish and cephalothorax length (CTL) of crayfish were measured with a caliper and W was measured using an electronic balance. In the laboratory, stomach contents were extracted, and prey items identified to the lowest possible taxon under a stereomicroscope (Sansoni, 1998; Schultz, 2010). We estimated number of stomachs containing each food item in relation to total number of full stomachs (frequency of occurrence, F%) and number of individuals of each food item with respect to the total number of individuals (number of occurrence, N%) for each food item in each respective species (Haubrock et al., 2019b). Despite the information DA provide on the direct feeding activity, they are frequently used to supplement stable isotope analyses as priors due to their often differing results (Pacioglu et al., 2019). However, from all species analyzed, several had empty stomachs (*S. glanis*: 20/39; *A. alburnus*: 2/10; *P. parva*: 4/10; *L. gibbosus*: 7/20). For isotopic analyses, muscle tissue was cleaned of fat, skin, scales, bones and carapace (for crayfish). For plants, different leaves were cut-off from the same individual and pooled in one sample to account for eventual differences. Due to their small size, insects, little crustaceans and tadpoles were treated as whole samples while, for molluscs, only the soft body tissue was analyzed without the shell. Samples were placed on separated glass trays and dried for 48 h at 60°C. Once dry, all samples were grinded into fine powder with an agate mortar and pestle. For each sample, two replicates of 0.20–0.30 mg for animals and 1.00–1.10 mg for plants, were weighed on a Mettler Toledo AG245 microscale and enclosed in a tin capsule to be analyzed in continuous flow—isotope ratio mass spectrometry (CF-IRMS) with a Thermo FlashEA 1112 elemental analyser and a Thermo Finnigan Delta Plus isotope ratio mass spectrometer at the Istituto di Geologia Ambientale e Geoingegneria (IGAG) of the National Research Council (CNR) in Montelibretti, Rome. Isotope compositions were expressed as ‰ with the δ notation (based on $\delta^{13}C$ or $\delta^{15}N = [(R_{sample}/R_{standard}) - 1) \times 1000$, where R is $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratios]. Results were referred to Vienna Pee Dee Belemnite for carbon and to atmospheric N_2 for nitrogen, with laboratory standards calibrated on IAEA international standards: N1, N2, and USGS25 for nitrogen and CH6, CH7, and USGS24 for carbon.

Statistical Analyses

The arithmetic mean ± SE was calculated, and eventual correlations between $\delta^{15}N$ and $\delta^{13}C$ values and TL, CTL and W measures were tested for each species using a linear model. The trophic position (TP) of each species was estimated using the R package "tRophicPosition," which incorporates a Bayesian model utilizing up to two baselines (Quezada-Romegialli et al., 2018). Hence, we included the combined plants (*Myriophyllum* sp., *Potamogeton* sp., *Phragmites australis*) as first, and molluscs (*Gyraulus chinensis*) as second baseline (n.adapt = 100,000; n.iter = 100,000; burnin = 10,000; n.chains = 5). These two groups were chosen due to the wide diet preferences known for alien fish species (Musil and Adámek, 2007; Almeida et al., 2009; Xu et al., 2011; Ribeiro and Leunda, 2012; Haubrock et al., 2019a,b).

To quantify intra-specific and community niche width, Layman metrics (Layman et al., 2007) were calculated with the R package "SIAR" (Stable Isotope Analysis in R; Parnell et al., 2010). Additionally, the corrected standard ellipse areas (SEAc, considering 40% of data plots) and SEAb (Bayesian standard ellipse areas, considering 95% of data plots) were calculated for all fish species with $n \geq 5$ as well as *Procambarus clarkii* using

TABLE 1 | Frequency of occurrence (%F), abundance (%N), and prominence value (PV) of food items analyzed for juvenile and mature *Ictalurus punctatus*, juvenile *Silurus glanis*.

Prey items	*Ictalurus punctatus* (Immature)		*Ictalurus punctatus* (Mature)		*Ictalurus punctatus* (Total)		*Silurus glanis* (Immature)		*Lepomis gibbosus*		*Pseudorasbora parva*		*Alburnus alburnus*	
	[F%]	[N%]	[F%]	[N%]	[F%]	[N%]	[F%]	[N%]	[F%]	[N%]	[F%]	[N%]	[F%]	[N%]
Detritus	0.30	0.08	0.25	0.04	0.30	0.06	0.10	0.09	0.00	0.00	0.00	0.00	0.00	0.00
Plants	0.45	0.09	0.60	0.14	0.45	0.12	0.13	0.11	0.00	0.00	0.10	0.50	1.00	1.00
Detergent	0.20	0.08	0.05	0.01	0.20	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Palaemon antennarius	0.25	0.18	0.15	0.04	0.25	0.10	0.03	0.02	0.05	0.01	0.05	0.25	0.00	0.00
Dikerogammarus villosus	0.18	0.14	0.20	0.03	0.18	0.08	0.05	0.07	0.13	0.03	0.00	0.00	0.00	0.00
Procambarus clarkii	0.10	0.01	0.15	0.03	0.10	0.02	0.00	0.00	0.13	0.03	0.00	0.00	0.00	0.00
Unid. Crustaceans	0.08	0.14	0.00	0.00	0.08	0.06	0.03	0.02	0.00	0.00	0.00	0.00	0.00	0.00
Terr. Insects	0.43	0.16	0.45	0.29	0.43	0.23	0.00	0.00	0.00	0.00	0.05	0.25	0.00	0.00
Aq. Insects	0.13	0.00	0.25	0.06	0.13	0.03	0.03	0.02	0.75	0.90	0.00	0.00	0.00	0.00
Hirudinea	0.03	0.00	0.05	0.01	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fish larvae	0.08	0.02	0.05	0.01	0.08	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Unid. Fish	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.02	0.25	0.05	0.00	0.00	0.00	0.00
Small cyprinids	0.25	0.05	0.30	0.08	0.25	0.06	0.18	0.16	0.00	0.00	0.00	0.00	0.00	0.00
Large cyprinids	0.03	0.00	0.05	0.01	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ictalurus punctatus	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.09	0.00	0.00	0.00	0.00	0.00	0.00
Molluscs	0.05	0.00	0.10	0.04	0.05	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Small mammals	0.03	0.00	0.05	0.01	0.03	0.00	0.03	0.02	0.00	0.00	0.00	0.00	0.00	0.00
Small birds	0.08	0.01	0.10	0.02	0.08	0.01	0.03	0.02	0.00	0.00	0.00	0.00	0.00	0.00
Testudines	0.03	0.00	0.05	0.01	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fishing baits	0.23	0.03	0.35	0.17	0.23	0.11	0.03	0.34	0.00	0.00	0.00	0.00	0.00	0.00

Lepomis gibbosus, Pseudorasbora parva, and Alburnus alburnus; "0.00" indicates that the specific prey items in the fish's diet were considerably low; "na" indicates that respective prey item was not present.

the R package "SIBER" (Stable Isotope Bayesian Ellipses in R; Jackson et al., 2011).

The application of scatter plot for δ^{15}N vs. δ^{13}C of consumer tissues and food sources enabled the determination of probable prey sources and combinations of prey contributing to the diet of predators (Phillips and Gregg, 2003). Dietary analyses (DA) for catfish species (juvenile and mature *I. punctatus* as well as *S. glanis*) were previously performed (Haubrock et al., 2019b), while DA for *Lepomis gibbosus*, *Pseudorasbora parva*, and *Alburnus alburnus* were conducted following the same protocol (**Table 1**). Stable isotope mixing models (SIMM; R package "SIMMr") were applied for all fish and crayfish without consideration of priors but considering potentially predated items, i.e., a predation on lower trophic levels. Thus, it was investigated how sampled species contribute to the isotopic signatures of each other, indicating the scenario with the highest probability (Parnell et al., 2013). Additionally, the same analyses were performed again for *L. gibbosus*, both life-stages of *I. punctatus* and *S. glanis* with results from DA as priors for the analyses (i.e., considering: the five most consumed prey items and then their number of occurrence N%; 4 prey items were used for *L. gibbosus*; **Table 1**). Priors from DA were not included into the mixing models for *P. parva* nor *A. alburnus* due to the low number of stomachs available (n = 10 for either species) of which the majority was empty (*P. parva* = 6; *A. alburnus* = 8). Prior based analyses were conducted with the R package "SIAR" (Parnell and Jackson, 2013). Results are presented as the average percent values with the possible range percentage for each prey item. With these, the trophic web of the studied ecosystem was reconstructed. Furthermore, using the software PRIMER (Clarke and Warwick, 2001) a Multi-Dimensional Scaling plot (MDS) was drawn and a Permutational Analysis of Variance (PERMANOVA; Resemblance: Euclidean distance; Sums of squares type: Type III (partial); Fixed effects sum to zero for mixed terms; Permutation method: Unrestricted permutation of raw data; Levels: 15; Number of permutations: 9999) was performed to test for significant differences in δ^{15}N and δ^{13}C among species and to eventually determine niche overlaps. Lastly, we compared the results among species to discuss the effect of an alien species origin in respect to indicated trophic interactions.

RESULTS

Overall, 9 invasive but no native fish species were sampled. There were complimented by 2 invasive and 1 native crustacean as well as 1 invasive mollusc. Additionally, 2 native insect, 1 native amphibian and 3 invasive plant species were collected (**Table 2**).

From these, some species were under-sampled due to their rarity (i.e., the locally alien species *Tinca tinca* and *Barbus barbus*) or status as endangered (i.e., *Padogobius* sp.), while only a minimum of *Cyprinus carpio* and no mature *S. glanis* (>80 cm) individuals were sampled to meet the demands of local anglers. Additionally, the alien omnivore *Squalius cephalus*, which is believed to be rare or locally extinct, was not sampled. The abundant invasive pond slider *Trachemys scripta* as well as the occasionally found alligator snapping turtle *Macrochelys temminckii* were not sampled as the sampling method was not suited to collect them. Moreover, only few macroinvertebrates were found and collected. Detailed results from DA are listed in **Table 1** and have been discussed previously for *I. punctatus* and juvenile *S. glanis* (Haubrock et al., 2019b). While the number of stomach contents analyzed for *P. parva* and *A. alburnus* were too low to be analyzed in depth or to be used as prior for subsequent mixing models, *L. gibbosus* showed a primary feeding preference for aquatic insects and secondarily for crustaceans (**Table 1**).

TABLE 2 | Morphological measures (mean ± SE), sex ratio (calculated as ratio of females, f. on males, m) and number of all sampled specimens. Length measured as total length (TL) for fishes and as cephalothorax length (CTL) for crayfish; n = number of specimens caught.

Species	Length [cm]	Weight [g]	Sex ratio [f:m]	*n*
Fish				
Silurus glanis	27.1 ± 1.1	122.8 ± 14.4	1:1	39
Ictalurus punctatus	33.8 ± 2.2	538.5 ± 106.3	1:1	40
Cyprinus carpio	31.2 ± 9.9	584.3 ± 506.7	3:1	4
Tinca tinca	9.8	11	–	1
Barbus barbus	17	36	–	1
Padogobius sp.	4.4 ± 0.1	2.3 ± 0.5	–	4
Lepomis gibbosus	9.1 ± 2.9	20.0 ± 16.6	1:1	20
Pseudorasbora parva	8.0 ± 1.3	4.8 ± 2.3	–	16
Alburnus alburnus	9.4 ± 1.0	7.3 ± 2.5	–	20
Crustaceans				
Procambarus clarkii	6.5 ±2.4	26.2 ± 1.7	1:1	10
Palaemon antennarius				16
Dikerogammarus villosus				11
Insects				
Odonata				15
Ephemeroptera				9
Amphibians				
Pelophylax kl. *esculentus* (tadpoles)				4
Molluscs				
Gyraulus chinensis				9
Plants				
Potamogeton sp.				6
Phragmites australis				5
Myriophyllum sp.				2

Statistically significant negative correlations were found between TL and δ^{13}C for *S. glanis* ($r_s = -0.512$; $p = 0.001$; $n = 39$), *I. punctatus* ($r_s = -0.665$; $p < 0.001$; $n = 40$) as well as *P. clarkii* ($r_s = -0.693$; $p = 0.026$; $n = 10$). A statistically significant positive correlation between TL and δ^{13}C were identified for *Pseudorasbora parva* ($r_s = 0.567$; $p = 0.022$; $n = 16$). Additionally, a statistically significant positive correlation between TL and δ^{15}N was found for *L. gibbosus* ($r_s = 0.917$; $p < 0.001$; $n = 20$) (**Supplement 1**).

Following the assumption of an isotopic enrichment of δ^{15}N by about 3.4‰ (Post, 2002), the entire isotopic community spanned over approximately three to four trophic levels (**Figure 1**). Considering as baseline both molluscs and plants

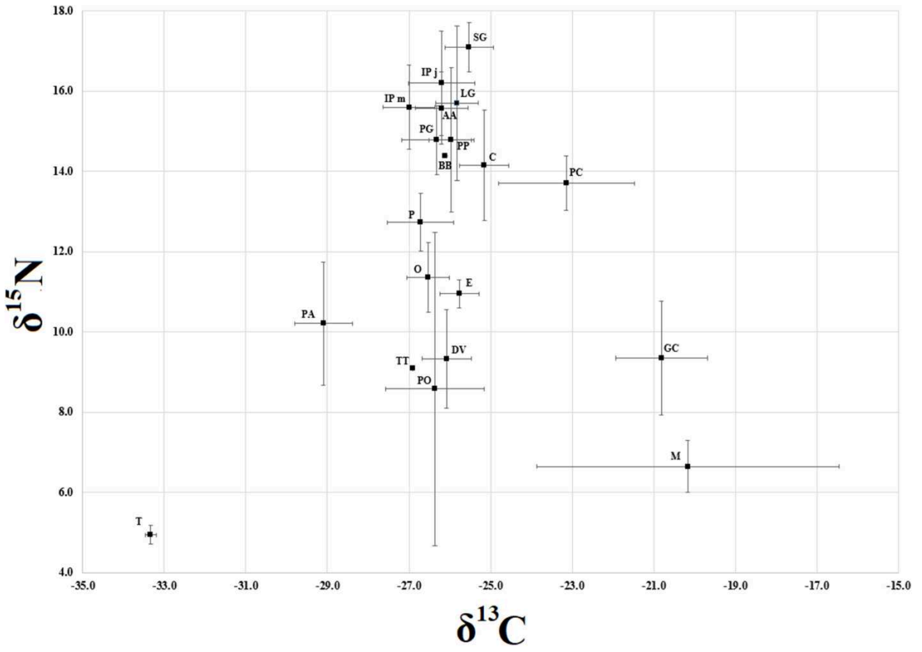

FIGURE 1 | Isotopic space scatterplot of the community. Species IDs: SG, *Silurus glanis*; IPj, *Ictalurus punctatus* (juveniles); IPm, *Ictalurus punctatus* (matures); LG, *Lepomis gibbosus*; AA, *Alburnus alburnus*; PG, *Padogobius* sp.; PP, *Pseudorasbora parva*; BB, *Barbus barbus*; C, *Cyprinus carpio*; PC, *Procambarus clarkii*; P, *Palaemon antennarius*; O, Odonata; E, Ephemeroptera; DV, *Dikerogammarus villosus*; GC, *Gyraulus chinensis*; PA, *Phragmites australis*; T, tadpoles (*Pelophylax* kl. *esculentus*); TT, *Tinca tinca*; M, *Myriophyllum* sp.; PO, *Potamogeton* sp. Each species is represented as mean (centroid), with bars representing standard deviation.

provided a well-structured hierarchy. The 4th apex level was constituted by *S. glanis* and both life stages of *I. punctatus*; the 3rd level by *C. carpio*, *B. barbus*, *Padogobius* sp., *L. gibbosus*, *P. parva*, *A. alburnus*, *P. clarkii*, and *P. antennarius*; the 2nd level by *T. tinca*, *D. villosus*, tadpoles, Odonata, and Ephemeroptera larvae.

Layman's metrics are shown in **Table 3**. Among fish species, the greatest N range (NR) was found for *P. parva* (7.51), followed by *L. gibbosus* (6.38) and adults of *I. punctatus* (4.60), while the narrowest values were presented by *Padogobius* sp. (1.79) and *S. glanis* (2.71). C range (CR) was largest for *A. alburnus* (3.24) and smallest for *C. carpio* (1.32). Total Area (TA, i.e., the convex hull area enclosing all individual points) was highest in *P. parva* (9.64), followed by *I. punctatus* juveniles (8.76) and *L. gibbosus* (7.81), and was narrowest in *Padogobius* sp. (0.13) and *C. carpio* (1.53). SEAc, which is less influenced by extreme values than TA, was highest in *L. gibbosus* (3.33), immediately followed by *P. parva* (3.27) then *I. punctatus* juveniles (2.20); the lowest values of SEAc were found for *Padogobius* sp. (0.17), *S. glanis* (0.87) and *C. carpio* (1.72). However, the small metrics for *Padogobius* sp. and *C. carpio* have to be considered with caution, due to the small number of collected individuals ($n = 4$ for both species). Among crustaceans, *D. villosus* had the highest NR (4.40), followed by *Palaemon* (2.11) and *P. clarkii* (2.07), while CR followed the exactly opposite hierarchy: *P. clarkii* = 3.96, *Palaemon* = 2.95 and *D. villousus* = 1.90. TA was comparable between *P. clarkii* and *D. villosus* (4.60 and 4.49, respectively), while smaller in *Palaemon* (3.38). In contrast, SEAc was highest for *P. clarkii* (3.59), followed by *D. villosus* (2.36), then *Palaemon* (1.39). Finally, tadpoles showed the lowest Layman's metrics of the whole community.

Considerable SEAc overlap was identified between juvenile *I. punctatus* and *A. alburnus* (0.50), matures *I. punctatus* and *L. gibbosus* (0.47), *L. gibbosus* and *P. parva* (0.49), and *A. alburnus* and *P. parva* (1.33). Overlap was generally low among all other fish species. *P. clarkii* showed particularly low overlap values with any fish species. The estimated SEAb overlaps indicated considerable overlap between life stages of *I. punctatus* (0.42), *P. parva* and *L. gibbosus* (0.59), and *A. alburnus* and *P. parva* (0.46) and minor overlap values between all other species. The ellipse overlaps between *A. alburnus* and *P. parva* estimated with SEAb was considerably lower than the SEAc overlap. Lastly, while the identified SEAb overlap was low between mature *I. punctatus* and juvenile *S. glanis* (0.09), juveniles *I. punctatus* and *L. gibbosus* (0.17), SEAc overlap did not indicate any overlap at all. Similarly, low SEAb overlaps between matures *I. punctatus* and *P. parva* (0.12), juvenile *S. glanis* and *A. alburnus* (0.22), and juvenile *S. glanis* and *P. parva* (0.16) were considerably higher than the respective SEAc overlap values, all being near 0 (**Table 4**).

Results of the applied SIMM without priors (**Figure 2**) indicated that both *I. punctatus* juveniles and adults expressed a great reliance on *A. alburnus* with a higher proportion in matures compared to juveniles, which consumed other items as well. *Silurus glanis* SIMM also showed a specialized piscivorous diet, focusing mostly on the consumption of *L. gibbosus*. In

TABLE 3 | Mean values of isotopic signatures, estimated trophic positions, Layman's metrics.

	SPECIES	TP	δ^{15}N (‰)	δ^{13}C (‰)	NR	CR	TA	CD	MNND	SDNND	SEAc	*n*
Fish	*Ictalurus punctatus* (Immature)	4.096	16.2	−26.2	4.12	2.96	8.76	0.94	0.46	0.49	2.20	20
	Ictalurus punctatus (Mature)	4.006	15.6	−27.0	4.60	1.96	5.39	1.06	0.43	0.25	1.84	20
	Silurus glanis (Immature)	4.366	17.1	−25.5	2.71	2.82	4.10	0.70	0.19	0.17	0.87	39
	Cyprinus carpio	3.481	14.1	−25.2	3.11	1.32	1.53	0.93	0.89	0.65	1.72	4
	Tinca tinca[†]	2.267	9.1	−26.9	na	1						
	Barbus barbus[†]	3.501	14.4	−26.1	na	1						
	Padogobius sp.	3.682	14.8	−26.3	1.79	1.76	0.13	0.80	0.45	0.46	0.17	4
	Lepomis gibbosus	3.956	15.7	−25.8	6.38	2.36	7.81	1.67	0.44	0.24	3.33	20
	Pseudorasbora parva	3.700	14.8	−26.0	7.51	2.13	9.64	1.41	0.69	0.71	3.27	16
	Alburnus alburnus	3.925	15.6	−26.2	3.01	3.24	5.27	0.95	0.37	0.29	1.80	20
Crustacean	*Procambarus clarkii*	3.217	13.7	−23.1	2.07	3.96	4.60	1.58	0.52	0.26	3.59	10
	Palaemon antennarius.	3.087	12.7	−26.7	2.11	2.95	3.38	0.93	0.30	0.20	1.39	16
	Dikerogammarus villosus	2.084	9.3	−26.1	4.40	1.90	4.49	1.20	0.51	0.42	2.36	11
Amphibian	*Pelophylax* kl. *esculentus* (tadpoles)	2.056	4.9	−33.3	0.58	0.27	0.05	0.17	0.18	0.13	0.07	4
Insects	Odonata	2.676	11.5	−26.2	2.01	0.89	0.92	0.55	0.36	0.50	0.71	15
	Ephemeroptera	2.578	11.0	−25.8	1.11	1.61	0.87	0.49	0.37	0.14	0.51	9
Molluscs	*Giraulus chinensis*[‡]	na	9.3	−20.9	4.15	3.48	7.67	1.51	0.97	0.34	5.13	9
Plants	*Potamogeton* sp.[‡]	na	8.6	−26.4	11.59	3.21	18.43	2.89	2.58	2.98	18.09	6
	Phragmites australis[‡]	na	10.2	−29.1	3.65	1.62	1.79	1.37	0.92	0.62	2.35	5
	Myriophyllum sp.[†/‡]	na	6.6	−20.2	na	2						
	Whole consumers community	na	12.14	12.53	62.86	3.29	1.53	2.12	25.83	232		

[†] number of samples not sufficient for the estimation of Layman's metrics; [‡] organisms used as baselines.

TP, trophic position.

n, samples number.

NR, δ^{15}N range.

CR, δ^{13}C range.

TA, convex hull area.

CD, mean distance to centroid.

MNND, mean nearest neighbor distance.

SDNND, standard deviation of the nearest neighbor distance.

SEAc, corrected Standard Ellipse Area.

contrast, *L. gibbosus* had a more generalist diet, based almost equally on tadpoles and various invertebrates: Ephemeroptera and Odonata larvae among insects, and *Palaemon* sp., *D. villosus* and the invasive *P. clarkii* among crustaceans. For *P. parva*, the estimated diet consisted mainly of *Palaemon* sp., followed by Ephemeroptera and then by Odonata, *D. villosus* and *P. clarkii*. The diet of *A. alburnus* is similar, but more balanced and with a marked preference for *Palaemon*. Finally, *P. clarkii* showed a preference for molluscs, followed by insects (Ephemeroptera and Odonata), *Palaemon*, plants (*Phragmites*), and tadpoles.

Mixing models refined using dietary priors (**Figure 3**) showed differing trends, highlighting a predation of *I. punctatus* juveniles on *P. antennarius*, *D. villosus*, as well as on small cyprinids (e.g., *A. alburnus*) and of *I. punctatus* (matures) on cyprinids, *P. antennarius* and *D. villosus*. As for *S. glanis*, *I. punctatus* and *D. villosus* constituted the preferred preys. Differing results were obtained for *L. gibbosus*; without numerical priors, *Palaemon* sp. and, with priors, aquatic insects majorly contributed to its trophic level. However, SIMMs including N% priors led to an increase in the estimated proportion of contributing prey items and less variability.

The applied PERMANOVA on carbon and nitrogen signatures highlighted an overall distinction among species [$F_{(14, 202)} = 135.34$, $p = 0.001$; **Table 5**]. Nonetheless, the applied pairwise *post-hoc* tests (**Supplement 2**) indicated that some fish species had no distinct isotopic niches. In particular, overlapping niches were found between *A. alburnus* and juvenile *I. punctatus* ($p = 0.054$), *A. alburnus* and *L. gibbosus* ($p = 0.46$), *A. alburnus* and *Padogobius* sp. ($p = 0.185$), *A. alburnus* and *P. parva* ($p = 0.085$), *C. carpio* and *L. gibbosus* ($p = 0.124$), *C. carpio* and *Padogobius* sp. ($p = 0.193$), *C. carpio* and *P. parva* ($p = 0.337$), juvenile *I. punctatus* and *L. gibbosus* ($p = 0.221$), mature *I. punctatus* and *Padogobius* sp. ($p = 0.146$), *L. gibbosus* and *Padogobius* sp. ($p = 0.35$), *L. gibbosus* and *P. parva* ($p = 0.145$), *Padogobius* and *P. parva* ($p = 0.803$).

TABLE 4 | Estimated ellipses overlap between all fish species with $n \geq 5$ and the crayfish *Procambarus clarkii*.

Pairwise niche comparison	SEAb overlap	SEAc overlap
Ictalurus punctatus total—*Silurus glanis* (Matures)	0.195	0.007
Ictalurus punctatus (Immature)—*Silurus glanis* (Matures)	0.278	0.224
Ictalurus punctatus (Mature)—*Silurus glanis* (Matures)	0.086	0
Ictalurus punctatus (Immature)—*Ictalurus punctatus* (Mature)	0.421	0.274
Alburnus alburnus—*Pseudorasbora parva*	0.462	1.326
Alburnus alburnus—*Lepomis gibbosus*	0.218	0.207
Pseudorasbora parva—*Lepomis gibbosus*	0.591	0.490
Ictalurus punctatus (Immature)—*Lepomis gibbosus*	0.167	0
Ictalurus punctatus (Mature)—*Lepomis gibbosus*	0.244	0.472
Ictalurus punctatus (Immature)—*Alburnus alburnus*	0.254	0.501
Ictalurus punctatus (Immature)—*Pseudorasbora parva*	0.168	0.152
Ictalurus punctatus (Mature)—*Alburnus alburnus*	0.187	0.191
Ictalurus punctatus (Mature)—*Pseudorasbora parva*	0.125	0.002
Procambarus clarkii—*Alburnus alburnus*	0.054	0
Procambarus clarkii—*Pseudorasbora parva*	0.057	0
Procambarus clarkii—*Lepomis gibbosus*	0.072	0
Procambarus clarkii—*Ictalurus punctatus* (Immature)	0.106	0
Procambarus clarkii—*Ictalurus punctatus* (Mature)	0.110	0
Procambarus clarkii—*Silurus glanis* (Matures)	0.003	0
Silurus glanis (Matures)—*Alburnus alburnus*	0.220	0.003
Silurus glanis (Matures)—*Pseudorasbora parva*	0.162	0.001
Silurus glanis (Matures)—*Lepomis gibbosus*	0.217	0.227

SEAc, corrected Standard Ellipse Area considering 40% of core data.
SEAb, Bayesian Standard Ellipse Area, considering 95% of data.

DISCUSSION

Multiple invaded communities are particularly interesting as most studies focus on the effects of a single introduced species (but see Preston et al., 2012; Jackson, 2015). In the Mediterranean area, to the best of our knowledge, only few studies explored the trophic interactions in highly invaded inland water communities despite the high frequency of species invasions (Galil, 2007; Di Castri et al., 2012). Furthermore, they both concern lentic waters and comprise generally fewer species. Like our study, combining dietary analyses and stable isotopes, Stellati et al. (accepted) described the trophic interactions of a pond community in Monterotondo (Rome, Central Italy), composed by almost exclusively invasive species except for top predators (*Natrix natrix* and *Natrix tesselata*. Using an identical approach, Haubrock et al. (2019a) investigated the relationships in Northern Spain in Arreo Lake dominated by invasive species. As shown by the studies above, such integrative approaches applied to a peculiar community can provide not only insights into trophic interactions among invasive species but can also improve our ability to understand their synergistic impacts onto communities. However, in the study presented here, investigations were conducted during spring season in a completely different ecosystem: a highly anthropogenically altered river with no seasonal drought and no native fish predators. While the dataset cannot be seen as enough to make a generalization on the entire year, we believe that the collected data are sufficient to analyse the interactions and subsequent interactions among alien invasive species in the Arno river during spring season.

Community Structure

The reconstructed community had a typical pyramidal shape, with the apex position being held by two invasive predatory fish, *I. punctatus* and juvenile *S. glanis*. Compared with the works of Stellati et al. (accepted) and Bissattini and Vignoli (2017), in which the community was composed—according to isotopic data—by approximately two trophic levels, in the Arno River it spanned over at least three trophic levels, indicating a higher complexity. However, it is interesting to note that this complexity is reached with a high number of species, while in Arreo Lake the same number of trophic levels was obtained with only four species, that had clearly separated niches, with a primary role of N isotopes signatures (and therein the trophic level) in this partitioning (Haubrock et al., 2019a). On the contrary, the Arno River community showed some overlap among trophic niches of fish species, but not a clear segregation of all niches as observed in Arreo Lake (Haubrock et al., 2019a), as the latter was the result of the low species number and a common geographic origin.

Estimations of trophic positions showed a strong top predator behavior for *S. glanis*, *I. punctatus*, especially in the juvenile stage, and also *L. gibbosus*, coherently with their known biology and other isotope studies (Werner et al., 1977; Vander Zanden and Rasmussen, 1999; Vander Zanden et al., 1999; Syväranta et al., 2010). Compared with Haubrock et al. (2019a), *L. gibbosus* was identified as a possibly secondary -not a primary- predator. This might be associated to the differences in ecosystem and community complexity. The functional category of primary predatory fish was represented by *P. parva*, *A. alburnus*, and *Padogobius* sp., although for the latter this position should be taken with caution due to the low number of sampled individuals ($n = 4$). *Cyprinus carpio* ($n = 4$) and the only specimen of *B. barbus* can be considered true omnivores as they had a TP intermediate between that of primary predators and herbivores. Commonly, *A. alburnus* and *P. parva* are known omnivores with a preference for zoobenthos (Politou et al., 1993; Gozlan et al., 2010). Nonetheless, they have the ability to exert wider, on higher trophic level focused feeding preferences (Vøllestad, 1985; Lammens and Hoogenboezem, 1991; Nagata et al., 2005), potentially explaining their comparatively elevated TPs (Syväranta et al., 2010; Jackson and Britton, 2013). However, the low number of individuals captured cannot allow other speculations, but rather give a rough indication of the positioning of these species trophic niche in the studied ecosystem. Among invertebrates, the invasive red swamp crayfish *P. clarkii* and the native *P. antennarius* were also included in this category. Compared with Arreo lake (Haubrock et al., 2019a), *P. clarkii* belonged to a higher trophic level, potentially due to the

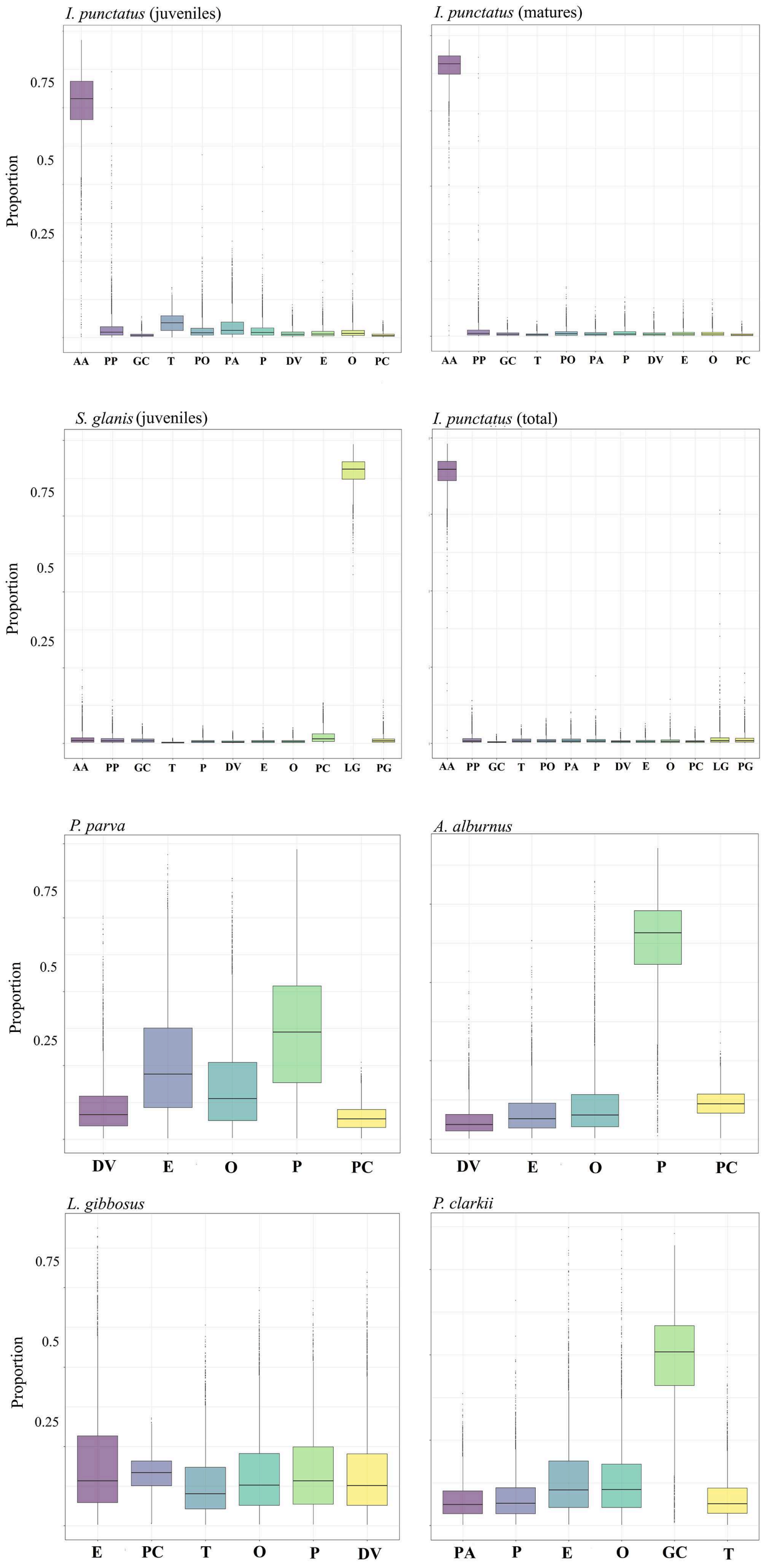

FIGURE 2 | Estimated proportions of prey items to the diet of: *Ictalurus punctatus* (juveniles), *Ictalurus punctatus* (matures), *Ictalurus punctatus* (total), juvenile *Silurus glanis*, *Pseudorasbora parva*, *Alburnus alburnus*, *Lepomis gibbosus*, and *Procambarus clarkii*. Prey items were considered those belonging to lower trophic levels without consideration of any prior. The analysis was performed using the R-package "simmr".

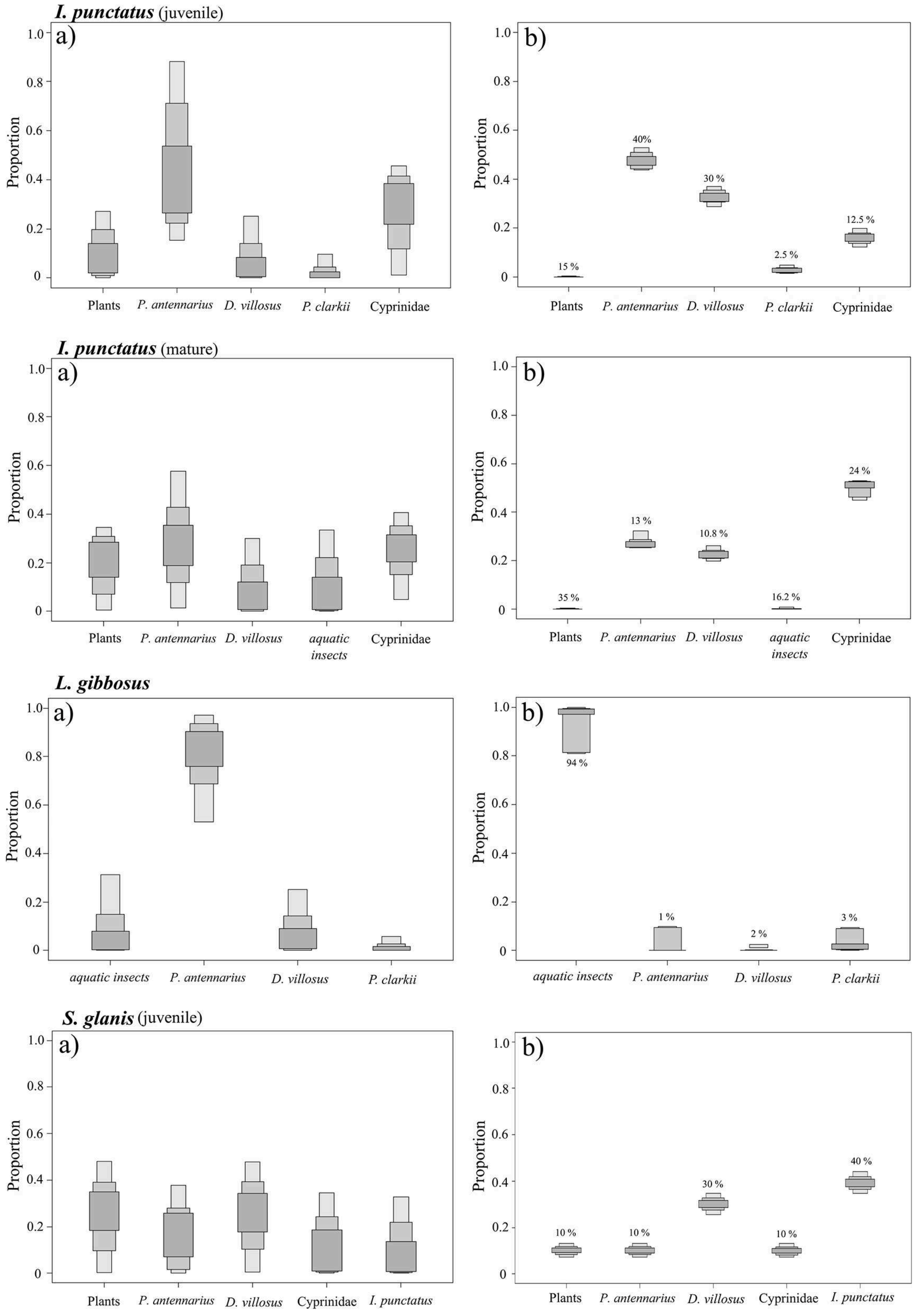

FIGURE 3 | Estimated contribution of consumed prey items to the diet of: *Ictalurus punctatus* (juveniles), *Ictalurus punctatus* (matures), *Lepomis gibbosus* and juvenile *Silurus glanis* using dietary priors. **(a)** Based on the 5* most consumed prey items; **(b)** including the number of occurrences (N%) of the 5* most consumed prey items (indicated in %). *: 4 in the case of *L. gibbosus*. The prior based analysis was performed using the R-package "SIAR" with credibility intervals of 95%.

TABLE 5 | PERMANOVA main test result on nitrogen and carbon isotopes signatures for factor SPECIES.

Source	df	SS	MS	Pseudo-F	P(perm)	perms	P(MC)
sp	14	3293.1	235.22	135.34	0.001	999	0.001
Res	202	351.07	1.738				
Total	216	3644.1					

greater complexity of the Arno River community and therefore higher number of potential prey items and advanced stage of eutrophication. As discussed for *B. barbus*, the trophic position of the omnivorous *T. tinca*, cannot be considered precise; however, both these samples are still indicative also for future studies on these two rare species in this or other ecosystems. Surprisingly, the larvae of predatory insects (Ephemeroptera and Odonata) as well as the invasive amphipod *D. villosus*, which is known to be a predator of other aquatic macroinvertebrates and fish eggs (Dick and Platvoet, 2000), fell in the same trophic level of primary consumers. This is maybe due to predation on unsampled invertebrate consumers, with a lower N isotopes signature than mollusks, leading to potential underestimations in TPs. Moreover, due to the generally high angling activity (using commonly float and feeder fishing) in the Arno river, an unpredictable influx of unnatural food sources cannot be excluded. These items could likely affect the trophic positioning of species like *T. tinca*. However, the angling activity in the Arno is considerably low during spring (pers. comm. Thomas Bussatto). Additionally, if such baited fishing would occur, used bait-items would have been observed within the diet of *I. punctatus* (Haubrock et al., 2018b, 2019b). Interestingly, juvenile *I. punctatus* had a slightly higher nitrogen signature than matures, which is uncommon for catfish species as they generally express higher N values with increasing total length (Syväranta et al., 2010). Considering the high level of eutrophication and the natural accumulation of nutrients (i.e., dead matter) in the river bottom, this observation can potentially be explained by the greater consumption of enriched detritus by juveniles (Hendrix et al., 1986; Hansen and Kristensen, 1998). Another explanation could be that adult *I. punctatus* lower their trophic position to avoid (or is confined to a lower position as a result of) high competition with other piscivorous fish like *S. glanis* (Fry, 1999) as they share similar niches. Because *S. glanis* is not a detrivorous species, an effect of N-enriched detritus can be neglected in this species' case. Nonetheless, it should be noted that the inclusion of adult specimens of *S. glanis* might lead to a significant correlation between TL and N signatures (Syväranta et al., 2010).

Moreover, several species expressed a negative correlation between TL and C signatures, suggesting consumption of prey items that feed on different aquatic or terrestrial vegetation. This underlines the scenario that these species change habits as they grow, with e.g., juveniles of *I. punctatus* living closer to the riverbank and relying on fallen terrestrial items more than matures, the latter feeding offshore. However, although Syväranta et al. (2010) argued that in *S. glanis* δ^{15}N increases with TL due to the ability to consume higher trophic prey, such an increase was only observed in *L. gibbosus*, indicating an ontogenetic size-based prey shift.

Layman Metrics and Niche Overlap

Among fish species, Layman's metrics (Layman et al., 2007) showed a great variability in N signatures, thus indicating the utilization of prey items from different trophic levels in *P. parva*, *L. gibbosus*, and adults of *I. punctatus*, while juvenile *S. glanis* were quite specialized in its trophic role as apex predator. Carbon variability was largest in *A. alburnus*, suggesting the direct or indirect consumption of prey from different origins. Niche width measures showed the widest niche for *L. gibbosus, P. parva*, and juveniles of *I. punctatus*, thus, these species express the greatest intra-specific variability. In contrast, a more specialized niche was found for *S. glanis* together with a niche partitioning among catfish species with the potential to compete due to the generalist diet of *I. punctatus* (see Haubrock et al., 2019b for a detailed discussion). Moreover, the comparison between *A. alburnus* and *P. parva*, two ecologically similar species belonging to the same trophic level and with a considerable niche overlap, showed a considerably wider niche width for *P. parva* based on a considerable variability in δ^{15}N values, probably due to a higher feeding variability and adaptability to many ecosystems, a key factor in its invasion history. Indeed, Rosecchi et al. (1993), Gozlan et al. (2010) and Yalçin-Özdilek et al. (2013) stated that *P. parva* is prone to predate, among other items, fish larvae as well as eggs. However, while these potential preys were present during the time of the sampling, they were not observed in the diets, as previously observed (Annamaria Nocita, unpubl. data). In contrast, *A. alburnus* showed significantly less inter-individual variability linked to feeding activity mostly relying on macroinvertebrates (Politou et al., 1993).

Similarly, it is interesting to compare the two size-matched crustaceans *D. villosus* and *P. antennarius*. The greater variability in δ^{15}N was found in the former, suggesting the consumption of prey items from different trophic levels. However, *D. villosus* unexpected low trophic position raises questions on its specialized predatory behavior, whilst *P. antennarius*, despite being an omnivore feeding also on detritus and algae, occupied a higher trophic position with low variability in δ^{15}N. Moreover, as for *P. parva*, *D. villosus* had a considerably wider niche than that of *P. antennarius*, a factor potentially related to the success of this invasive species. Finally, *P. clarkii* showed a wider niche compared with the other two crustaceans, particularly due to its variability in carbon sources. This was not surprising, giving the opportunistic and generalist diet, especially in invaded areas (Souty-Grosset et al., 2016).

Permutational analysis of variance of species niches identified considerable overlaps mainly between predatory fish species, confirming SEAc and SEAb overlap estimations. The result is a community structure more packed at higher than at lower trophic levels (**Figure 1**). Therefore, we speculate that, in the Arno River community, a diminished predator pressure could be not caused by the presence of a keystone species (such as in Bissattini et al., 2018), but by the potential competition among predators and potentially occurring competition- avoidance mechanisms (Haubrock et al., 2018b).

Mixing Models and Resource Utilization

Standard mixing models under the assumption of predation on lower trophic positions (**Figure 2**) presented limited, but representative, information, as this theoretical predation scenario is estimated based on the potential availability of prey items and the possibility of their consumption (Phillips, 2001; Phillips et al., 2005). However, including the observed prey and their occurrences in the diet in these models, considerable differences emerged (**Figure 3**). When using DA based priors, *I. punctatus* presented more precise proportions of prey contributions, while the diet of *L. gibbosus* is based more on aquatic insects, which is directly mirrored by DA results (**Table 1**). For *P. parva* and *A. alburnus*, where no suitable DA were applicable due to the low number of stomach contents, mixing models without priors showed a higher predation of *P. antennarius* by *A. alburnus*, but a wider more generalistic feeding by *P. parva*. In addition, using the five most commonly present prey items in the diet of *S. glanis* led to a considerable role of plants, which did not majorly contribute to its TP. Nonetheless, it has to be stated that only 19 of 39 *S. glanis* had full stomachs. In this regard, the presence of plants in the diet of *S. glanis* is particularly interesting, as firstly, *S. glanis* is only feed on plant matter during early invasion (Carol et al., 2009), thus life stages, and secondly, because symbiotic bacteria are needed for fish to be able to digest plants (Vejríková et al., 2016). However, accidental digestion of plant matter while preying on living prey may occur, the potential competition with *I. punctatus* cannot be excluded of having caused diet shifts within *S. glanis* immature life stage due to this species known generalism and adaptability to new prey sources (Vejrík et al., 2017b; Haubrock et al., 2019b).

Concluding, this latter prior-based method can be considered more accurate, due to the combination of two different time mediated methods (Moore and Semmens, 2008). Nevertheless, when information on the local diet are not available, the first method without prior can be considered as very informative (Pacioglu et al., 2019).

A Case Study on Alien Species Communities?

The study river is a peculiar ecosystem, as the majority of the inhabiting species are alien. Moreover, the communities' hierarchical order indicates a typically intact community and highlights the foregone interactions that have led to this structure (McQueen et al., 1989; Polis, 1994). The main predatory species were observed to mostly feed on other alien species, and thus, resulting in complex and manifold species interactions. These differing interactions underline the need for studies on a case by case basis. Interestingly, combining SIA and DA revealed differing interactions according to whether alien species shared a common life-history or not.

In the case of *I. punctatus* and *L. gibbosus*, as these two species co-occur in the same ecosystems in North America (Werner et al., 1977; Smith, 1981) and thus, share a common life-history and overlapping native range as well as history of co-evolution, some niche overlap was identified. Moreover, the applied PERMANOVA did not indicate a statistically significant difference between juvenile catfish and pumpkinseed trophic niches. The diet of both included a considerable percentage of aquatic insects, but the mixing models with and without prior showed a different contribution of prey items. More importantly, *I. punctatus* did not show any indication of predation on or direct competition with *L. gibbosus*. Hence, competition could be not direct, but rather based on the opportunistic nature of *I. punctatus* or other factors that lead to a slightly lower trophic position in mature than juvenile *I. punctatus*. Moreover, these species likely avoid competition with their different feeding activities (benthivorous generalist vs. pelagic opportunist). On the other hand, where potentially competing alien species came from different geographical ranges, observations were slightly different. *Pseudorasbora parva* and *A. alburnus*, two species with similar behavior and activity pattern, showed major isotopic niche overlap confirmed also by the PERMANOVA *post-hoc* test, suggesting that, if resources become limiting, these species are likely to compete.

As for the two apex predators, the results indicated that while both, mature and juvenile *I. punctatus* show a medium to high diet overlap with *S. glanis*, a minor isotope overlap was only observed between juvenile *I. punctatus* and *S. glanis* but not among life-stages of *I. punctatus* (Haubrock et al., 2019b). It has therefore to be considered that *I. punctatus* is a primarily benthic forager, a factor that likely limits the competition with other species. These results led to the conclusion that both life-stages of *I. punctatus* show a potential to compete with juvenile *S. glanis* but not with each other, likely due to diet- and behavioral-based intra-specific avoidance mechanisms. However, it should be noted that including mature individuals of *S. glanis*, as shown in Syväranta et al. (2010) and Vejrík et al. (2019), might have an effect of this species' niche occupation and the observed niche partitioning (Haubrock et al., 2019b). With the use of mixing models, different preferred prey items were identified for both species, while the use of priors, when applicable, indicated a considerable feeding activity by *S. glanis* on *I. punctatus*.

Finally, the presence of *P. clarkii*, often described as a keystone species (Liu et al., 2018), was shown to lower the impact of *L. catesbeianus* on native amphibians due to *P. clarkii*'s availability as prey (Bissattini and Vignoli, 2017). However, in the Arno River it occurred at low densities. For this reason, the more abundant *P. antennarius* and *D. villosus*, both highly present in the diet of multiple species as well as indicated by the mixing models, probably reside in a similar function within the community. Therefore, in our study site, the presence of *P. clarkii* seemed not to lower impacts on present species by predatory fish, but rather contributed to it.

Not all alien species that originate from roughly the same geographic region share a common history of co-evolution. However, in this studies case, North American species indeed share the same ecosystems. As such, it was shown that not only alien species do differ in their ecological niche occupation, but the existence and type of interactions may differ according to the involved species and their respective histories of co-evolution. This is true despite the close clustering of fish species within the 4th and 3rd trophic level. Indeed, our combined SIA and DA results suggest that, when two alien species originate from the same geographical range, competition seemed to be possible due to some diet overlap, but was neglectable due to varying feeding preferences, occupied niche space and potential avoidance mechanisms (Haubrock et al., 2018b), as indicated by prior (DA) - based mixing models and $\mathrm{SEA}_{b/c}$. Nonetheless, when species originated from different geographic ranges but occupied the same trophic level, i.e., similar trophic positions, shared niche space and isotope overlap were more likely to occur. Resultant, due to the variability of occurring interactions, niche shifts or increasing competition could lead to an increased impact on native species, while predetermined interactions among species with common life-history could lead to a lowered feeding pressure. However, more studies are therefore needed in this direction to confirm these findings and to increase information on these alien species' impacts. These future sampling campaigns should also consider summer and autumn because behaviors and thus, interactions may differ (Vejrík et al., 2017a), potentially further enhancing our comprehension of mechanisms underlying biological invasions and species interactions.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because this study was carried out in accordance with the principles of and under the restriction of the laws currently effective in Tuscany, Italy, following the recommendations of Dr. Annamaria Nocita. The protocol was approved by the local governmental authorities.

AUTHOR CONTRIBUTIONS

PH, PB, and ET designed the study. PH, PB, MA, and WG performed research. PH, MA, and AI analyzed data. LV contributed new models. PH and PB wrote the first draft. All authors contributed to the paper and approved it.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2019.00355/full#supplementary-material

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The Introduced Fanworm, *Sabella spallanzanii*, Alters Soft Sediment Macrofauna and Bacterial Communities

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The Mediterranean fanworm, *Sabella spallanzanii*, is listed as an introduced and established "unwanted species" in New Zealand, subject to nationwide targeted surveillance in port, marina, urban and natural environments. *Sabella spallanzanii* has the potential to change soft-sediment benthic habitats due to the physical presence of the fanworm's tube and associated biological activities, particularly suspension feeding and bio-deposition. A 6-month field experiment was conducted to investigate the impacts of *S. spallanzanii* on the structure and diversity of existing communities within invaded soft-sediment habitats. Macrofaunal communities were assessed using traditional sampling and identification via microscopy, while microbial and eukaryotic communities were characterized using metabarcoding of 16S and 18S ribosomal genes, respectively. Live and mimic *S. spallanzanii* were transplanted at different densities (10–50 individuals per m^2) into experimental plots with existing assemblages, to test for potential biological and/or physical effects on benthic communities. Analyses revealed significant differences in macrofaunal, eukaryote, and bacterial assemblages in the presence of live *S. spallanzanii* and mimics, underpinned by changes in the relative abundance of several taxa, indicating that these effects are brought about by biological and physical functions. The presence of *S. spallanzanii* did not alter total abundance and taxa richness of benthic assemblages but resulted in compositional differences. We found no effect of live or mimic worm density on the structure and diversity of the studied communities. Changes in the structure of native benthic communities, as indicated by this study, can potentially impact functioning of soft-sediment habitats, through alterations to nutrient cycling, bioturbation and benthic-pelagic coupling. Quantitative measurements of impacts are crucial to understand the trajectory of marine invasions, their roles in re-structuring communities, and to guide management efforts.

Keywords: introduced species, soft sediment, invasive species, impact study, field experiment, environmental DNA, Mediterranean fanworm

INTRODUCTION

Non-indigenous species (NIS) can have profound impacts on marine coastal ecosystem functioning, by altering community structure, native species richness and ecological processes (Ruiz et al., 1999; Molnar et al., 2008). The magnitude and extent of these impacts vary across temporal and spatial scales (Carlsson et al., 2009), but can be extensive and irreversible (Edelist et al., 2013). Some NIS can regulate the availability of resources to other species by changing the physical state of the ecosystems they invade. Such species are termed "ecosystem engineers" (Jones et al., 1994) or "transformier species" (Richardson et al., 2000), which have the potential to disproportionately affect the functioning of coastal habitats (Cuddington and Hastings, 2004). Ecosystem engineers modulate invaded environments through physical and biological mechanisms. Physical mechanisms are associated with the creation of habitats and modification of the local environment by the presence of body structures of the engineers themselves. For example, introduced mussels or oysters are well-recognized ecosystem engineers that form dense beds or reefs, which can increase local diversity by providing novel hard habitat in soft-sediment environments for many species to colonize (Sousa et al., 2009). On the other hand, biological mechanisms alter the flux of resources through mechanical or chemical functions, such as filtration and bio-deposition that can shift the flow of energy between pelagic and benthic habitats. For example, the proliferation of an invasive filter-feeding clam in San Francisco Bay (USA) caused persistent changes in planktonic food webs and benthic macrofaunal community structure through alteration of benthic-pelagic coupling (Peterson, 2002; Cloern and Jassby, 2012).

The Mediterranean fan worm, *Sabella spallanzanii* (Gmelin, 1791), a large sabellid tube worm, is considered an ecosystem engineer owing to its capacity to form dense canopies of tubes extending to 50 cm above the sediment. The physical structure provided by the leathery tubes and spiral feeding fans of *S. spallanzanii* canopies can alter near-seabed hydrodynamics and reduce light penetration below. In Australia, changes in hydrodynamics caused by dense aggregations of invasive *S. spallanzanii* have been linked to alterations in understory invertebrate larval abundance and recruitment patterns of epifaunal assemblages (Holloway and Keough, 2002a,b). *Sabella spallanzanii* canopies can also provide structurally complex habitat for other species to inhabit and seek refuge from predators, thus potentially changing the structure of benthic assemblages. Additionally, *S. spallanzanii* is a suspension feeder that can filter large quantities of water (ca. 12 m^3 per day, Stabili et al., 2006). Its efficient removal of organic matter from the water column, and conversion to fecal material and other waste products, may alter bentho-pelagic coupling in invaded habitats. Previous studies conducted in south-eastern Australia revealed changes in benthic community structure and composition associated with *S. spallanzanii* (O'Brien et al., 2006; Ross et al., 2007). The impact of NIS is frequently context dependent (Didham et al., 2007; Thomsen et al., 2011), making it difficult to extrapolate findings from one location to another. This merits further efforts to understand the generality of their effects or to add context across regions that may attempt to manage their incursion.

Sabella spallanzanii is native to the Mediterranean and east Atlantic coast of Europe and has established introduced populations along the southern coast of Australia and in New Zealand, and has also been recorded in Brazil (Currie et al., 2000). *Sabella spallanzanii* was first detected in New Zealand in 2008 (Read et al., 2011) and has since become established in several regions around the country. It occurs subtidally at a range of densities in multiple geographic locations (100s of km apart) including on man-made structures and in natural reef and soft-sediment habitats. Although *S. spallanzanii* is an epifaunal species that requires a solid substrate on which to attach, it can successfully invade soft sediments by using hard substrate within the sediment as anchoring points, such as a fragment of cobble, shell or debris. The density of an introduced species often drives the magnitude of impact. Worm density is generally higher in artificial than natural habitats, and in subtidal soft sediment environments it generally increases with depth, up to a maximum of 30 m. In these natural soft sediment habitats worms typically grow in clumps (50–200 worms) attached to dead shells or live mussels and ascidians (Currie et al., 2000). Its reproductive biology, including high fecundity rates, "sperm-casting" fertilization strategy, and extended reproductive season (Giangrande et al., 2000), contribute to it being found in dense aggregations as a numerically dominant, habitat-forming species (Currie et al., 2000). It has been designated an Unwanted Organism under New Zealand's Biosecurity Act 1993 and is subject to targeted surveillance in harbors, ports, marinas, and high-value natural environments around the country. However, there is still relatively scarce knowledge about the associated adverse effects of established populations. Given *S. spallanzanii's* wide environmental tolerance and its rapid spread potential into natural habitats, there is a need for a better understanding of its impacts on ecosystem diversity and functioning. This information is critical to understanding resource allocation and benefits of management.

This study aimed to elucidate the ecological impacts of *S. spallanzanii* on soft-sediment ecosystems in New Zealand. We used a manipulative field experiment to quantify density dependent effects of *S. spallanzanii* on the structure of soft sediment macrofaunal, eukaryote, and microbial assemblages. We manipulated the density of live and mimic *S. spallanzanii* to separate biological and physical effects of the worms on macrofaunal and microbial assemblages.

METHODS

Experimental Design, Sample Collection, and Processing

A field experiment was conducted from September 2017 until March 2018 in subtidal soft-sediment habitats along the Rangitoto Channel, Waitemata Harbor, Auckland, New Zealand (−36.813, 174.8396). The site had a water depth of 8 m and consisted of relatively flat soft-sediment habitat. The site is situated just outside Waitemata Harbor and is located approximately 1 km outside the commercial shipping lane to the Port of Auckland. An earlier survey revealed the presence of *S. spallanzanii* in this area, at varying densities (0–20 individuals per m^2).

A two-factor experimental design was used to examine the influence of fanworms on soft sediments at the study location. A first factor, "Treatment," had three levels: "*Sabella*," corresponding to the addition (via transplants, see below) of live *S. spallanzanii* worms to experimental plots; "Mimic" corresponding to the addition of mimic *S. spallanzanii* worms; and "Control" corresponding to plots without worms. Live *S. spallanzanii* were obtained by divers from adjacent soft sediment invaded areas and transplanted into the plots using garden pegs. During a 2017 pilot study conducted at a nearby marina, the use of U-shaped steel garden pegs had been identified as a reliable method to anchoring and ensuring the viability of transplanted live and mimic worms (Atalah, unpubl. data). Only healthy undamaged worms with intact tubes were transplanted for this treatment. Many of the live worms collected at the study site were attached to shell fragments, sometimes with several individuals per shell, and thus treatments were created by transplanting naturally occurring clusters of worms into experimental plots to achieve the target densities. The average tube length of transplanted worms was 32.1 ± 0.66 cm (mean ± standard error), with a range of 10–57 cm. *Sabella spallanzanii* mimics was made from elastic bungee cord (of a length and diameter similar to real *S. spallanzanii*) with frayed ends that resembles the worms' crown structure. These were threaded onto pegs in clumps of two, three or five individuals, similar to the natural aggregations observed at that site (**Supplementary Video 1**).

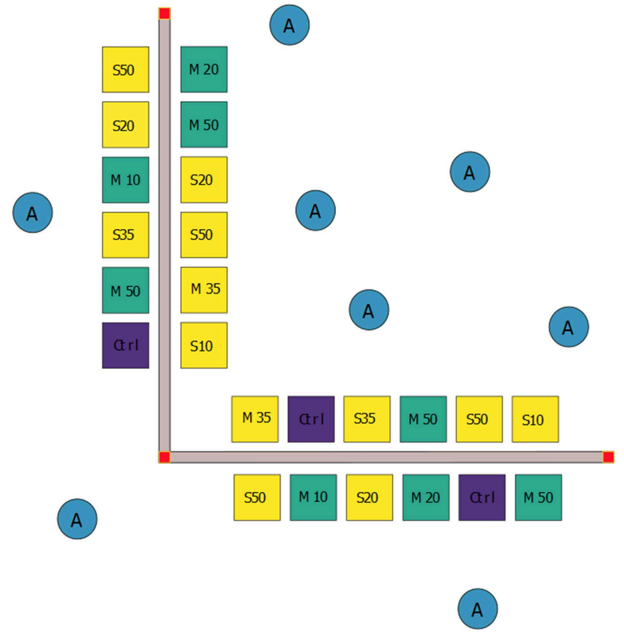

FIGURE 1 | Schematic diagram of the design and set-up of the field experiment showing the random allocation of treatments to 1 m^2 experimental plots: live *Sabella spallanzanii* worms (S, yellow squares), mimic *S. spallanzanii* worms (M, green squares) and controls (Ctrl, purple squares) without worms. Live and mimic worms were transplanted at four different densities (i.e., 10, 20, 35, and 50 worms (or mimics) per plot). Additionally, eight ambient samples (A, blue circles) were collected from adjacent uninvaded areas as procedural controls.

A second factor, "Density," had five levels: zero (control), 10, 20, 35, and 50 worms (or mimics) per plot. Twenty-four 1 m^2 plots, separated by approximately 2 m, were arranged along two perpendicular 30 m transect with a common vertex (**Figure 1**, $n = 12$ plots per transect). Plots were positioned on each side of the transects, separated by a corridor to allow divers access. Four replicate plots were assigned to Controls, two replicate plots were assigned to 10, 20, and 35 density level plots (both *Sabella* and Mimic), and four replicates to the 50 individual density level (**Figure 1**). Extra replicates in the end member treatments (i.e., four replicates each in the control and 50 density treatments, rather than two each) limited the influence of potential outliers and provided greater statistical power. To ensure interspersion, experimental plots were randomly assigned to treatment combinations. Control plots were treated in the same manner as *Sabella* and Mimic plots (i.e., garden pegs inserted into the sediment), but no worms were transplanted.

Experimental plots were established on 19th September 2018 and left undisturbed until 21st March 2019, when destructive sampling of macrofaunal assemblages and sediment characteristics was carried out to conclude the experiment. Two small sediment cores (3 cm internal diameter, 2 cm deep) were collected from each experimental plot: one for sediment grain size and organic matter content analysis (Gatehouse, 1971; Mook and Hoskin, 1982), the other for sediment chlorophyll-*a* and phaeopigment content (Sartory and Grobbelaar, 1984). One core (13 cm internal diameter, 15 cm deep) was collected from a randomly selected area within each of the 24 plots and the material retained on a 500 µm mesh sieve preserved in 70% isopropyl alcohol for macrofaunal community analysis. Eight additional set of samples were collected from non-invaded adjacent "Ambient" areas (i.e., without *S. spallanzanii*) to serve as procedural controls. Macrofauna were separated from sediment and shell hash after staining with Rose Bengal and identified to the lowest feasible taxonomic level and their abundance quantified using a binocular dissecting microscope. A random subset of samples was quality checked by an independent taxonomy expert. All samples and vouchers specimens of the identifications were kept for later verification. The macrofauna dataset was dominated by infaunal taxa, but by nature of the sampling method also encompassed surface-dwelling organisms (e.g., epifauna growing on shell hash or coarser sediment fractions). All organisms were included in the analyses, as we were interested in changes in all components of soft-sediment communities. Molecular samples were collected in parallel with macrofauna samples to enable direct comparison of the metabarcoding results (i.e., microbial and eukaryotic communities) with the traditional macrofaunal methods. Specifically, triplicate 50 g sediment samples were obtained by scraping the top 2 cm layer from haphazardly selected intact areas within each of the 24 plots using sterilized plastic vials. Molecular samples were frozen and transported to the laboratory and immediately stored at −80°C until further processing.

High-Throughput Sequencing and Bioinformatics Analyses

The microbial and eukaryotic community compositions of the triplicate sediment samples collected at the 24 plots, represented by 10 Mimic plots ($n = 30$ samples), 10 *Sabella* plots ($n = 30$ samples), 4 control plots ($n = 12$ samples), and 7 ambient plots ($n = 21$ samples), were assessed using DNA metabarcoding and following a similar high-throughput sequencing library preparation method as outlined in Keeley et al. (2018). Briefly, environmental DNA was extracted from pre-homogenized 0.2 g of surface sediment using the DNeasy PowerSoil^{TM} DNA extraction kit (Qiagen, Hilden, Germany), including DNA extraction blanks (i.e., extraction controls), followed by DNA quality and purity verification using a spectrophotometer (Eppendorf, Leipzig, Germany). A two-step tailed Polymerase Chain Reaction (PCR) amplicon procedure (Kozich et al., 2013) was applied to all DNA samples ($n = 95$; including pooled extraction and pooled PCR blanks), targeting ca. 400 base-pair (bp) fragments of a) the nuclear 16S rRNA bacterial gene (V3-4 region) and b) the nuclear 18S rRNA eukaryotic gene (V4 region), and using the primers, reagents and PCR thermocycling conditions described in von Ammon et al. (2018). Detailed information on sequence library preparation, demultiplexing and follow-up bioinformatics analyses are provided in **Supplemental Material 2**.

Data Analyses

All three datasets (i.e., macrofauna, eukaryotes and bacteria) were analyzed in a similar manner using R software (R Core Team, 2018). Amplicon sequence variants (ASVs) of bacteria and eukaryotes were rarefied at a sequencing depth of 5,000 reads per sample using the "rarefy_even_depth" function in the *phyloseq* package (McMurdie and Holmes, 2013). This resulted in two and one triplicate samples removed for bacteria and eukaryotes, respectively, because they contained fewer reads than the sample size. Additionally, ASVs represented with a mean $< 1^{-4}$ and present <3 times in at least 20% of the samples in each dataset were removed. Rarefaction curves were calculated for each sample using the *vegan* package (Oksanen et al., 2018). Differences in assemblage structure between treatments were tested using permutational analysis of variance (PERMANOVA, Anderson et al., 2002) based on Bray-Curtis similarities of the log (x + 1) transformed abundance data using the "Adonis" function in the *vegan* package. "Treatment" was considered a fixed factor and "Density" included as a continuous covariate representing the final density of fanworms per plot. Significant terms were then investigated using pair-wise comparisons with the PERMANOVA t statistic and 999 permutations. Assemblage differences among treatment levels were visualized using canonical analysis of principal coordinates (CAP, Anderson and Willis, 2003). Indicator taxa for each treatment were determined using the R package "indicspecies" (Cáceres and Legendre, 2009). Indicator species were selected if they were significantly associated with each treatment and their relative abundance visually compared among treatments. Univariate analyses of covariances (ANCOVA) were used to test changes of taxa richness of all three datasets and macrofaunal total abundance using the same experimental design described above for the multivariate data. Tukey tests with Bonferroni adjustment for multiple comparisons were used to compare means when factor "Treatment" was found to be statistically significant in the ANCOVA analyses. In all cases the "Treatment × Density" interaction was not significant ($P > 0.3$), thus it was removed from the ANCOVA models. Model assumptions were checked by inspecting the model residuals. Type III sums of squares (and its direct multivariate analog) were used to analyse the unbalanced designs.

The relationship between species data and sediment environmental variables was analyzed using multivariate multiple regression (McArdle and Anderson, 2001), more specifically using the *adonis* routine in the *vegan* package. A conditional test was used where individual variables were fitted separately to test their relationship with the assemblage data (ignoring other variables) based on Bray-Curtis dissimilarities of the log (x + 1) transformed abundance data. Models were visualized using redundancy analysis ordination biplots (Borcard et al., 2011).

RESULTS

Macrofaunal, Eukaryote, and Bacterial Diversity

Overall, the macrofaunal data comprised 75 different taxa, classified into 10 different phyla, 13 classes, 22 orders, 45 families, and 68 genera. Species level identifications were made for 27 of the 75 unique taxa. Macrofaunal assemblages were largely dominated by annelids, molluscs and crustaceans (52, 19, and 16% of all specimens, respectively, **Figure 2A**), with the non-indigenous bivalve *Theora lubrica* numerically dominant, followed by polychaetes from the Paraonidae and Cirratulidae families.

The illumina^{TM} MiSeq sequencing run generated a total of 12,717,386 sequences, consisting of 5,592,988 bacterial 16S rRNA gene sequences and 7,124,398 eukaryotic 18S rRNA gene sequences. Following filtering (denoising, merging, and chimera removal), the number of high-quality 16S and 18S sequences retained for taxonomic assignments of Amplicon Sequence Variants (ASVs) were 1,848,310 and 3,111,615, respectively. Following reads rarefaction and the removal of ASVs represented with a mean $<1^{-4}$ and present <3 times in at least 20% of the samples, the number of ASVs retained for downstream statistical analyses for the eukaryote and bacterial datasets were 1,210 and 6,947, respectively; with rarefaction of samples indicating that all samples reached saturation (**Supplemental Material 3**). Eukaryotic ASVs were assigned to 29 different phyla, 64 classes, 137 orders, 232 families, 304 genera, and 339 distinct species. The output of the metabarcoding identification resulted in a somewhat similar abundance-based partitioning of higher taxa (**Figure 2B**) with phyla Annelida, Mollusca and Nematoda dominating the eukaryote dataset (26,

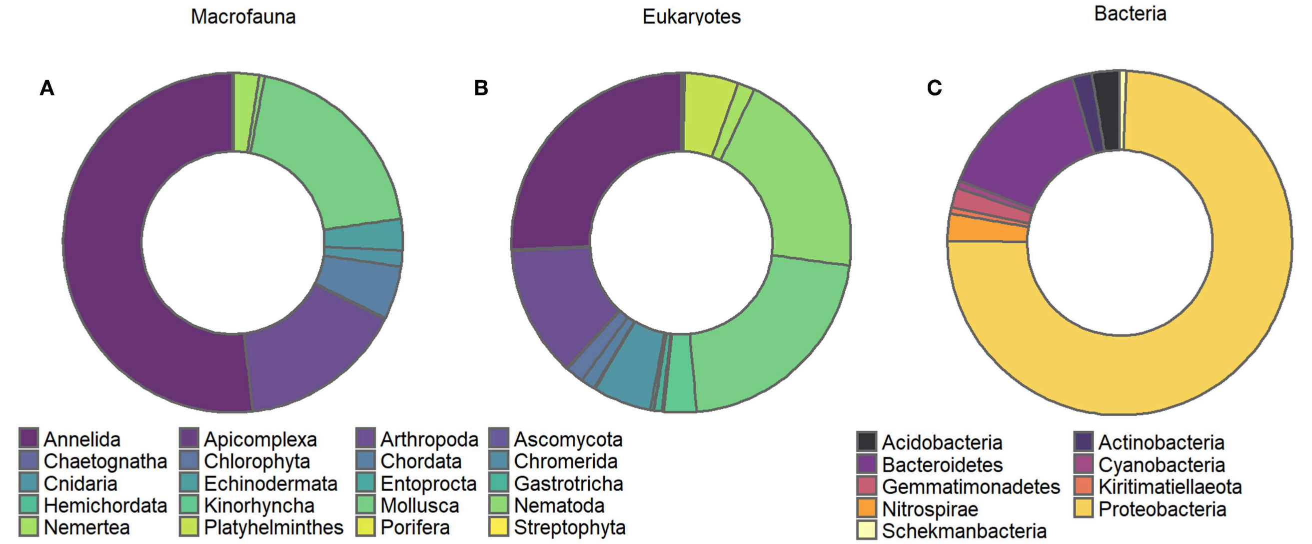

FIGURE 2 | Chart of proportional abundance of different: **(A)** macrofauna, **(B)** eukaryote, and **(C)** bacterial phyla identified from soft sediment assemblages inhabiting experimental plots.

21, 20% of all detected specimens, respectively, **Figure 2B**). Bacterial ASVs were assigned to 44 different phyla, 92 classes, 206 subclasses, 281 families. Bacterial assemblages were largely dominated by Protobacteria, in particular from the family Woeseiaceae and Desulfobulbaceae, followed by bacteria from the phylum Bacteroidetes (**Figure 2C**). All sequence data are deposited in GenBank's Short Reads Archive, BioProject PRJNA552692, Accession number SRR9644676 to SRR9644588.

Treatment Effects on Assemblage

We found no differences between control plots and ambient (procedural) sediment samples (i.e., the effects of garden pegs and manipulation) on macrofaunal total abundance (t-test, $P > 35$), richness (t-test, $P > 0.25$) or assemblage structure (PERMANOVA, $P > 0.05$) for all three communities (i.e., macrofauna, eukaryotes, and bacteria, **Supplemental Material 4**). Therefore, results measured from ambient plots were excluded from all further analyses. There was a significant effect of treatment on assemblage structure consistent across macrofaunal, eukaryote and bacterial datasets ($P < 0.05$, **Table 1**), although there was no main or interactive effect of the factor Density for all three datasets ($P > 0.1$, **Table 1**). The CAP plots illustrate this pattern, showing a clear separation between assemblages from the three treatments in all three datasets (**Figure 3**). For the macrofaunal data, pair-wise comparisons distinguished between *Sabella* and Control ($P < 0.05$); and *Sabella* and Mimic ($P < 0.05$), but not between Control and Mimic ($P > 0.1$). The most significant indicators for Control plots included the brushworm *Phylo novazealandiae*, amphipods, the burrowing crab *Pilumnus novaezealandiae*, the burrowing holothurian *Taeniogyrus dendyi*, and the polychaete *Aglaophamus* sp. Significant indicator taxa for Mimic plots included the bivalves *Monia zelandica* and *Limaria africana*, the polychaetes *Armandia maculata*, unidentified worms from the family Spionidae and *Heteromastus filiformis* (**Figure 4A**). *Sabella* plots were associated with polychaete family Paraonidae, nemerteans and the polychaete *Prionospio* sp. (**Figure 4A**).

For the eukaryote communities, pair-wise comparisons found significant differences among all three treatments ($P < 0.05$). Controls were significantly associated with higher abundances of polychaetes from the family Lumbrineridae and nematodes from the family Microlaimidae (**Figure 4B**). Most significant indicator eukaryote taxa for the Mimic treatment included polychaetes from the families Paraonidae and the non-indigenous Serpulidae tubeworm *Hydroides elegans* and ascidian *Molgula manhattensis*, whereas tellinid bivalves, harpacticoid copepods and dorvilleid polychaetes were more abundant on *Sabella* plots (**Figure 4B**).

For bacterial assemblages, pair-wise comparisons also revealed significant differences among all three treatments ($P < 0.01$ for Control vs. Mimic and Mimic vs. *Sabella*, and $P < 0.05$ for Control vs. *Sabella*) and 55 indicator taxa were identified as being significantly associated with these differences. Most significant indicator taxa for the Control treatment included families in the phyla Actinobacteria, Bacteroidetes and Proteobacteria (**Figure 4C**), whereas for the Mimic treatment they included bacteria in the phyla Acidobacteria, Gemmatimonadetes, Nitrospirae, and Proteobacteria (**Figure 4C**). Significant indicator bacterial taxa for the *Sabella* treatment most notably included families in the phyla Kiritimatiellaeota (e.g., Kiritimatiellaceae), Spirochaetes (e.g., Spirochaetaceae) and Proteobacteria (**Figure 4C**), including significant higher abundance of Desulfobacteraceae.

Total abundance and richness of macrofaunal and bacterial assemblages did not vary significantly among Treatment or Density (**Figure 5** and **Table 2**), although there was a non-significant trend for higher numbers of macrofauna taxa richness

TABLE 1 | Results of PERMANOVA analyses to test the effect of Treatment ("*Sabella*," "Mimic," and "Control") and *Sabella spallanzanii* Density on macrofaunal, eukaryote and bacterial assemblages.

	Macrofauna				Eukaryote				Bacteria			
	df	**MS**	***F***	***P***	***df***	**MS**	***F***	***P***	***df***	**MS**	***F***	***P***
Treatment	2	0.3	1.4	0.04	2	0.6	2.3	<0.001	2	0.3	1.3	<0.001
Density	1	0.2	0.9	0.66	1	0.2	1	0.45	1	0.2	0.9	0.6
Residuals	20	0.2			68	0.2			68	0.2		

df, degrees of freedom and MS, mean square.

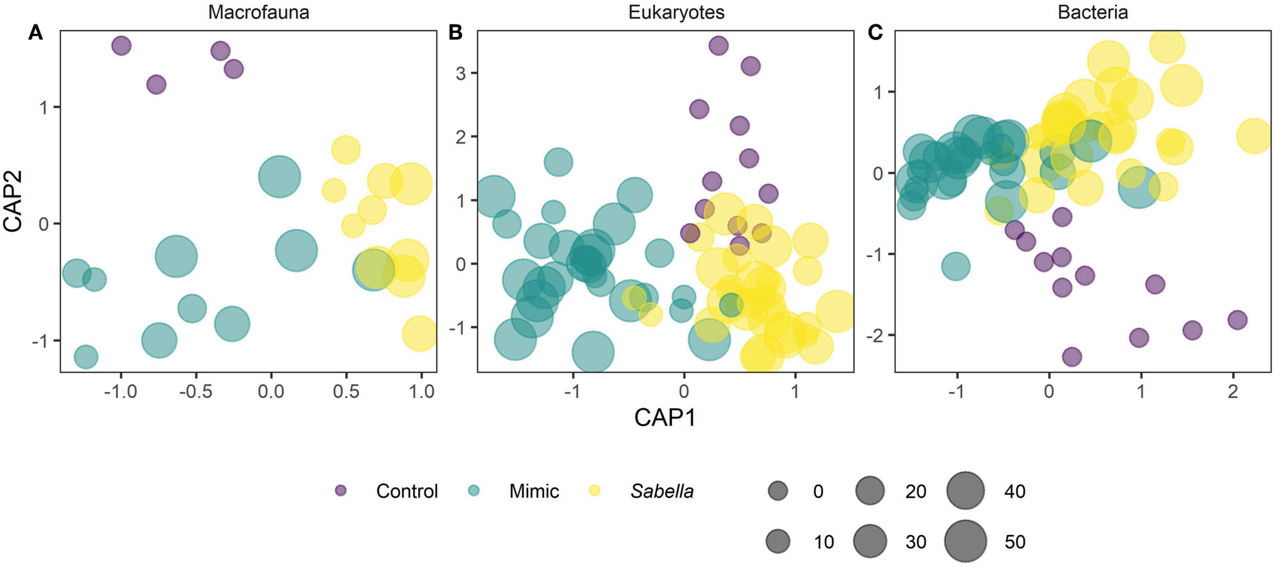

FIGURE 3 | Principal coordinate ordination (PCO) and Canonical analysis of principal coordinates of assemblages in "Control" (purple circles), "Mimic" (green circles) and "*Sabella*" (yellow circles) experimental plots on the basis of Bray–Curtis similarities of the log (x +1) transformed abundance of macrofauna **(A)**, eukaryotes **(B)**, and bacteria **(C)**.

in the Mimic compared to the Control plots. Treatment had a significant effect on eukaryote taxa richness, with Mimic having higher number of taxa compared to Control plots ($P < 0.05$, **Figure 5** and **Table 2**).

Relationship Between Soft-Sediment Assemblages and Environmental Variables

The sediment at the site was relatively poor in terms of organic content (mean 2.5% LOI ± 0.1 S.E.) and was dominated by very fine sand (63–500 µm, 46.6% ± 0.9), silt particles (< 63 µm, 29.3% ± 1.1) and clay (11% ± 1.1) grain size fractions. Mean chlorophyll-*a* and phaeopigments contents were on average 1.92 $\mu g\ g^{-1}$ (± 0.1) and 4.5 $\mu g\ g^{-1}$ (± 0.3), respectively. *Sabella* treated plots had higher fine sand, chlorophyll-*a*, organic and phaeopigments compared to Mimic and Control plots (**Figure 6**). On the other hand, Mimic and *Sabella* plots had higher content of medium and coarse sediment in relation to the controls (**Figure 6**). The multivariate multiple regression analysis showed that gravel, medium and fine sediment had a significant relationship with macrofaunal assemblage structure, explaining 8, 7, and 9% of the variation in the data, respectively. Eukaryote assemblage structure was significantly related to sediment chlorophyll-*a* concentration and marginally to the fine sediment fraction and total organic content; whereas bacterial assemblage structure was not significantly related to sediment characteristics. Although no significant relationships were detected between bacterial assemblages and sediment characteristics, there was a trend of bacterial associations with the fine sediment fraction and phaeopigment concentration ($P < 0.1$). The dbRDA plots (**Figure 7**) illustrate that all three benthic assemblages were distinguishable among treatments constrained to the variation in sediment characteristics. In all three cases *Sabella* plots assemblages were generally well separated from the other treatments and correlated with higher phaeopigment, chlorophyll-*a*, organics and the fine sediment fraction, whereas Mimic plots were related to larger sediment fractions (i.e., medium and gravel).

DISCUSSION

Soft-sediment communities in Waitemata differed significantly in the presence and absence of Mediterranean fanworms

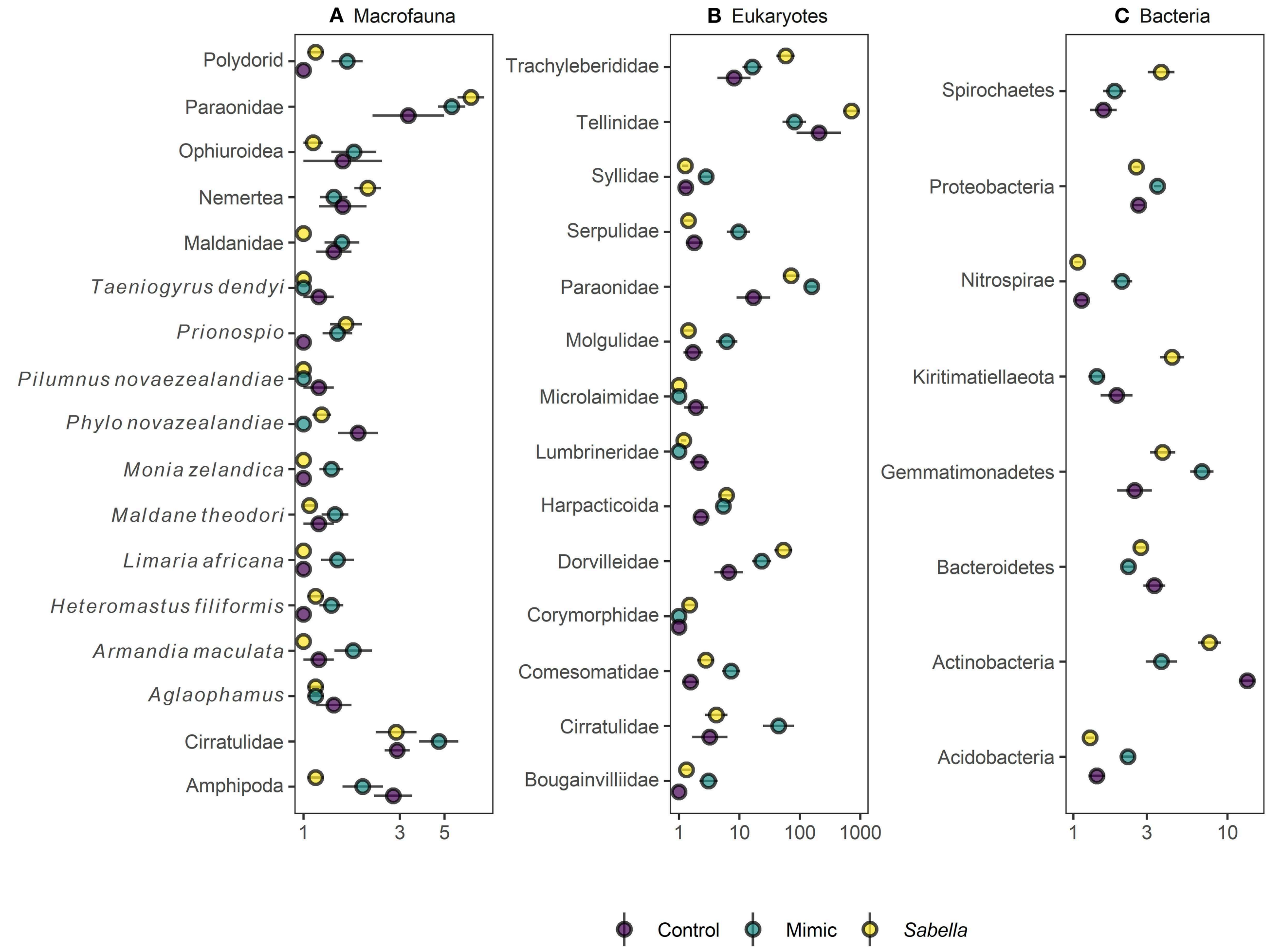

FIGURE 4 | Mean (± SE) relative abundance (%) of indicator taxa for **(A)** macrofaunal, **(B)** eukaryote, and **(C)** bacterial communities in "Control" (purple circles), "Mimic" (green circles) and "*Sabella*" (yellow circles) experimental plots.

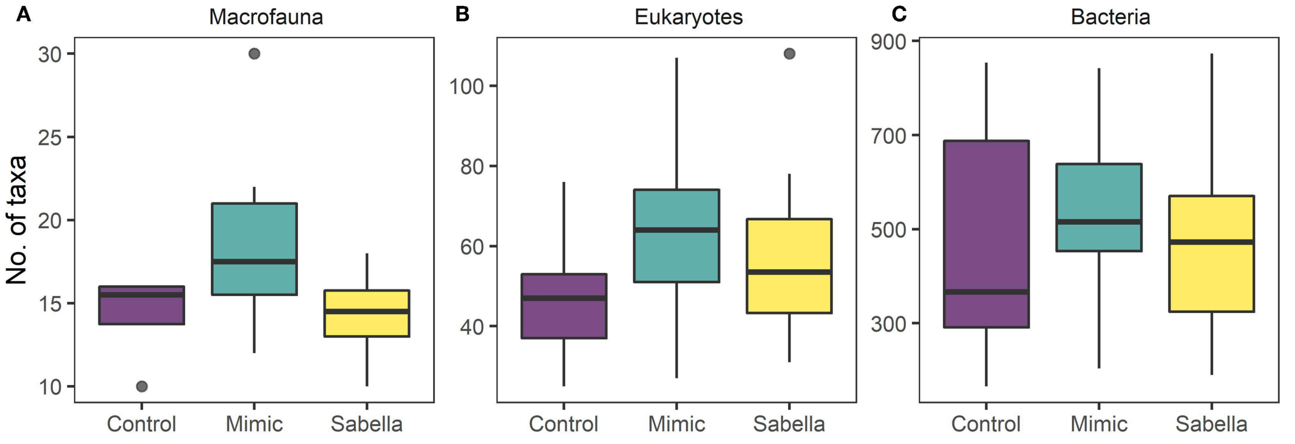

FIGURE 5 | Boxplots of taxa richness for **(A)**, macrofauna **(B)**, eukaryote **(C)**, and bacterial communities in "Control" (purple), "Mimic" (green) and "*Sabella*" (yellow) experimental plots.

TABLE 2 | Results of univariate analyses of covariance (ANCOVAs) to test the effect of Treatment ("*Sabella*," "Mimic" and "Control") and *Sabella spallanzanii* Density on macrofaunal, eukaryote, and bacterial univariate indices.

	Macrofauna abundance				Macrofauna richness			Eukaryote richness				Bacterial richness			
	df	*MS*	*F*	*P*	*MS*	*F*	*P*	*df*	*MS*	*F*	*P*	*df*	*MS*	*F*	*P*
Treatment	2	0.4	0.8	0.48	54.2	3.2	0.063	2	1170	4.2	0.02	2	36,042	1.0	0.37
Density	1	0.0	0.0	0.86	0.2	0.0	0.914	1	88	0.3	0.57	1	13,150	0.4	0.55
Residuals	19	4.5			320			67	18528			66	2,367,367		

df, degrees of freedom and MS, mean square.

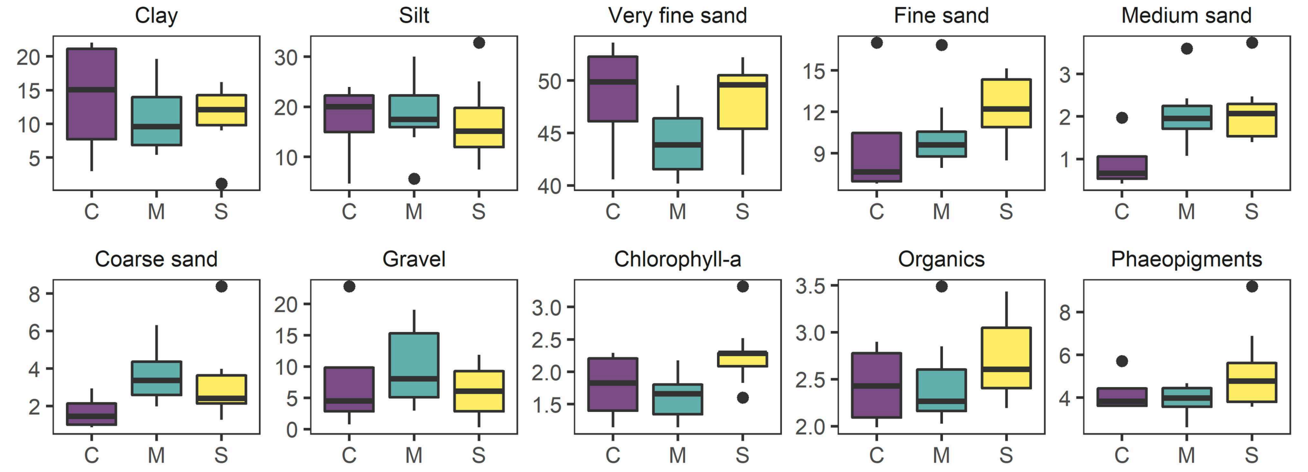

FIGURE 6 | Boxplots of soft sediment physico-chemical environmental variables: clay (< 0.98 µm), Silt (3.9–62.5 µm), very fine sand (62.5–125 µm), fine sand (12–250 µm), medium sand (0.28–0.5 mm), coarse sand (0.5–1.0 mm), gravel (>1 mm), chlorophyll-*a* (µg g^{-1}), organic content (% LOI), and phaeopigments (µg g^{-1}) in relation to experimental treatments: C, "Control"; M, "Mimic"; S, "*Sabella*".

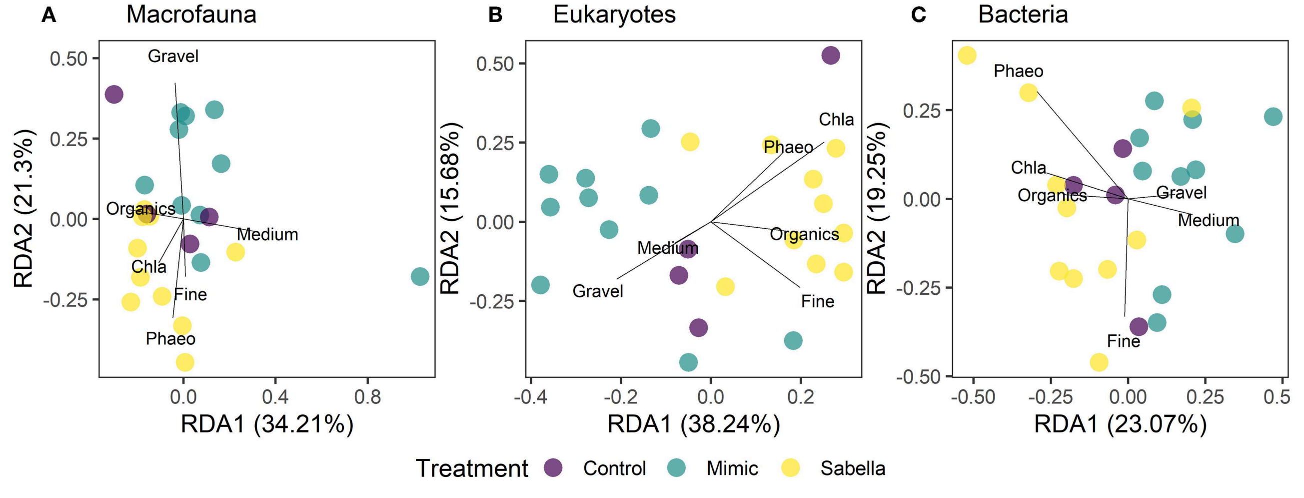

FIGURE 7 | Distance-based redundancy analysis (dbRDA) ordination of first and second fitted axes relating the environmental variables to the faunal data **(A)** macrofaunal, **(B)** eukaryotes, and **(C)** bacterial communities in "Control" (purple circles), "Mimic" (green circles) and "*Sabella*" (yellow circles) experimental plots. Biplot projections are given for the sediment environmental variables. Vectors are plotted showing the correlations of environmental variables with dbRDA axis 1 and dbRDA axis 2, with the length and direction of the arrow representing the strength and direction of the relationship.

and their mimics. This effect was consistent across benthic macrofaunal, eukaryote and bacterial assemblages, and indicates that *S. spallanzanii* may affect benthic community structure through both biological and physical processes and mechanisms. The measured differences among treatments were based on relative abundances of several taxa between plots with transplanted live and mimic *Sabella* and control plots rather than differences in overall abundance or richness. These differences in community structure can have important consequences for ecosystem functioning of soft-sediment habitats (Thrush et al., 2017) based on shifts in feeding, burrowing and sediment irrigation intensity that can drive crucial functions, such as nutrient and organic matter processing.

Small mobile taxa (mainly amphipods) and larger bioturbator taxa such as ophiuroids, a burrowing crab (*Pilumnus novaezealandiae*), and a burrowing holothurian (*Taeniogyrus dendyi*) were less abundant, or absent, in treatment plots compared to controls. This may be a direct or indirect consequence of both physical presence and biological activities of fanworms. Resident communities may be affected by small-scale changes in hydrodynamics linked to the physical structures of *S. spallanzanii* canopies, as changes in water velocities can alter sediment stability and concentrations of key solutes (e.g., oxygen, sulfide; O'Brien et al., 2006). Mobile bioturbator taxa can substantially change the physical and chemical properties of soft sediment habitats and thus are crucial for the functioning of these ecosystems (Lohrer et al., 2004). On the other hand, *S. spallanzanii* plots in our study were also associated with significantly higher abundance (compared to controls) of direct deposit-feeders, such as polychaetes from the family Paraonidae, Dorvilleidae and Spionidae, and Nematoda. The biodeposition activity of the fanworm may has the potential to change nutrient cycling and the microbial community, with knock-on effects on deposit feeders. Suspension feeders, such as *S. spallanzanii* can increase sedimentation of organic material and produce abundant feces and pseudofeces rich in organic matter, which may serve as food sources for deposit feeding organisms (Commito and Boncavage, 1989). This biological activity may explain the observed patterns of higher concentration of chlorophyll-*a*, organics and phaeopigments in *Sabella* treated plots. As such, there has been some concern about the effect of *S. spallanzanii* on nitrogen cycling including changes in nutrient availability and its consequences for community structure (Harris et al., 1996).

Changes in bacterial communities in response to experimental treatments were consistent with those observed for macrofaunal and eukaryote communities. Soft-sediment dwelling bacteria play a crucial role in the functioning of these habitats and are recognized for their sensitivity to environmental change. Recent studies have highlighted the ability of *S. spallanzanii* to filter, accumulate and concentrate bacteria from the surrounding environment (Stabili et al., 2006; Licciano et al., 2007). *Sabella spallanzanii* have also been shown to exude mucus with antibacterial properties, which may influence the composition of microbial biofilms on the worm's tube and surrounding sediments (Stabili et al., 2011). In contrast, nutrient enrichment and increased primary production observed in *Sabella* treated plots may decrease the redox potential in sediments and favor sulfide-oxidizing and sulfate-reducing bacteria for example, as illustrated by the observed increase in the abundance of Desulfobacteraceae. Regardless of the mechanisms that may be responsible for these effects, the observed changes in bacterial communities may have important consequences for the functioning of soft-sediment habitats, most notably on nutrient and organic matter cycling.

It is often assumed that the impacts of invasive species are density-dependent, although few studies have assessed this contention (Thomsen et al., 2011) and this may not always be the case. We found no effect of density in our simulated invasion of such an engineer throughout our study, which may indicate that no real effect occurred, that the range of experimental densities was too small to have an effect, or that the duration of the study was insufficient for density-based differences to emerge. Non-indigenous filter-feeder species, such as *S. spallanzanii*, are more likely to affect communities over longer time scales than provide instantaneous shifts or impacts (Byrnes and Stachowicz, 2009). Nonetheless, the simple presence of fanworms or mimics (≥ 10 m^{-2}) appeared to prompt community differences compared to plots without them. One mechanism is through secondary colonization of *S. spallanzanii* tubes that can provide additional habitat for a range of epifauna typically associated with hard substrates (O'Brien et al., 2006). Although no significant changes in total macrofaunal abundance were detected, the presence of *S. spallanzanii* and mimics was associated with higher eukaryote taxa richness and higher abundance of encrusting and non-indigenous organisms such as tube worms (*Hydroides elegans*), ascidians and hydroids, evidenced by the macrofaunal and eukaryote datasets. These changes were reflected in a non-significant trend for an increase in macrofauna taxa richness in mimic plots compared to the controls. Although metabarcoding identification resulted in similar taxonomic partitioning, estimated taxa richness was an order of magnitude higher than that using morphological identification. Metabarcoding provides a more holistic view of the metazoan taxonomic diversity, regardless of the size and developmental stage (e.g., including eggs or larvae). The eukaryote data not only included macrofaunal taxa that dominated morphological samples, but also small-sized meiofaunal species (<1 mm), extending the scope of the analysis. Novel epifaunal taxa facilitated by the presence of *S. spallanzanii* may have important implications in the functioning of invaded soft-sediment habitats, as the combined filtering capacity of these organisms is considerably enhanced compared to uninvaded habitats (Lemmens et al., 1996). Furthermore, positive and potentially synergistic interactions among invasive species may lead to accelerated impacts on native ecosystems (Simberloff and Von Holle, 1999).

The results reported here are generally consistent with previous studies looking at the impacts of *S. spallanzanii* in soft sediments assemblages (O'Brien et al., 2006; Ross et al., 2007). However, the effects recorded in these Australian studies from Port Phillip Bay were dependent on spatial distribution and density of *S. spallanzanii*, with negligible effects recorded at low densities (<1 individual per m^2). However, similarly to this study, localized dense clumps (15 individuals per m^2), caused changes in benthic infauna structure characterized by lower abundances of cumaceans, ostracods and harpacticoid copepods (O'Brien et al., 2006). Ross et al. (2007) found no effect of *S. spallanzanii* on the resident macrofauna, with the exception of lumbrinerid polychaetes and gammarid amphipods; however, these taxa only represented a small proportion of those present. Ross et al. (2013) found no difference in the total abundance of macrofauna between *S. spallanzanii* and control plots. However, the composition of assemblages did change significantly in the presence of *S. spallanzanii*, with a significant increase in the abundance of echinoderms (largely attributable to brittle stars) in the presence of *S. spallanzanii* at all three sites investigated (Ross et al., 2013).

Our study site experiences relatively high velocity tidal currents (average 1.25 m s^{-1}, Greig and Proctor, 1988), which may pose physical stress on organisms and are unfavorable for the accumulation of organic matter. It is likely that these prevailing hydrographic conditions and resulting sedimentary physical conditions may exert a stronger meso-scale effect on biological communities compared to the relatively subtle observed response to the experimental treatments. In this context, the generality of observed effects to a regional scale or other locations is difficult to gauge. On the other hand, conditions that promote a mixed particle size benthic surface (larger fragments within a sandy-silt matrix) and regular water flow may be ideal for *S. spallanzanii* settlement and growth. *Sabella spallanzanii* is also among the only or very few organisms in this area's benthic community that can extend as high as 50 cm above the seafloor into the water column to take advantage of passing food-laden currents. A key factor for future work is to monitor population density, especially clusters of high density, because the magnitude of effects would undoubtedly increase if such novel canopies of this species proliferated.

Shallow subtidal soft sediment habitats are an extensive component of bays and estuaries and provide an important environment for *S. spallanzanii* establishment and spread. This species represents a relatively novel introduction as an epibenthic (rather than infaunal) invader that is not (almost) exclusively associated with hard-bottom habitats, like a majority of marine introduced species in New Zealand (Cranfield et al., 1998) and elsewhere (Byrnes et al., 2007; Ruiz et al., 2011). Given that these habitats are a dominant and important feature in the study area and wider region, it would be valuable to replicate this study in other areas to better understand the generality of effects of this invasion. As it is a listed "unwanted" organism under New Zealand legislation, there are active campaigns to (a) prevent its anthropogenic spread (mainly via shipping and boating) and (b) locally extirpate any nascent populations that are detected in discrete new areas using targeted removals (Read et al., 2011). These efforts, combined with monitoring hotspot populations that are not being managed, provide a precautionary approach to *S. spallanzanii* in New Zealand (Champion, 2018). An eradication attempt in the Auckland region is considered too uncertain and potentially expensive to initiate at present using existing tools and strategies. This view may change as marine eradication matures and if impacts of NIS on benthic communities, functioning, and maritime industries (e.g., aquaculture) proliferate (Edwards and Leung, 2009; Soliman and Inglis, 2018).

NIS are generally considered among one of the greatest threats to marine ecosystems, however our understanding about their overall impacts is often insufficient (Thomsen et al., 2011), partly because of the lack of adequate studies and context-dependency of their effects. Elucidating and predicting context-dependent impacts of NIS are crucial for assessing the associated risks to biodiversity and ecosystem functioning, and to underpin management efforts. At a global scale it has been shown that NIS have significant, although modest in magnitude, ecological effects (Anton et al., 2019). Using a combination of morphological and molecular taxonomical techniques, our study provides insights into the nature and magnitude of local-scale effects on different communities inhabiting soft-sediment habitats, which may inform the implementation of effective knowledge-based management and mitigation measures that reduce the impacts of NIS.

DATA AVAILABILITY STATEMENT

All data and R scripts used for the analyses are available at https://github.com/jatalah/sabella_impacts_experiment.git.

AUTHOR CONTRIBUTIONS

JA, OF, MT, LT, and AL: conceived and designed the experiments. JA, OF, MT, LT, and AL: performed the experiments. JA: analyzed the data. XP: analyzed molecular samples. JA, XP, and OF: wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2019.00481/full#supplementary-material

Supplementary Video 1 | Video footage of the field set-up of the experiment in conducted in Waitemata Harbour, Auckland, New Zealand showing the treatments to 1 m^2 experimental plots: live *Sabella spallanzanii* worms, mimic *S. spallanzanii* worms and controls without worms. Live and mimic worms were transplanted at four different densities [i.e. 10, 20, 35 and 50 worms (or mimics) per plot].

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BRIEF RESEARCH REPORT
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Greenhouse Gas Emissions From Native and Non-native Oysters

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Non-native species introductions are associated with a range of ecosystem changes such as habitat destruction, competition with native species, and biodiversity losses. Less well known is the role non-native species play in altering biogeochemical processes, such as the emission of greenhouse gases (GHGs). In this study we used laboratory incubations to compare seasonal (spring, summer, fall) emissions of the GHGs nitrous oxide (N_2O), methane (CH_4), and carbon dioxide (CO_2) from native (*Crassostrea virginica*) and non-native (*Ostrea edulis*) oysters collected from a northern temperate estuary (Duxbury Bay, Massachusetts, USA). We observed strong seasonal signals in GHG fluxes, where *C. virginica* was the higher GHG emitter, and produced on average twice as much N_2O (0.39 nmol g^{-1} dry tissue weight hr^{-1}) and 20 times as much CH_4 (1.31 nmol g DTW^{-1} hr^{-1}) compared to *O. edulis* (0.16 nmol N_2O g DTW^{-1} hr^{-1} and 0.07 nmol CH_4 g DTW^{-1} hr^{-1}). *C. virginica* also had significantly ($p < 0.001$) higher summer maximum production rates of CO_2 compared to *O. edulis* (53.4 μmol g DTW^{-1} hr^{-1} and 45.4 μmol g DTW^{-1} hr^{-1}, respectively). Despite these differences, chlorophyll-a consumption rates between the species were similar ($p = 0.95$). These results suggest that the non-native *O. edulis* is a lower GHG emitter than the native *C. virginica* and highlight that, at least in terms of GHG emissions, this non-native species introduction may not be detrimental to the environment.

Keywords: methane, nitrous oxide, exotic species, coastal biogeochemistry, carbon dioxde

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INTRODUCTION

The introduction of non-native species to both terrestrial and aquatic ecosystems is a growing global concern (Vitousek et al., 1996; Castro-Díez et al., 2019; Seebens et al., 2019). In some cases, these non-native species have led to dramatic declines in biodiversity (Molnar et al., 2008) and displacement of native species (Byers, 2000; Rossong et al., 2006). In other cases, they appear to have little to no impact (e.g., Grosholz, 2005). The effect of non-native species on marine ecosystems is not well constrained. A recent meta-analysis examined the impact of 76 exotic marine species (just 6% of the listed exotics) on marine communities and found that these species caused a small, but significant decrease in ecological variables such as primary producers and multi-trophic assemblages (Anton et al., 2019). Of note, they reported that mollusks were one of just four taxonomic groups related to declines in the ecological function of many native marine taxa (Anton et al., 2019). This finding agrees with a variety of studies on the ecological impacts of exotic bivalves (Sousa et al., 2009; Helmer et al., 2019). One of the best-known examples is the introduction of the Pacific oyster, *Magallana gigas* (formerly *Crassostrea gigas)* to Europe. *M. gigas* has reportedly changed sediment properties (Green et al., 2012), shifted macrozoobenthic communities (Kochmann et al., 2008), and altered food web structure (Souchu et al., 2001). Alternatively, other studies have reported minimal ecosystem impacts following the introduction of *M. gigas* (Krassoi et al., 2008; Wilkie et al., 2012; Zwerschke et al., 2016).

In North America, the European Flat Oyster (*Ostrea edulis*) was intentionally introduced to the state of Maine (United States, US) with aims to establish an oyster fishery in the 1950s (Laing et al., 2006). Today, *O. edulis* has expanded its range south, and is now established from Maine to Rhode Island (FAO, 2009). The impact of this non-native species on ecosystem function is largely unknown. In this study, we compared emissions of nitrous oxide (N_2O) and methane (CH_4) from *O. edulis* and *C. virginica*, as previous research has shown that bivalves can be sources of these two powerful greenhouse gases (GHG) which have global warming potentials 298 and 25 times more than carbon dioxide (CO_2), respectively (Myhre et al., 2013).

Microbial processes occurring on (e.g., shell epibiota), or within (e.g., mantle cavity, digestive system) oysters can produce or consume N_2O. For example, both nitrification (which can produce N_2O) and denitrification (which can produce or consume N_2O) occur on *C. virginica* shells, although shell denitrification rates are higher from living oysters (Caffrey et al., 2016). Arfken et al. (2017) used a metabolic approach to demonstrate that the relative abundance of denitrifying bacteria was higher in *C. virginica* whole oysters and shells compared to nearby sediments, and concluded that oysters appear to be denitrifying "hot spots." Denitrification has also been confirmed in the epibiota of the Sydney Rock Oyster (*Saccostrea glomerata*; Erler et al., 2017). Most recently, significant N_2O emissions have been observed from the *C. virginica* digestive system (Ray et al., 2019a). Additionally, experimental incubations of living *C. virginica* individuals showed oysters switched from being a N_2O sink to a N_2O source when incubation water was enriched with dissolved inorganic nitrogen (DIN; Gárate et al., 2019). Together, these studies demonstrate that oysters support dynamic microbial ecosystems capable of altering N_2O fluxes in coastal ecosystems.

Compared to N_2O, we know much less about CH_4 cycling in oysters. Previous research shows that bivalve gut cavities can house archaeal methanogenic symbionts and methanotrophs can live in the mantle cavities of bivalves (Childress et al., 1986; Bonaglia et al., 2017). Thus, it seems reasonable to propose that oysters too may host CH_4 producing and consuming organisms. However, a recent study reported only very low CH_4 emissions from *C. virginica* ($0.00038 \pm 0.00046\ \mu\text{mol CH}_4\ \text{gDW}^{-1}\ \text{hr}^{-1}$), rates not statistically different from zero (Ray et al., 2019b). Thus, CH_4 emissions from oysters are largely unknown, but potentially small.

Within this context, we quantified the net fluxes of N_2O and CH_4, as well as chlorophyll-a by *C. virginica* and *O. edulis* over an annual temperature cycle. We also measured dissolved oxygen uptake and used it to estimate CO_2 release for each species using a respiratory quotient from the literature (Galtsoff et al., 1964). Our primary goals were to quantify GHG emissions from a native (*C. virginica*) and non-native (*O. edulis*) oyster species, to examine how these GHG fluxes varied with environmental parameters (e.g., temperature, salinity) and oyster characteristics (i.e., dry tissue and shell weight), and to add to the growing literature on the potential role non-native species play in altering biogeochemical cycling. We hypothesized that each species would release N_2O, but little to no CH_4, and that GHG fluxes would increase with temperature.

METHODS

Site Description

We collected oysters and site water from Duxbury Bay (Massachusetts, USA) on six occasions between September 2017 and August 2018 (**Figure S1**). Duxbury Bay is a shallow system with an average water depth of 3 m at high tide, and several exposed mudflats at low tide (Feinman et al., 2018). The system exchanges 70% of its water volume with the Atlantic Ocean twice daily (Lawson, 2011).

Sample Collection

During each sampling event, we measured water column dissolved oxygen (DO) concentration, pH, salinity, and temperature using a Hach HQ40d, equipped with LDO101, PHC301, and CDC401 probes (**Table S1**). We measured water column chlorophyll-a concentrations in duplicate with a 60 mL polypropylene syringe and glass fiber filters (Whatman GF/F, 0.70 μm pore size). Chlorophyll-a filters were stored at −80 °C until analysis.

Upon collection, oysters were immediately placed in a cooler filled with site water. We also collected unfiltered site water in carboys. Both were transported back to the laboratory, and kept in a dark environmental chamber set to *in situ* field temperature. Once in the environmental chamber, air-stones were placed in the oyster filled cooler and the carboys and were gently bubbled overnight (>12 h) to keep the overlaying water oxygenated.

Oyster GHG Incubation

C. virginica and *O. edulis* individuals were randomly assigned to an incubation chamber filled with site water using a random number generator (Randomness and Integrity Services Ltd.). Each treatment (i.e., native: *C. virginica* and non-native: *O. edulis)* had three clear polyvinyl chloride (PVC) chambers (2.106 L) per incubation. In cool months (September 2017, March 2018, April 2018, June 2018), we placed four oysters in each chamber. In warmer months (July 2018, August 2018) we placed three oysters in each chamber to ensure DO would not drop too rapidly during the incubation due to elevated oyster respiration. We changed the number of oysters used in summer incubations to reduce the risk of hypoxic conditions in the chambers during warm temperatures and higher rates of respiration. For each incubation, we also incubated triplicate chambers containing site water alone (i.e., water column control chambers) to account for any fluxes occurring in the water column.

Before sealing the chambers with gas tight lids, we collected samples for the initial chlorophyll-a concentrations in each chamber using the methods described above for chlorophyll-a collection in the field. We repeated this sampling at the end of the incubation. We then sealed each chamber without any air headspace using gas-tight acrylic lids equipped with inflow and outflow ports (Ray et al., 2019a). Magnetic stir bars fixed to the core tops provided gentle mixing of the overlying water (~40 revolutions min^{-1}). Incubation time was determined by balancing the following criteria: achieving at least a 62.5 μM (2 mg O_2 L^{-1}) drop in DO (Foster and Fulweiler, 2016) over the course of five sampling time points at intervals of ~20–60 min, and maintaining DO concentrations above the hypoxic threshold (>62.5; Heiss et al., 2012). DO concentrations were measured at three time points (initial, middle, and final) using an optical dissolved oxygen sensor (Hach LDO101). Gas samples for N_2O and CH_4 were collected in duplicate water samples at five time points allowing for overflow for each sample vials into 12 mL Labco Limited Exetainer® with gastight septa. Sample vials were filled from the bottom with ~3 times the overflow volume and preserved with 25 μL of saturated zinc chloride solution (Ray et al., 2019b). These samples were immediately transferred to a refrigerator (4–6 °C) until further analysis.

During our six incubations, some chambers did drop below the hypoxic threshold. In the July incubation, all three of the *O. edulis* chambers went hypoxic, and in the August incubation, two *O. edulis* chambers went hypoxic. Regardless, our flux data remained linear, so we included these chambers in our analysis. Lights in the environmental chamber were left on during the gas incubations due to short sampling windows. At the start and end of incubations, we visually checked that oysters were open and actively filtering water.

Within 24 h of the incubation, we measured the shell length, width, and depth (cm) of each oyster. We then shucked the oysters, weighed their shell and tissue wet mass (g), and placed them in a drying oven set to 60°C until a constant weight was reached.

Calculating Shell Surface Area

In order to estimate shell surface area, we used photogrammetric processing software (Agisoft Metashape Professional 1.5.2) to create three-dimensional mesh renderings of a subset of specimens ($n = 36$ for *C. virginica* and $n = 32$ for *O. edulis*). Each mesh was crafted from a series of ~50 images of each specimen top, curved shell only, taken with a digital camera.

Sample Analysis and Flux Calculations

Chlorophyll-a analysis was conducted by extracting the sample filter in 10 mL of 90% acetone, followed by fluorometric analysis using a Turner Model AU-10 Digital Fluorometer with Optical Kit P/N 10-040R (Arar and Collins, 1997; Carey and Fulweiler, 2013).

We measured concentrations of dissolved N_2O and CH_4 gas directly using a headspace equilibration technique followed by analysis of the sample headspace using gas chromatography (GC) following published methods (Kling et al., 1991; Foster and Fulweiler, 2016). All standard curves had $R^2 \geq 0.995$ for six data points used. The detection limits during sample analysis were 0.383 μM for N_2O and 5.188 μM for CH_4.

Flux rates of each GHG were determined by calculating the slope of the linear regression of analyte concentration over the incubation time (Giblin et al., 1997; Hopkinson et al., 1999; Heiss et al., 2012) A flux was only considered significant when $R^2 \geq 0.65$ and $p < 0.10$ (Prairie, 1996; Foster and Fulweiler, 2016). If these criteria were not met, the flux was determined to equal zero, and indicated that either no flux occurred, there was a balance between production and consumption processes in the chamber, or fluxes occurred at rates below the detection limit (Foster and Fulweiler, 2016). The resulting fluxes were scaled by total dry tissue mass and all GHG fluxes are reported as nmol hr^{-1} g DTW^{-1}. Chlorophyll-a and DO flux rates were calculated as the difference in final and initial concentrations over the incubation time, scaled by total dry tissue mass, and are reported as μmol hr^{-1} g DTW^{-1}. We calculated oyster CO_2 production using a respiratory quotient (RQ) of 0.83 for *C. virginica* (Galtsoff et al., 1964) and *O. edulis* because we were unable to find an RQ value for *O. edulis* in the literature (**Table S3**). In all cases, a positive flux indicates production of an analyte by the oyster while a negative flux indicates the consumption of analyte from the water column by the oysters (Fulweiler et al., 2008; Heiss et al., 2012).

Statistical Analysis

All statistical analyses were conducted using R studio (version 3.4.4) and were considered statistically significant when $p \leq 0.05$. We compared shell area per g DTW, shell dimensions, and tissue and shell mass between species using Wilcoxon rank-sum tests. We compared whether fluxes were significantly differently from zero using one-sample Wilcoxon signed rank tests. Before comparing fluxes between species, we identified the distributions that best described each flux using the *fitdistrplus* package (Marie et al., 2015). N_2O fluxes best fit a lognormal distribution, CH_4 and chlorophyll-a fluxes were normally distributed, and CO_2 fluxes best fit a gamma distribution.

To compare net fluxes between species we used a mixed model approach. In order to best meet model assumptions, we first shifted all of the N_2O and O_2 data so that all values were positive, and then applied a log transformation to the N_2O data. CH_4, CO_2, and chlorophyll-a fluxes were not transformed. Next, we constructed a Generalized Linear Model (GLM) for each flux, using the *lme4* package (Bates et al., 2015) with oyster species and temperature as fixed effects. For the CO_2 data, we used a gamma family in the model. We then compared whether the two species differed using a pairwise least-square mean test of the GLM with the *emmeans* package (Lenth, 2018). Additionally, we tested whether fluxes differed between seasons by constructing models that included oyster species and season as fixed effects, and then compared seasons using pairwise least-square mean tests. We also compared fluxes between species using this model (**Table S6**).

Correlations between GHG fluxes and salinity, respiration, feeding rates, and shell characteristics for each species were calculated using Pearson correlation tests. We did not run correlations for shell surface area and GHG flux because we did not have area measurements for all of our specimens. We used Pearson correlation tests to quantify if any biotic (e.g., feeding rates, shell characteristics) or abiotic factors (e.g., salinity) we measured were related to GHG emissions from each species. Additionally, we tested for correlations between fluxes of the three GHGs to see if there were any common flux patterns. Finally, we used linear regressions to test if temperature could be used to predict the magnitude of GHG flux for each species.

RESULTS

Physical Characteristics of Oyster Species

C. virginica and *O. edulis* shells had statistically different shell widths ($p < 0.001$) and depths ($p < 0.001$) but not shell lengths ($p = 0.08$). On average, *O. edulis* used in the experiment were longer (mean length of 8.93 ± 0.25 cm) than *C. virginica* (mean length 8.22 ± 0.13). *O. edulis* was also wider (mean width 8.25 ± 0.15 cm) than *C. virginica* with mean widths (mean width 5.78 ± 0.13 cm). However, *C. virginica* had a deeper cup (2.19 ± 0.07 cm) than *O. edulis* (1.91 ± 0.06 cm) (**Tables S2, S4**). The average dry tissue mass per gram dry shell mass (DTW per g DSW) was significantly different between treatments ($p < 0.01$) where *O. edulis* had more tissue per gram of shell than *C. virginica* (mean 0.11 ± 0.02 DTW g DSW^{-1} and 0.08 ± 0.01 DTW g DSW^{-1}, respectively). For the subset of shells we measured, shell surface area was not significantly different between species ($p = 0.33$). However, *C. virginica* had significantly ($p < 0.001$) more shell area per tissue mass (110.34 ± 8.7 cm^2 shell g DTW^{-1}) than *O. edulis* (67.91 ± 4.02 cm^2 shell g DTW^{-1}).

GHG Fluxes and Correlation Analysis

Both oyster species emitted N_2O at rates significantly different from zero (*C. virginica* $p = 0.02$ and *O. edulis* $p = 0.02$). On average, *C. virginica* emitted more than twice as much N_2O than *O. edulis* (0.39 ± 0.16 nmols hr^{-1} g DTW $^{-1}$ and 0.16 ± 0.06 nmols hr^{-1} g DTW^{-1}, respectively), but these rates were not statistically different ($p = 0.276$). In general, net N_2O production was observed during spring (March and April 2018) and fall (September 2017) while little N_2O flux occurred during the summer (June, July and August 2018) (**Figure 1A**). N_2O fluxes were statistically higher in spring compared to summer ($p < 0.01$) or fall ($p = 0.05$), and higher in the fall compared to summer ($p < 0.01$) (**Table S5**). There was no significant relationship between N_2O fluxes and temperature for either species (**Figures 2A,B**). Additionally, N_2O fluxes were only correlated to salinity for *C. virginica* (**Table 1**) and to g DTW for *O. edulis* (**Table 2**; **Figure S2**).

Neither *C. virginica* ($p = 0.06$) nor *O. edulis* ($p = 0.22$) released CH_4 at rates statistically different from zero when all incubations were pooled. CH_4 fluxes also did not differ significantly between species ($p = 0.08$). Despite not being statistically significant, *C. virginica* produced, on average, more than twenty times as much CH_4 as *O. edulis* (mean 1.31 ± 2.62 nmol hr^{-1} g DTW $^{-1}$ and 0.07 ± 1.41 nmol hr^{-1} g DTW $^{-1}$, respectively). Generally, CH_4 release occurred during the summer (June, July and August 2018), while small rates of CH_4 production, or oxidation, were observed during the spring (March and April 2018) and fall (September 2017) (**Figure 1B**), though these seasonal changes were not statistically different (**Table S5**). Similarly, temperature was not a significant predictor of CH_4 flux (**Figures 2C,D**). *C. virginica* CH_4 fluxes were positively correlated with CO_2 fluxes (**Table 1**) while CH_4 fluxes from *O. edulis* were not correlated any of the parameters we measured (**Table 2**; **Figure S2**).

CO_2 production rates were not significantly different between *C. virginica* and *O. edulis* ($p = 0.38$) and mean rates of production were 24.77 ± 18.88 μmols hr^{-1} g DTW $^{-1}$ and 19.44 ± 14.52 μmols hr^{-1} g DTW $^{-1}$, respectively. However, *C. virginica* had a higher maximum production rate of CO_2 than *O. edulis* during the summer (53.4 μmols hr^{-1} g DTW^{-1} and 45.4 μmols hr^{-1} g DTW $^{-1}$, respectively; **Figure 1C**). CO_2 release was lower in the spring compared to summer ($p < 0.01$) or fall ($p < 0.01$),

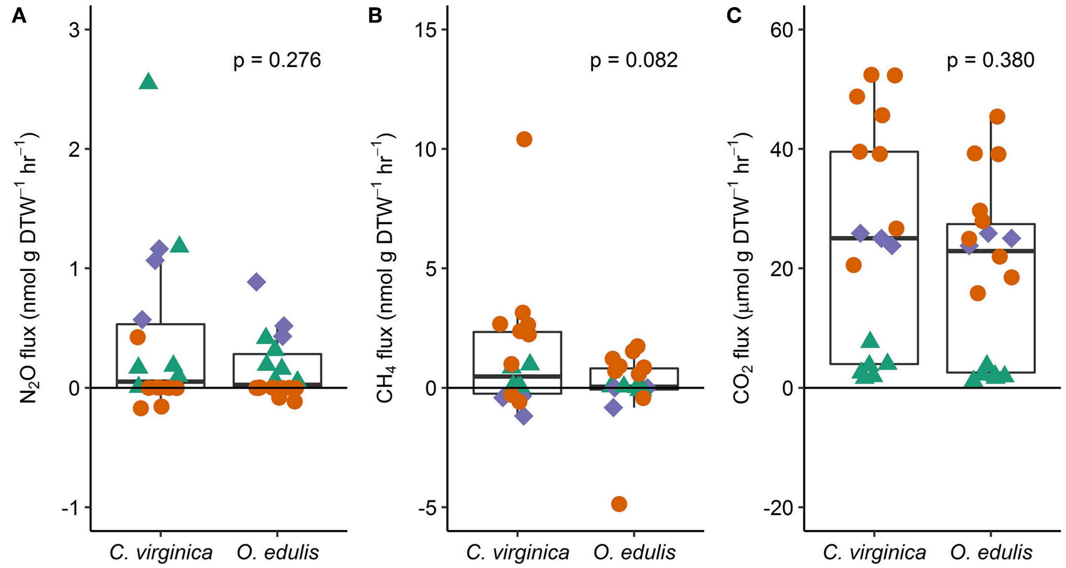

FIGURE 1 | Fluxes of nitrous oxide **(A)**, methane **(B)**, and carbon dioxide **(C)** from native (*C. virginica*) and non-native oysters (*O. edulis*); $n = 18$ for each species and flux, except $n = 17$ for *C. virginica* CO_2 fluxes. *P*-values show the result of least squares means tests comparing the mean flux between the native and non-native oysters. Each point represents an individual flux measurement (spring: green triangles, summer: orange circles, and fall: blue diamonds). Points below the zero-line show net consumption, and points above show net production.

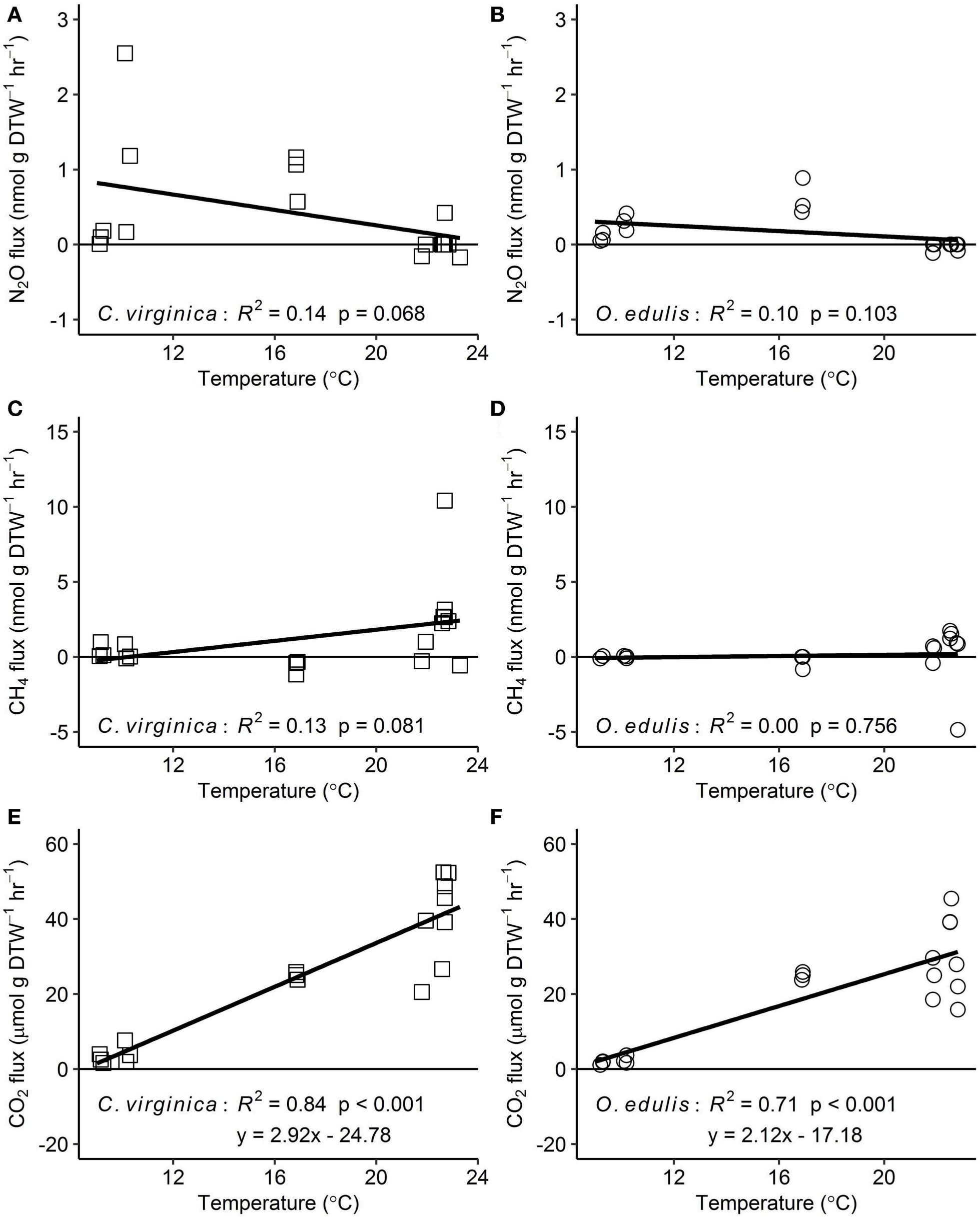

FIGURE 2 | Relationships between temperature and nitrous oxide **(A,B)**, methane **(C,D)**, and carbon dioxide **(E,F)** fluxes for native (*C. virginica*: open squares) and non-native oysters (*O. edulis*: open circles); $n = 18$ for each species and flux, except $n = 17$ for *C. virginica* CO_2 fluxes. R^2 and p-values from linear regressions. Points below the zero-line show net consumption, and points above show net production.

and there was no difference in CO_2 release between summer and fall ($p = 0.31$) (**Table S5**). Temperature was a significant predictor of CO_2 release from both species (**Figures 2E,F**). *C. virginica* CO_2 production was positively correlated with CH_4 flux and salinity (**Table 1**), while *O. edulis* CO_2 production was positively correlated with salinity and negatively correlated with chlorophyll-a consumption (**Table 2**; **Figure S2**).

Chlorophyll-a consumption rates for *C. virginica* and *O. edulis* were both significantly different from zero ($p < 0.01$ for each species), but were not significantly different between species ($p = 0.95$). Chlorophyll-a consumption was lower in the spring compared to summer ($p = 0.03$) or fall ($p = 0.02$), but consumption was not different between summer and fall ($p = 0.64$) (**Table S5**). *C. virginica* chlorophyll-a consumption rates were negatively correlated with DTW (**Table 2**) and *O. edulis* chlorophyll-a consumption rates were negatively correlated to CO_2 production and temperature (**Table 2**).

Pairwise comparisons between species using a model with season and species as fixed effects yielded the same results as the model with temperature and species as fixed effects (**Table S6**).

TABLE 1 | Pearson correlations between greenhouse gas fluxes, g dry oyster tissue (DTW) and g dry oyster shell (DSW), and salinity for *C. virginica*.

	CO_2 flux	**CH_4 flux**	**N_2O flux**	**Chl a flux**	**DTW**	**DSW**	**Salinity**
CO_2 flux	1.00	**0.50 (0.04)**	−0.34 (0.19)	−0.22 (0.39)	−0.08 (0.77)	−0.16 (0.54)	**0.84 (<0.01)**
CH_4 flux		1.00	−0.14 (0.58)	0.05 (0.86)	−0.44 (0.07)	−0.45 (0.06)	0.31 (0.21)
N_2O flux			1.00	−0.19 (0.46)	0.32 (0.20)	−0.23 (0.37)	**−0.48 (0.04)**
Chl a flux				1.00	**−0.72 (0.001)**	−0.36 (0.16)	−0.46 (0.06)

CO_2 flux is reported in μmol hr^{-1} g DTW^{-1} and N_2O and CH_4 flux are reported in nmol hr^{-1} g DTW^{-1}. Chlorophyll-a (Chl a) fluxes are reported in (μg^{-1} hr^{-1}). For each pair of variables, the top number represents the r-value while the value in parentheses beneath it is the p-value. Significant relationships ($p \leq 0.05$) are indicated in bold (except for 1:1 correlations).

TABLE 2 | Pearson correlations between greenhouse gas fluxes, g dry oyster tissue (DTW) and g dry oyster shell (DSW), and salinity for *O. edulis*.

	CO_2 flux	**CH_4 flux**	**N_2O flux**	**Chl a flux**	**DTW**	**DSW**	**Salinity**
CO_2 flux	1.00	0.28 (0.26)	**−0.16 (0.05)**	**−0.68 (< 0.01)**	0.09 (0.73)	−0.44 (0.07)	**0.84 (<0.01)**
CH_4 flux		1.00	−0.08 (0.76)	−0.33 (0.18)	−0.16 (0.53)	−0.12 (0.63)	0.05 (0.84)
N_2O flux			1.00	0.17 (0.51)	**0.80 (<0.01)**	−0.18 (0.46)	−0.26 (0.30)
Chl a flux				1.00	0.10 (0.70)	0.05 (0.85)	−0.44 (0.07)

CO_2 flux is reported in μmol hr^{-1} g DTW^{-1} and N_2O and CH_4 flux are reported in nmol hr^{-1} g DTW^{-1}. Chlorophyll-a (Chl a) fluxes are reported in (μg^{-1} hr^{-1}). For each pair of variables, the top number represents the r-value while the value in parentheses beneath it is the p-value. Significant relationships ($p \leq 0.05$) are indicated in bold (except for 1:1 correlations).

DISCUSSION

Here we demonstrate that both the native *C. virginica* and the non-native *O. edulis* produce significant amounts of N_2O and CO_2, but not CH_4. Mean annual CH_4 fluxes were not statistically different between the two oysters however, they varied temporally with consumption in the fall and spring and notable pulses of production in the summer months (**Figure 1B**). Although not significantly different, *C. virginica* produced twice as much N_2O and ~twenty times more CH_4 than the non-native *O. edulis.* Further, we show that GHG fluxes and chlorophyll-a consumption rates were not driven by a common set of environmental parameters, nor did fluxes vary consistently with oyster characteristics. These chlorophyll consumption rates may be an underestimate as the chamber was closed and thus we did not introduce new food overtime.

Because metabolic processes scale with temperature, we anticipated GHG fluxes to be highest during warm summer months, but this pattern only held for CO_2. Perhaps this is not surprising, as we measured net fluxes and thus N_2O and CH_4 production and consumption processes could both be stimulated by warmer temperatures. Regardless, the overall net effect of temperature on N_2O and CH_4 fluxes appears to be zero, an effect previously observed for sediment N_2O fluxes (Foster and Fulweiler, 2016). Instead, we observed highest N_2O fluxes at the intermediate temperatures during March and September, suggesting that during these time periods other factors may be more important in regulating oyster N_2O flux. For example, Gárate et al. (2019) found that the addition of DIN to incubations of *C. virginica* more than tripled rates of N_2O release. Previous research has proposed that N_2O is released from bivalves due to inefficient nitrification in the shell biofilm (Rossong et al., 2006; Svenningsen et al., 2012; Heisterkamp et al., 2013; Ray et al., 2019a), or as a byproduct of denitrification in the gut (Stief et al., 2009; Heisterkamp et al., 2010; Svenningsen et al., 2012; Ray et al., 2019a). Sediment N_2O fluxes also increase with higher concentrations of DIN in the water column as nitrification and denitrification become less efficient (Seitzinger and Nixon, 1985; Beaulieu et al., 2011). In this study, we did not measure DIN concentrations in the site water. However, we can estimate periods of high DIN at our sampling sites using daily discharge data from the closest United States Geological Survey gage (USGS 01105870) located on the Jones River, which empties into Duxbury Bay. River discharge is typically positively correlated with DIN loading to estuaries (e.g., Fulweiler and Nixon, 2005). The period of highest river discharge—and therefore likely highest DIN loading—during the course of our study was in March 2018. This was followed by a decrease in discharge through the summer months, and another peak in discharge in September 2018. These times also correspond with the highest oyster N_2O emissions we measured. Further, we recorded a significant negative correlation between N_2O flux and salinity for *C. virginica*. Thus, we hypothesize that during periods of relatively low salinity, riverine water that is higher in DIN drives the emission of N_2O from both the native and non-native oyster, and suggest that in estuarine systems, seasonal patterns of DIN loading from river discharge are more important in regulating oyster N_2O release than temperature or oyster species.

Similar to N_2O, CH_4 fluxes were not predicted by temperature alone, though fluxes of the largest magnitude, regardless of release or uptake, occurred during the warmest incubations. We have a poor understanding of how bivalves regulate CH_4 fluxes, and the conditions that promote or inhibit CH_4 release. We know that the Baltic Clam (*Limecola balthica)*, hosts archaeal methanogenic symbionts inside its gut cavities (Bonaglia et al., 2017), and deep-sea mussels (*Bathymodiolus thermophilus)* have methanotrophic symbionts in their mantle cavities (Childress et al., 1986), demonstrating that microbes responsible for regulating CH_4 in marine environments do associate with bivalves. Our results show that there is both production and consumption of CH_4 by oysters, yet the environmental drivers of these processes are unclear. It is likely that microbially-mediated methanogenesis and methanotrophy both proceed simultaneously in oysters, and occurrences of CH_4 release or uptake are due to environmental conditions turning these pathways on or off. We recorded a significant positive correlation between CO_2 and CH_4 release in *C. virginica*, suggesting that when more CO_2 is present (and less O_2 is available) near the oyster, oxygen levels are low enough to slow methanotrophy and allow some CH_4 to escape. Alternatively, with lower O_2, methanogenesis may become more rapid, with no change in methanotrophy.

Ray et al. (2019b) measured GHG emissions from *C. virginica* and the sediments beneath them, and then compared the CO_2-equivalent cost per g protein from oysters with terrestrial livestock. In their estimation, which only required N_2O and CH_4 emissions from the oyster as CO_2 from respiration is not included in estimates of other animal GHG release, they assumed that N_2O and CH_4 release from oysters was highest in the summer, and used summer GHG release values across the whole life-cycle of the oyster as a conservative approach. They estimated an oyster GHG cost of 0.13 kg CO_2-eq kg protein^{-1}. While their approach was correct for assuming no difference in flux due to temperature, here we demonstrate that N_2O and CH_4 emissions do not necessarily proceed most rapidly during warm summer months. While the mean rate of N_2O release in this study (0.39 nmol g DTW^{-1} hr^{-1}) is relatively close to that reported by Ray et al. (2019a: 0.12 nmol g DTW^{-1} hr^{-1}), it is slightly higher due to the spring and fall samples. Similarly, Ray et al. (2019b) reported a flux of 0 for CH_4. Yet in this study we measured instances of release and consumption, and only reported a value of zero for *C. virginica* as the *p*-value comparing the CH_4 flux against zero was equal to 0.06, instead of the ≤ 0.05 value we selected as "statistically significant" before beginning the experiment. If we use the slightly higher mean values reported in this study and the same methods as Ray et al. (2019b), who estimated a GHG cost of 0.13 kg CO_2-eq kg protein^{-1}, we estimate that *C. virginica* has a GHG cost of 1.3 kg CO_2-eq kg protein^{-1} and *O. edulis* has a GHG cost of 0.52 kg CO_2-eq kg protein^{-1}. While higher than the previous estimate, these values are still much lower than the GHG cost of beef, which is 465.5 kg CO_2-eq kg protein^{-1} (Opio et al., 2013; ARS, 2018; Ray et al., 2019b). It is likely that N_2O releases by oysters is only a small portion of their nitrogen budget (Ray et al., 2019a). Additionally, the CH_4 observed emissions were <0.01% of the average CO_2 emissions and generally, oyster reefs are net carbon sinks (Dame et al., 1989).

This study adds to our overall understanding of the role oysters in general, and a native vs. non-native oyster species in particular, play in regulating GHG emissions from coastal ecosystems. It demonstrates that, at least in terms of GHG fluxes, the non-native oyster species appears to produce less GHGs compared to the native species. This study is just a first step in understanding how oysters alter estuarine GHG emissions. Future research could address how oyster mediated GHG fluxes vary with changes in oyster physiology and behavior, as well as with different ecosystem parameters (e.g., seasonal nutrient concentrations and phytoplankton assemblages). Overall, this study highlights that ecological impacts of non-native species introductions are complex and vary by the ecological parameter being studied.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

RF provided the funding and facilities for this research. GM and NR completed the field, laboratory, and data analyses. All authors designed the research, contributed to writing and editing of this manuscript, and agree to be listed and accept responsibility for the manuscript.

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In a Pinch: Mechanisms Behind Potential Biotic Resistance Toward Two Invasive Crayfish by Native African Freshwater Crabs

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It is essential to incorporate mechanisms of biotic resistance into predictions of ecological impact conferred by invasive species. Trophically and functionally analogous native species have high potential to confer biotic resistance or to be susceptible to competitive exclusion by the invading species. In species with dominance hierarchies and high aggression the role of weapons, such as chelae, is emphasised and selected for. Differences in traits such as crushing capacity can indicate prey handling capabilities, while correlations between closing force and morphology can be used to understand the role of signaling in agonistic contests. Closing force strength can be used to infer the outcomes of both direct (predation) and indirect (competition) trophic interactions. Southern Africa has been invaded by two freshwater crayfish species (*Cherax quadricarinatus* and *Procambarus clarkii*). Biotic resistance of freshwater crabs toward crayfish invasions varies between geographic location and co-evolutionary history, thus comparing invasion histories without incorporating geographic context can produce unequivocal conclusions. We compared the closing force and chelae morphology of both crayfish species with a native trophically analogous freshwater crab, *Potamonautes perlatus*. Closing force increased significantly with mass for all species. There was significant interaction between sex and species on closing force. *Potamonautes perlatus* females showed significantly stronger maximum chela closing force than male *P. perlatus,* both sexes of *P. clarkii* and female *C. quadricarinatus*. Contrastingly, male *C. quadricarinatus* had significantly higher closing forces than both sexes of *P. clarkii* and female *C. quadricarinatus*, however, there was no difference between female *P. perlatus*. Native *P. perlatus* has the capacity to hold a competitive mechanical advantage over both invaders, but this varies with sex. Chelae length was not a significant predictor for closing strength in any of the species, which may be related to dishonest signaling in decapod species. This makes it imperative to assess whether factors such as closing force actually translate to resource holding potential in a contest scenario. We thus provide evidence that African freshwater crabs may exhibit biotic resistance toward invasion and the first measurements for *C. quadricarinatus* closing force strength.

Keywords: *Procambarus clarkii*, *Cherax quadricarinatus*, *Potamonautes perlatus,* invasive species, competition, strength

INTRODUCTION

Non-native species introductions and subsequent establishment continues to threaten global biodiversity (Seebens et al., 2017; Meyerson et al., 2019). The likelihood of a particular species successfully establishing and persisting in a novel environment is influenced by both abiotic and biotic factors (Byers, 2002; Pearson et al., 2018; Kinney et al., 2019). For example, evidence toward the environmental matching hypothesis suggests that when the climate of the incumbent ecosystem matches the climate of the native range, the invader has a higher potential to both persist and perform better than when the climate does not (Capinha et al., 2013; Iacarella et al., 2015). The community assemblage of the invaded system can also facilitate or impede establishment via biotic resistance (deRivera et al., 2005; Alofs and Jackson, 2014). Theoretically, areas with high species richness are more likely to have species that are strong competitors or, indeed, predators, which make the system less invadable than those with low species richness (Elton, 1958; Tilman, 1999). Although, in areas where species are functionally similar there is a likelihood that the analogous native species are outcompeted or niche excluded by the invader (Dick et al., 2017; Zeng et al., 2019).

Species interactions shape community dynamics through consumptive effects, but also in non-consumptive manners such as intraspecific and interspecific competition for resources such as food, shelter, and reproduction (Sih et al., 2010; Lopez et al., 2019; Mofu et al., 2019; Zeng et al., 2019). Biotic resistance can thus be exhibited in both consumptive and competitive manners, however, in freshwater systems biotic resistance is driven overwhelmingly by consumption (Alofs and Jackson, 2014). Aquatic environments exhibit a higher occurrence of generalist feeding and omnivory leading to a lack of intraspecific and interspecific competition (Alofs and Jackson, 2014). Moreover, dietary plasticity and frequency dependent predation (i.e., prey switching) are common traits of successful invaders as it allows persistence of species via trophic niche separation and capacity to consume new resources when one is over-exploited (Snyder and Evans, 2006; Olsson and Nyström, 2009; Havel et al., 2015). Often, successful invasive species also outcompete and competitively exclude native species for resources through agonistic interactions, though, aggressiveness does not always equate to resource holding potential (Camerlink et al., 2015; Lopez et al., 2019).

Freshwater crayfishes are highly successful invaders, having established widespread invasive populations. While the impacts, both positive and negative, have been reported for various crayfish species (see review by Lodge et al., 2012), the mechanisms that drive species persistence and increased impact are still somewhat unclear. The African continent is devoid of native crayfish species. This is potentially due to evolutionary competition with freshwater crabs (Ortmann, 1902; Lodge et al., 2012; Nunes et al., 2017a). Freshwater crabs of the genus *Potamonautes* are, however, present in almost all African freshwater habitats where they are trophically analogous to freshwater crayfish and provide essential nutrient cycling services (Hill and O'Keeffe, 1992; Dobson, 2004; Cumberlidge and Daniels, 2009; Peer et al., 2015). Potamonautid species typically exhibit high degrees of endemism and range restriction. These traits make them vulnerable to the impacts of habitat destruction and invasive species introductions (Cumberlidge and Daniels, 2009; Zeng and Yeo, 2018). Crabs and crayfish are polytrophic benthic omnivores that are both opportunistic scavengers and direct predators (Hill and O'Keeffe, 1992; Grey and Jackson, 2012). Due to the trophic similarities between crayfish and freshwater crabs it is likely that they will either provide an important component of biotic resistance or be competitively excluded by crayfish invasions (Lodge et al., 2012; Dick et al., 2017). Indeed, in Tanzania the red swamp crayfish, *Procambarus clarkii* (Girard, 1852) has replaced a native freshwater crab *Potamonautes neumannii* in many systems (Ogada, 2007), and has replaced *Potamonautes loveni* as the primary food source in African clawless otter diets in Lake Naivasha (Ogada et al., 2009). Similar trends are reported in Singapore where Australian redclaw crayfish *Cherax quadricarinatus* competitively excludes smaller native freshwater crabs from shelter resources (Zeng et al., 2019). In Cyprus the invasive *P. clarkii* shows shelter holding dominance over native freshwater crab *Potamon potamios* despite both species being equally aggressive (Savvides et al., 2015). Contrastingly, the European river crab *Potamon fluviatile* shows dominance in aggression and resource holding capacity toward the invasive *P. clarkii* but this may be driven by co-evolutionary history with the native *Astropotamobius italicus* crayfish (Cioni and Gherardi, 2004; Mazza et al., 2017). Again, there are similar reports that populations of native pseudothelphusid crabs persisting despite the *C. quadricarinatus* invasion in Mexico, likely due to the co-evolutionary history with native crayfish (Bortolini et al., 2007). Predicting the impacts of invasive crayfish species on native biota via consumptive and non-consumptive effects by way of comparing invasion histories can thus produce unequivocal conclusions due to geographical context.

Southern Africa is suffering from an over-invasion scenario by functionally similar crayfish species which have been introduced primarily through aquaculture ventures and the pet trade (Lodge et al., 2012; Russell et al., 2014; Nunes et al., 2017a,b; Weyl et al., 2020). *Cherax quadricarinatus*, the Australian redclaw crayfish and *P. clarkii* both have established invasive populations in South Africa (Nunes et al., 2017a,b). *Cherax quadricarinatus* is also present in Zambia (Nakayama et al., 2010; Nunes et al., 2016), Swaziland (Nunes et al., 2017a) and Zimbabwe (Marufu et al., 2018) while *P. clarkii* is invasive in Zambia, Uganda, Kenya, Egypt, Sudan, and Rwanda (Hobbs et al., 1989; Mikkola, 1996; Cumberlidge, 2009). Both species are likely to spread into ecologically and economically integral streams and wetlands, which will potentially threaten the stability of aquatic systems that provide refuge habitat for imperilled species such as Potamonautid crabs (Ahyong and Yeo, 2007; Belle et al., 2011; Nunes et al., 2017a,b; Zeng et al., 2019). To test whether there could be some degree of biotic resistance exhibited by African freshwater crabs, we measure and compare the maximum closing force of native *Potamonautes perlatus* to the invasive crayfish *C. quadricarinatus* and *P. clarkii*. Weapon performance can usually be assumed to be an honest signal and is correlated with aggression (Lappin and Husak, 2005; Wilson et al., 2007; Bywater and Wilson, 2012). We hypothesised that the invasive crayfish species would have a higher maximum closing force than *P. perlatus*. Closing force can indicate prey handling capacity (Meers, 2002; Miranda et al., 2016), but also, if closing force is related to morphology it can contribute to important signaling in agonistic contests (Wilson et al., 2007; Bywater et al., 2008; Bywater and Wilson, 2012). Thus, we also determined whether morphological and biological factors can be used to predict closing force.

METHODS

Animal Collection and Maintenance

Cherax quadricarinatus is native to Northern Australia and southeastern New Guinea. Populations have established in southern African freshwater systems where they were introduced and are spreading (Nunes et al., 2016, 2017a; Douthwaite et al., 2018). *Cherax quadricarinatus* tolerates a wide variety of habitats and environmental conditions (Masser and Rouse, 1997). One hundred and fifty live *C. quadricarinatus* samples (mean ± sd carapace length: 63.20 ± 4.93 mm, mass: 67.34 ± 11.26 g) were collected from sugarcane irrigation ponds in Nkomazi, Komatipoort (Mpumalanga Province) supplied by the Komati River (S 25.55°, E 31.90°). Komatipoort has been invaded by *C. quadricarinatus* since 2002 and represents an invasion core for the species in South Africa (de Moor, 2002; De Villiiers, 2015). A standard gear for trapping the redclaw crayfish was used, this consists of Promar collapsible traps baited with dry dog food. Traps were deployed at 1,600 h and retrieved at 800 h. Permits to transport and keep *C. quadricarinatus* were issued by the DEA (Permit Numbers: 50869181001115242, 50869181001120608). The crayfish caught were transported to a biosecure facility at the South African Institute for Aquatic Biodiversity (SAIAB) in insulated cooler boxes with source water from the dam and constantly aerated with battery pumps.

Procambarus clarkii is native to southern and south-eastern USA and northern Mexico. In Africa, *P. clarkii* was introduced to South Africa, Zambia, Uganda, Kenya, Egypt, Sudan, and Rwanda (Hobbs et al., 1989; Mikkola, 1996; Cumberlidge, 2009). *Procambarus clarkii* lives in a variety of freshwater habitats, including lakes, ponds, rivers, canals, streams, seasonally flooded swamps and marshes, and ditches with mud or sand substrata and plenty of organic debris (Huner and Barr, 1991). Fifty-six live *P. clarkii* crayfish samples (carapace length: 58.62 ± 6.86 mm, mass: 59.54 ± 7.05 g) were collected from Mimosa Dam (S 27.88°, E 26.69°) in Free State Province South Africa, where there has been a recent invasion ((DEA), 2018) using traps baited with fish heads and dry dog food. Traps were deployed at 1,600 h and retrieved at 800 h. Mimosa Dam represents an invasion core population of *P. clarkii*. The crayfish caught were transported from Mimosa Dam to the biosecure facility at SAIAB in insulated cooler boxes with source water from the dam and constantly aerated with battery pumps. Permits to transport and keep *P. clarkii* were issued by the Department of Environmental Affairs (DEA) (Permit Numbers: 50869181001113030, 50869181002121045).

The same gear was also used to trap *P. perlatus* samples from Eastern Cape Dams (S 33.32°, E 26.52°; S 33.32°, E 26.52°; S 33.29°, E 26.51°; S 33.41°, E 26.50°). Traps were deployed at 1,600 h and retrieved at 800 h. Permits to sample crabs were issued by the Eastern Cape Department of Economic Development, Environmental Affairs and Tourism (CRO 19/18CR and CRO 21/18CR). Larger crabs were selected to most closely match the mass of the crayfishes, however, there were few females caught in the traps. Twenty crabs (carapace length: 53.27 ± 4.55 mm, mass: 96.29 ± 22.15 g) were caught and placed in 60 L cooler boxes with fresh dam water with battery powered air pumps and transported to the biosecure facility at SAIAB.

Not all collected animals were used in this experiment as they were in use for other research purposes at the time, therefore the animals used were a random subsample of the total collected animals (**Table 1**). Animals were maintained in species specific and sex specific holding tanks (60 L) with constantly filtered and aerated aged tap water which was replaced twice a week to maintain good water quality. Water temperature was maintained at 23 ± 1°C by a computer controlled recirculating heating: cooling air conditioner unit. The laboratory was held under a 12:12 light:dark regime. All animals were maintained on cabbage leaves, broad leaved pondweed *Potamogeton nodosus* and fennel-leaved pond weed *Stockenia pectinatus* and cultured *Eisenia* sp. worms.

Experimental Setup

Individual animals were selected from the holding tank haphazardly and patted dry before measurements were taken. Animals were weighed (to the nearest g), the chelae length (propodus) of both left and right chela, and cephalothorax length were measured with Vernier calipers (to the nearest mm), and sexed (**Table 1**). Animals with regenerated claws and females with eggs were excluded due to differences in energy conservation.

TABLE 1 | Median and range of morphometric values and closing force (*N*) values for *P. perlatus*, *C. quadricarinatus* and *P clarkii* females and males.

Species	Sex (n)	Mass (g) (median and range)	Carapace length (mm) (median and range)	Left chela length (mm) (median and range)	Right chela length (mm) (median and range)	Left closing force (N) (median and range)	Right closing force (N) (median and range)
Potamonautes perlatus	F (*n* = 8)	95.1, 54.6–123.5	75, 59–87	33, 25–39	37, 27–45	11.0, 6.7–42.7	49.8, 10–153.4
Cherax quadricarinatus	F (*n* = 27)	76.2, 22.0–132.0	69, 45–86	31, 24–41	33, 24–44	6.0, 2.0–26.0	4, 2.0–30.0
Procambarus clarkii	F (*n* = 18)	50.4, 28.7–64.5	61, 47–66	36, 28–49	35, 31–45	3.3, 2.0–10.0	3.3, 2.0–5.3
Potamonautes perlatus	M (*n* = 6)	70.7, 46.3–117.2	65, 58–76	28, 21–35	32.5, 26–41	6.0, 2.7–10.0	14.67, 3.3–25.3
Cherax quadricarinatus	M (*n* = 25)	98.9, 38.1–144.6	71, 51–81	32, 25–41	34, 26–41	18.7, 3.3–136.7	19.43, 2.6–116.7
Procambarus clarkii	M (*n* = 21)	52.1, 25.7–76.8	60, 48–65	41, 30–52	40, 25–50	2.6, 2.0–37.4	3.1, 2.0–9.3

Chelae closing force measurements were completed *in vivo* in the laboratory using the Kistler system and the protocol outlined in Herrel et al. (1999), Singh et al. (2000), and Lailvaux et al. (2009). Animals were allowed to grip from the proximal region of the chela onto plates set at a gap of 6 mm, to allow comparison with Miranda et al. (2016). Closing force (*N*) was measured five times per individual, per chela, resulting in 10 measurements taken for each individual. Animals were given a rest period of 5 min between measurements. The laboratory, and holding tank water, were both held at (23 ± 1°C) throughout the entire acclimation and experimentation time.

Analyses

Differences in mass and cephalothorax length between species was determined using one way non-parametric Kruskal-Wallis tests and Dunn test *post-hoc*, with *p*-values adjusted for multiple comparisons using Holm-Bonferroni corrections. Handedness was assessed separately for each species, by comparing left and right chela closing force measurements using Friedmans test to account for repeated measures.

Closing force data were log-transformed and linear mixed-effects models were fitted, based on maximum likelihood. Initially the models were fitted separately to each left and right chelae. It was assumed that chela length would affect closing force, and to account for some individuals having asymmetrical chelae lengths between the left and right claws, we first ran a model to account for this rather than taking data from only one chela. Fixed factors were "species," "mass," "sex" and "chela length," while "individual identity" was used as a random factor nested within "species." A full model was fitted, containing "species," "mass," "sex" and "chela length," and their interactions. Chela length was not a significant predictor of closing force so was removed from the model (see results) and only the maximum closing force obtained per individual was selected, regardless of left or right chelae. Cephalothorax length was significantly correlated with mass and thus not included in the analysis (**Figure 1**). If either the fixed factors, or the interaction did not have a significant effect, in subsequent steps the model was re-run with non-significant terms removed, starting with the interaction term. The final model only contained significant terms to obtain the most parsimonious model. Differences were calculated *post-hoc* using χ^2 to communicate effect size. All analyses were performed in an R environment (R Core Team, 2018), using the package nlme (Pinheiro et al., 2018).

RESULTS

There were significant differences in mass of each species ($\chi^2 = 39.03$, $df = 2$, $p < 0.001$; **Table 1**, **Figure 1**), where *P. clarkii* weighed less than both *C. quadricarinatus* ($z = 5.83$, $p < 0.001$; **Table 1**, **Figure 1**) and *P. perlatus* ($z = 4.11$, $p < 0.001$; **Table 1**, **Figure 1**), however there was no difference between *P. perlatus* and *C. quadricarinatus* mass ($z = 0.08$, $p = 0.93$; **Table 1**, **Figure 1**).

Potamonautes perlatus had significantly stronger right chela closing force than left (Friedmans $\chi^2 = 15.05$, df = 1, $p < 0.0001$; **Supplementary Figures S1**, **S2**). Contrastingly, both *C. quadricarinatus* and *P. clarkii* had significantly stronger left chela closing force (respectively: Friedmans $\chi^2 = 5.68$, df = 1, $p < 0.05$, Friedmans $\chi^2 = 5.09$, df = 1, $p < 0.05$; **Supplementary Figures S1**, **S2**). Female *P. perlatus* had a higher left and right chela closing force than males (**Table 1**, **Figure 2**) whereas, male *C. quadricarinatus* had a higher left and right chela closing force than females (**Table 1**, **Figure 2**). There were no sex differences in left or right chela closing force in *P. clarkii* (**Table 1**, **Figure 2**). Chelae length did not affect closing force in any species on either left or right chelae (left: $\chi^2 = 0.19$, $df = 1$, $p = 0.65$; right: $\chi^2 = 0.83$, $df = 1$, $p = 0.36$).

There was a significant interaction between species and sex on maximum closing force (**Table 2**, **Figure 3**; **Supplementary Figure S3**). Species and sex both had significant main effects on closing fsorce (**Table 2**, **Figure 3**; **Supplementary Figure S3**). Closing force increased significantly with mass for all species (**Table 2**, **Figure 3**; **Supplementary Figure S3**). Opposite trends in maximum closing force were seen between male and female *P. perlatus* and

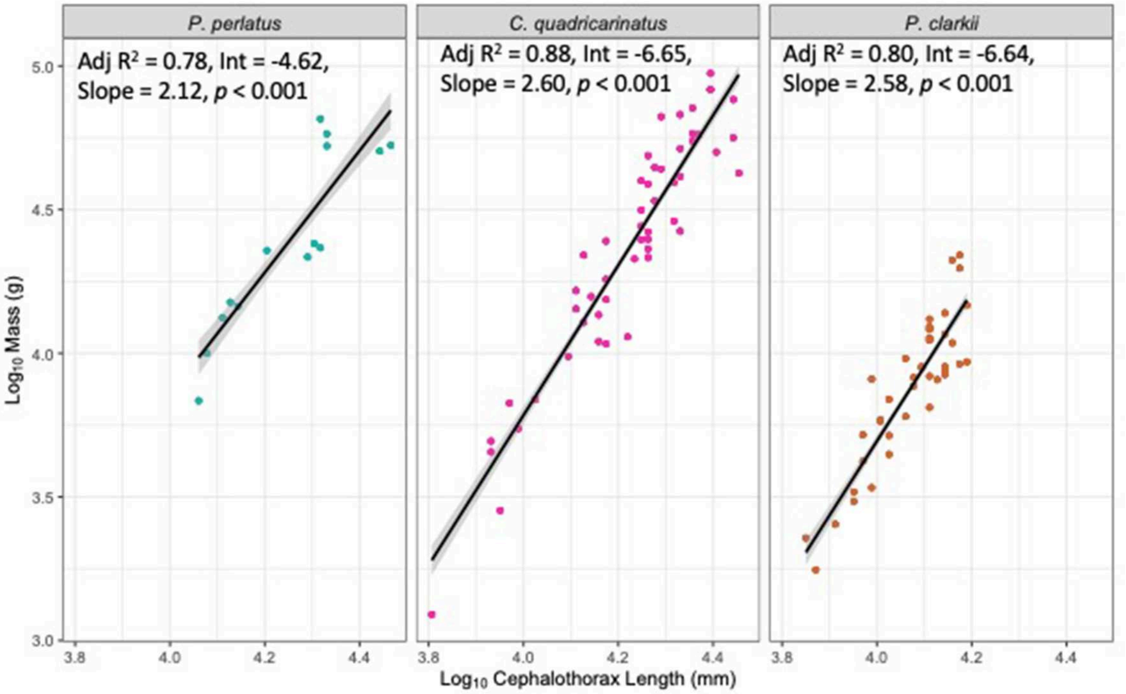

FIGURE 1 | Linear regressions and SE of Log_{10} mass (g) and Log_{10} Cephalothorax length (mm) of *P. perlatus, C. quadricarinatus* and *P. clarkii*.

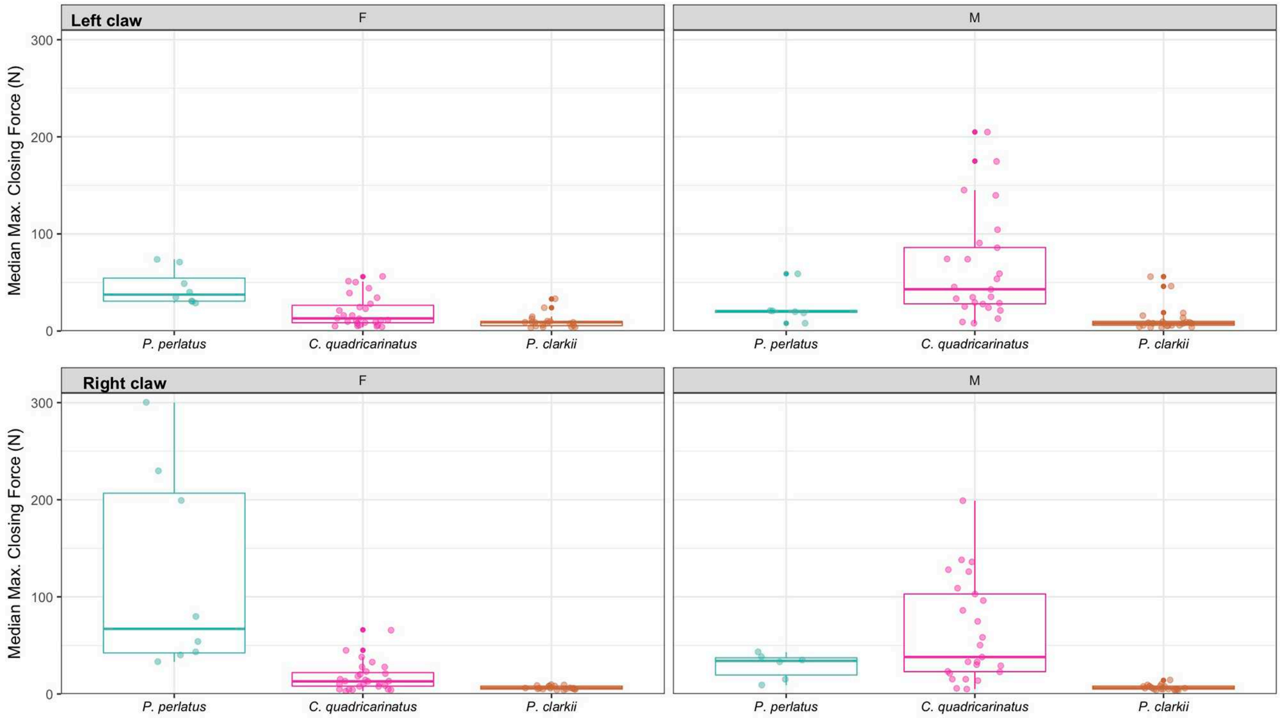

FIGURE 2 | Median maximum closing force (N) of left and right chelae of female and male *P. perlatus*, *C. quadricarinatus*, and *P. clarkii.* Individual points represent raw data. Boxplots represent median value and 25–75% interquartile range.

TABLE 2 | Model terms for all factors from a linear mixed effects model used to determine differences in maximum closing force (*N*) with regards to fixed factors "species," "sex," and "mass," using a Type 3 Anova and $\chi 2$ to report the effect size of a factor on the dependent variable.

Predictor	Maximum closing force (*N*)		
	χ^2	df	*p*-value
Species	39.25	2	<0.001
Sex	32.94	1	<0.001
Mass	23.02	1	<0.001
Species*Sex	29.86	2	<0.001

C. quadricarinatus, however there was no difference between male and female closing force in *P. clarkii* ($p = 0.46$). Female *P. perlatus* had a significantly stronger closing force than male *P. perlatus*, female *C. quadricarinatus,* and both sexes of *P. clarkii* (all $p < 0.01$). There was no difference between female *P. perlatus* closing strength and male *C. quadricarinatus* or between male *P. perlatus* and female *C. quadricarinatus* (both $p > 0.05$). However, male *C. quadricarinatus* had significantly higher maximum closing forces than male *P. perlatus* ($p < 0.01$). Male *P. perlatus* had significantly stronger maximum closing forces than both sexes of *P. clarkii* (all $p < 0.01$). There was no difference between maximum closing force of *P. clarkii* females and *C. quadricarinatus* females ($p = 0.58$).

DISCUSSION

Determining what makes systems resilient to biological invasion is a many faceted challenge (Holling, 1973; Havel et al., 2015). Nonetheless, by understanding basic differences in physical capacity it is possible to infer how performance can relate to species interaction outcomes within an invasion scenario (Griffen and Mosblack, 2011). We predicted that the invasive crayfish species would have a higher maximum closing force than native crabs, however, this proved to be unequivocal at least when considering animals of the same size. Given that there have been reports of invasive crayfish reducing abundance of trophically analogous decapods (Ogada, 2007; Ogada et al., 2009; Zeng et al., 2019), these results suggest that *P. perlatus* has the capacity to hold a competitive mechanical advantage over both invaders, but that this varies with sex. Unfortunately, our results are based on a small sample size of native *P. perlatus* which may confound results with regards to sex based differences. Nonetheless, there was a more even amount of males and females amongst smaller *P. perlatus* individuals (50–80 g) whereas the heavier individuals (>100 g) were predominantly female. Our results indicate that there are other mechanisms at play that may cause freshwater crabs to be competitively excluded by invasive crayfish species, rather than brute strength. Although, population size structure, growth rates, and maximum attainable mass of each species will affect competitive exclusion. For example, larger individuals will have a higher resource holding potential over smaller individuals of any species. Resultingly, it is possible to determine maximum chelae strength for these species through correlation with mass and sex but it is unclear as to whether chelae strength can actually be a predictor for resource holding potential or success in agonistic contests.

Closing force relates to ability of an individual to pinch down onto a subject. Whether this is for direct predation, during agonistic contests, reproductive purposes, or even to withstand abiotic disturbances such as high flow rates (Gherardi, 2002; Ion et al., 2019). It can be assumed that the larger the closing force the more damage may be conferred to the recipient despite high force conferring a high energy cost to the individual (Herrel et al., 1999; Wilson et al., 2007). Our results of chela closing force fall well within the range reported for *P. perlatus* (18–598 N) (Miranda et al., 2016), and for *P. clarkii* (males: 1.35 ± 0.41 N/g; females: 2.22 ± 0.89 N/g; see **Supplementary Figure 3** for N/g results) (Claussen et al., 2007; Malavé et al., 2018). Similar to our results (Claussen et al., 2007) and Malavé et al. (2018) found sexual dimorphism in chela length where male *P. clarkii* had longer chela than females but this did not relate to closing force. This is likely related to reproductive activities and the cost of signaling during male-male contests (Stein, 1976). Contrastingly, *P. perlatus* females were stronger than males indicating a difference in resource holding potential between sexes and species. These differences may also be exacerbated in a natural setting when individuals are in or out of reproductive status. For example, female *P. clarkii* are more aggressive and more successful when they are maternal compared to non-maternal females and males (Peeke et al., 1995; Figler et al., 2005). Another native African crab species (*Potamonautes sidneyi*) had a significantly weaker closing force than *P. perlatus* (8–43 N) (Miranda et al., 2016). There is no prior published data on the closing force of *C. quadricarinatus,* but for comparative purposes the closing force of the largest terrestrial arthropod, the coconut crab (*Birgus latro*), is 29.4–1765.2 N (Oka et al., 2016), of which the lower ranges all overlap with the three species in the present study. In a first attempt to incorporate *C. quadricarinatus* into impact assessments, Zeng et al. (2019), show that larger bodied *C. quadricarinatus* have a competitive advantage over a native freshwater crab (*Parathelphusa maculata*) when competing for shelter space.

The presence of heterochely or "cutter vs. crusher" is well-established in marine decapods but it is less evident in freshwater crayfish species (Govind, 1989; Schenk and Wainwright, 2001; Lele and Pârvulescu, 2019). In essence it describes potential handedness between left and right chelae. *Potamonautes perlatus* had stronger right chela, whereas *C. quadricarinatus* and *P. clarkii* had stronger left chela. As claw length was not an effective predictor of strength it suggests a degree of ambidexterity between left and right chelae in all of these species as a response to likelihood of losing chelae during agonistic bouts (Kouba et al., 2011; Lele and Pârvulescu, 2019). The lack of strong morphometric predictors reinforces the concept of dishonest signaling in crayfish species (Wilson et al., 2007; Malavé et al., 2018). This combination of potential dishonest signaling and opposite trends in dominant chelae could be

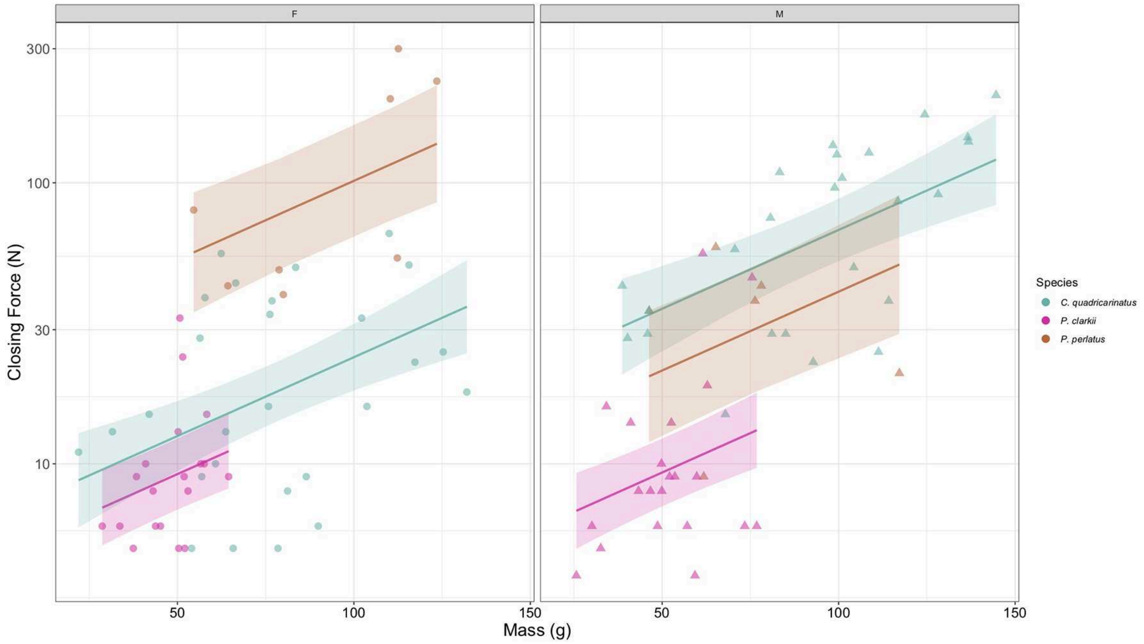

FIGURE 3 | Predicted maximum closing force correlation with mass (g) of female (left panel) and male (right panel) *P. perlatus*, *C. quadricarinatus*, and *P. clarkii* with 95% confidence intervals. Individual points indicate raw data points for each species.

a factor in competition between crab and crayfish species despite *P. perlatus* females dominating mechanically. Potential for biotic resistance, either consumptive or competitive, is likely to be species specific and still further regulated by other biotic and abiotic parameters (deRivera et al., 2005). In this case, size mis-matches, differential spatial ecology, abundance, and type of resources present in a system are all possible factors that could be driving the likelihood of biotic resistance.

Freshwater crabs and crayfish are polytrophic keystone consumers that occupy an unusually large dietary niche breadth as a result of generalist feeding strategies (Jackson et al., 2014, 2016). Chelae are regularly used in decapod feeding to assist in subduing, capturing, holding, and manipulating resource items (Loya-Javellana et al., 1993; Mariappan et al., 2000). This is particularly important when considering durophagous feeding, as crushing predators are thus limited by their strength to process prey items such as snails, but also limited by the shell resistance and predator induced phenotype changes (DeWitt et al., 2000; Evers et al., 2011; Miranda et al., 2016). There is limited information on crush resistance of African gastropod species, but the invasive snail *Tarebia granifera* has a resistance of 100 ± 6 (mean ± se) N, while native *Melanoides tuberculata* has a resistance of 31 ± 4 N (Miranda et al., 2016). However, in its invasive range in Lake Malawi *M. tuberculata* has a crush resistance range of 18.63–94.73 N (Evers et al., 2011). *Bulinus globosus* and *Bulinus nyassanus* are native gastropods of Lake Malawi, which have crush resistance ranges of 2.29–4.79 N and 8.33–117.82 N, respectively (Evers et al., 2011). All three of the species represented in this study, besides female *P. clarkii,* have the capacity to handle all of these gastropod species. The relatively high crush resistance of the invaders suggests that this may facilitate their persistence in a system, however it should also be considered that if the southern African crayfish invasion persists there could be an invasion meltdown scenario where predation is concentrated on the native gastropods and facilitates population expansion of the invasive gastropods (Ricciardi, 2001; Simberloff, 2006). An invasion meltdown scenario may also be facilitated in the wild by *Potamonautes* sp. via differences in biotic resistance. Although, the cost-benefit of undertaking crushing activities for food, rather than reproductive efforts, should be investigated as it is likely that predators select for forage with low handling demands (Murdoch, 1969; Behrens Yamada and Boulding, 1998). The present study focuses on the relative differences in closing force with respect to resource utilisation and non-consumptive competition but direct predation by either crabs or crayfish upon heterospecific juveniles is also likely. Therefore, addressing actual contest outcomes and consumption rates between different sized individuals of each species would further our understanding of competitive interactions.

Handling vegetation and crushing prey items require differences in closing force and dentition patterns (Sibbing, 1991; Herrel et al., 1999). Thus, differences in closing force could also relate to niche separation between the species when they occur in sympatry, which could in turn facilitate species persistence of both natives and invaders. *Procambarus clarkii* exhibit this pattern of niche breadth reduction when found in sympatry with *P. loveni*, where they affect leaf litter breakdown due to direct consumption (Jackson et al., 2016; Nishijima et al., 2017), possibly related to differences in chela morphology (Sibbing, 1991). Further, *P. clarkii* is capable of exerting predatory pressure on planktonic prey items which do not need strong crushing capacity to handle (South et al., 2019). Little work has been completed on the diet and trophic niche of *C. quadricarinatus* in either its invasive or native range. Nonetheless, Marufu et al. (2018) found the main diet components of the Lake Kariba *C. quadricarinatus* population to be predominantly macrophytes, detritus and macroinvertebrates. In Lake Kariba the trophic niche of crayfish differed with size class, wherein macroinvertebrate consumption increased with size, which could potentially be due to the positive relationship between mass and closing force (Marufu et al., 2018). Comparative functional morphology of decapod chelae and feeding apparatus should thus be incorporated into invasion risk assessments as ecomorphology can help to predict impact (Nagelkerke et al., 2018).

Due to the complex nature of trophic interactions and food web structuring, particularly within a stochastic aquatic environment, it is difficult to determine mechanisms of biotic resistance *in situ* (Havel et al., 2015). Comparing species traits is a first step in assessing whether native species will exhibit some degree of either competitive or consumptive resistance toward invaders (Funk et al., 2008; Kumschick and Richardson, 2013; Zeng et al., 2015). The results presented here indicate that chelae closing force can be predicted by body mass and sex of the individual for all three decapod species but in order to correctly predict biotic resistance these must be validated further by assessing actual resource holding potential. Consequently, when trait based analysis should be complemented with other predictive assessments such as the comparative functional response and relative impact potential metrics (Dick et al., 2017; Dickey et al., 2018; South et al., 2019; but see Vonesh et al., 2017), but also with contest based experiments (Lopez et al., 2019; Zeng et al., 2019). Unfortunately, there is a severe paucity of data on the ecological impact of *C. quadricarinatus* but also on the basic ecology of Potamonautid crabs in southern Africa. Further, the specific dynamics of invasion and the recipient system can mediate trait expression in populations across the invasion gradient, whereupon crayfish at the invasion front can be more aggressive (Pintor et al., 2009), or they have smaller and less heavy chela as a response to reduced competition (Messager and Olden, 2019). Therefore, a considerable amount of baseline assessment (i.e., abundance, size structure, fecundity, distribution, and diet) is needed to be completed in order to effectively assess the risk that both invasive crayfish species pose toward functionally similar and ecologically important species such as freshwater crabs. Further, assessing actual interaction frequency and habitat or resource use overlap between different sized native and invasive decapod species would determine the potential degree of biotic resistance in the environment.

DATA AVAILABILITY STATEMENT

The raw data generated and used in the analysis are publicly available at https://doi.org/10.6084/m9.figshare.11993961.

ETHICS STATEMENT

This research was given ethics clearance by the Animal Ethics Subcommittee, Rhodes University (Ethics No. DIFS2718) and SAIAB Ethics Committee (#25/4/1/7/5_2018_06).

AUTHOR CONTRIBUTIONS

JS, TM, JM, and OW conceived the study. JM provided the equipment. OW provided the funding. JS, NT, and TM conducted the experiments. JS analysed the data and wrote the first draft. JS, TM, NT, JM, and OW all contributed toward manuscript editing and final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00072/full#supplementary-material

Supplementary Figure 1 | Median maximum closing force (N) per mm of chelae right chelae for *P. perlatus*, *C. quadricarinatus*, and *P. clarkii*. Individual points represent raw data. Boxplots represent median value and 25–75% interquartile range.

Supplementary Figure 2 | Median maximum closing force (N) per mm of chelae left chelae for *P. perlatus*, *C. quadricarinatus*, and *P. clarkii*. Individual points represent raw data. Boxplots represent median value and 25–75% interquartile range.

Supplementary Figure 3 | Median maximum closing force (N) per g of animal for *P. perlatus*, *C. quadricarinatus*, and *P. clarkii*. Individual points represent raw data. Boxplots represent median value and 25–75% interquartile range.

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Seasonal and Spatial Variation in Growth and Abundance of Zebra Mussel (*Dreissena polymorpha*) in a Recently Invaded Artificial Lake: Implications for Management

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The control of the highly invasive zebra mussel (*Dreissena polymorpha*) has been flagged as a priority but success has been variable. A better understanding of the growth and drivers of settlement of zebra mussel is necessary for a more efficient management of this invasive species, but seasonal data are still relatively scant. We monitored the seasonal changes in settlement rates, density, and growth of zebra mussel in artificial substrates over 1 year in Cardiff Bay (United Kingdom), an artificial amenity lake invaded by zebra mussels in 2004, where the species is rapidly expanding. Mean settlement rates varied from 4,200 to 6,200 mussel m^{-2} over June to September mirroring changes in water temperature, and peaked at 17,960 mussels m^{-2}, the highest density reported in Britain. Density was highest at the deepest panels (3 m). Growth varied significantly among sampling stations, taking place during the summer and ceasing during winter and spring. Mixture analysis reveals the existence of multiple cohorts displaying different growth and settlement rates that follow different density dependent mechanisms, being positive density-dependent at low densities, and negative density-dependent at high densities. We suggest this creates the conditions necessary for source and sink metapopulations to develop which may need to be considered in management. Targeting mussels for removal in deep waters during the summer and early autumn might prove beneficial, but the existence of contrasting density-dependent mechanisms suggests that removal may be beneficial or counterproductive depending on local conditions.

Keywords: aquatic invasive species, population dynamics, density-dependence, colonization, bivalves (mussels), growth

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INTRODUCTION

The zebra mussel (*Dreissena polymorpha*) is one of the most damaging aquatic invaders (Strayer, 2010), having been included in the list of the 100 world's worst alien species (Lowe et al., 2000). Zebra mussels can drastically reduce the biomass of phytoplankton, and change its community composition, which can in turn change water parameters, resuspend nutrients into the water column (Bastviken et al., 1998), and increase water transparency (Holland, 1993; Fahnenstiel et al., 1995). The species can compete for food and space with native freshwater mussels and drive them to extinction through epizootic colonization, disruption of their valve functionality, smothering of their siphons, and impairing of their movement through deposition of metabolic waste (Schloesser et al., 1996; Baker and Hornbach, 1997). A strong association has been found between density of zebra mussels and mortality of native unionid mussels (Ricciardi et al., 1995).

The economic losses caused by zebra mussels can be considerable. They can block pipes and water supplies, intakes from nuclear, hydroelectric and industrial facilities (O'Neill, 1997), and clog the cooling systems of power boats (Johnson et al., 2001). Navigation buoys have been sunk under the weight of zebra mussels, and dock pilings can become severely weakened due to zebra mussel encrustations (Minchin and Moriarty, 1998). In the United States, the cost of cleaning a single hydroelectric plant of zebra mussels may amount to $92,000 per year, and the combined costs may have reached $6.5 billion over 10 years (Lovell et al., 2006). Not surprisingly, the control of zebra mussel has become a priority (Aldridge et al., 2004, 2006; Strayer, 2010). However, eradication measures are seldom successful (Whitledge et al., 2015; Lund et al., 2018), may require repeated treatments, and may have to be extended over several years (**Table 1**). The only known completely successful eradication program was reported in Millbrook Quarry (United States) using 12% potassium (Fernald and Watson, 2013). In Lake George (United States), manual SCUBA diving removal during eight consecutive years eradicated zebra mussel from a private marina, but new small colonies appeared in other parts of the lake that required further control (Wimbush et al., 2009). OAFB (2009) reported the successful eradication of zebra mussel in lake Offutt (United States) using copper sulfate, however, in 2014 the species was found again in the lake (Britton, 2015). A local eradication program in the Grand Canal (Ireland) using the molluscicide Zequanox failed to remove zebra mussel (Meehan et al., 2014). Similar failures have been reported in Deep Quarry (United States) (Whitledge et al., 2015) and in Christmas Lake (United States); in the last case the local eradication was successful, but zebra mussel were subsequently found outside the treated area (Lund et al., 2018).

Some traits of the zebra mussel make their eradication particularly challenging: (1) the species is highly fecund (up to 1 million eggs per female), (2) has a planktonic veliger stage which can travel over great distances and survive for weeks (Minchin et al., 2002), (3) displays high tolerance to a wide range of temperatures (−10.3 to 28.0°C; Spidle et al., 1995; Gallardo et al., 2013), salinities (0–18.4‰; Kilgour et al., 1994; Karatayev et al., 1998; Orlova et al., 1998) and pH values (6.5–11.1; Claudi and Mackie, 1993; Bowman and Bailey, 1998; Bodamer and Bossenbroek, 2008), and (4) has a tendency to aggregate in enormous beds (up to 32,500 individuals/m^2, Berkman et al., 1998) on different types of substrates. Zebra mussel invasions have been facilitated by many anthropogenic actions, including the building of canals and channels that connect formerly isolated water bodies (Decksbach, 1935), boat trading (Kearney and Morton, 1970) and aquatic leisure activities (Kinzelbach, 1992). These can make eradication particularly difficult, as the risk of recolonization from connected water ways is always high (Mari et al., 2011). In Britain, the first record of zebra mussel dates back to 1824, but it is only since 2000 that the species has started to spread rapidly and cause widespread ecological damage, a pattern that cannot be explained simply by increasing public awareness (Aldridge et al., 2004) and that remains unclear.

One pressing issue with the control of zebra mussel is to assess to what extent incomplete eradication (i.e., partial removal) is useful in controlling population growth and limit dispersal, or on the contrary, may cause more harm than good if populations simply bounce back in greater numbers. Under controlled environments, mitigation measures can help reduce the abundance of zebra mussel (Luoma et al., 2018; Waller and Bartsch, 2018), which could help reduce impacts (Wimbush et al., 2009; Fernald and Watson, 2013). However, information on natural systems is very scant and models have typically low predictive power to predict zebra mussel dispersal patterns (Rodríguez-Rey et al., 2019). The ability of zebra mussel to recover from partial removal will likely depend on seasonal patterns of growth and recruitment, which have been correlated with seasonal temperatures (Allen et al., 1999), and chlorophyll-a (Churchill et al., 2017), but also with calcium, alkalinity, and total hardness (Hincks and Mackie, 1997). Mortality and recruitment appear to be influenced by fluctuations in temperature, but also by population size structure (Allen et al., 1999), and there is some evidence that settlement of new juveniles is negatively affected by the density of established adult mussels (Nalepa et al., 1995), suggesting the existence of negative density-dependence processes.

The recruitment and demography of zebra mussel have been well studied in North America (Martel, 1993; Nalepa et al., 1993; Martel et al., 1994; Chase and Bailey, 1999) but there is relatively little information on the dynamics of the species in recently invaded waters in Europe (MacNeil et al., 2010; Alix et al., 2016). Nor is it clear how populations are structured during the initial stages of the invasion, when boom and bust dynamics might be expected (Strayer et al., 2017), and where a better understanding of growth and recruitment could make control measures more efficient.

We monitored the colonization and growth of zebra mussel in experimental panels submerged at different depths in Cardiff Bay (Wales, United Kingdom), an amenity lake where the species is spreading and causing increasing damage. Zebra mussels were first recorded in Cardiff Bay in 2004 (although they may have been introduced a year earlier), and have spread rapidly since, being now present throughout the Bay (Alix, 2010; Wood et al., 2015; Alix et al., 2016).

Zebra mussels cause a major management issue in Cardiff Bay. To comply with dissolved oxygen standards contained within the Cardiff Bay Barrage Act, an aeration system consisting of 26 km of pipework pumps compressed air into the lake to ensure oxygenated water is mixed through the water column and anoxic water pockets are not formed. The aeration system is colonized by zebra mussels which clog the diffusers and reduce oxygenation, requiring regular cleaning. Zebra mussel also foul many boats found in the area, as well as two fish passes constructed within

TABLE 1 | Examples of eradication programs of zebra mussel.

Site	Type	Method	Time after first record	Duration	Cost	Success	References
Lake George United States	Localized eradication	Mechanical removal SCUBA divers	Few months	8 years	NA	Y	Wimbush et al., 2009
Lake Offutt United States	Whole site eradication	Copper Sulfate	2 years	4 days	NA	Y	OAFB, 2009
Millbrook Quarry United States	Whole site eradication	12% Potassium	4 years	3 weeks	$800,000	Y	Fernald and Watson, 2013
Grand Canal Ireland	Localized eradication	Zequanox	NA	1 treatment	NA	N	Meehan et al., 2014
Deep Quarry United States	Localized eradication	Zequanox	3 year	2 treatments	NA	N	Whitledge et al., 2015
Christmas Lake United States	Whole site eradication	Zequanox, EarthTec QZ Potassium chloride	23 days	8 months	$64,000	N	Lund et al., 2018

the barrage, that require annual maintenance to remove zebra mussel build-up.

The objectives of the study were (1) to assess the extent of seasonal and spatial variation in the growth and settlement rates of zebra mussel in a recently colonized artificial lake area, (2) to identify the conditions that are most favorable for zebra mussel production, and (3) to test for the existence of density-dependent growth. Ultimately, the aim of our study was to inform the development of more efficient control measures for zebra mussel by acting on those periods and locations where zebra mussel production is highest.

MATERIALS AND METHODS

Study Area

The study site, Cardiff Bay, is a 2.0 km^2 amenity lake (depth = 4–7 m) located in Cardiff (Wales, United Kingdom) and fed by two rivers (River Taff and River Ely). It was built between 1994 and 1999 as part of a regeneration project of the old docklands areas of Cardiff and Penarth. The site has been described in detail by Alix (2010), Alix et al. (2016). The lowermost section of the rivers Taff and Ely, in the vicinity of the Bay, contain zebra mussels, most likely transported upstream by boats. However, weirs and low bridges prevent extensive upstream movement. The National Biodiversity Network Atlas for Wales shows no upstream records of zebra mussels on the rivers Taff or Ely (NBN Atlas Wales, 2020), and the species was never reported in the rivers before it appeared in the Bay.

Sampling Strategy

We deployed four experimental buoys in different parts of Cardiff Bay (**Figure 1**), each buoy consisting of a weighted rope and three white plastic panels (A4 size, 210 × 297 mm) set at the surface (0 m), 1 m, and 3 m depth (**Figure 2**). The buoys were deployed on June 2017 and were monitored monthly until May 2018. The sites varied in depth between 3.3 m and 7.3 m (mean = 5.1 m, SE = 0.84), had moderate to slow flows, little aquatic vegetation, and differed in the abundance of veligers and adult zebra mussels found in a previous study (**Table 2**), who also reported monthly means (SE) of Chlorophyll *a* (μg.l-1) ranging between 4.3 ± 1.7 in May and 10.5 ± 2.7 in June and water velocity in February ranging between 4.4 and 12.2 cm/s (Alix et al., 2016).

An area corresponding to 25% of each panel (i.e., 156 cm^2) was scraped clean every month and all attached mussels were counted and measured. These are referred to as "clean samples" and provide data on the number and size of new recruits. A total of 141 scrape samples were collected in this way over the 12 months of the study, 43 of which contained zebra mussels (30.5%). In addition, 81 scrape samples were obtained from colonized sections of the panel (i.e., let undisturbed, never scraped before) 5 months after the start of the study; each month a different area of the panel was scraped, 67 of which contained zebra mussel (82.7%). Specimens were preserved in 70% ethanol and brought to the laboratory where they were counted and measured (shell length along the longest axis, mm).

During the monthly monitoring, water parameters (temperature, DO concentration, DO%, salinity, conductivity, pH, turbidity) were measured at 0 m, 1 m, and 3 m depth at each sampling station with a probe (YSI Water Quality Sonde,6600 EDS V2, United States). Buoy B was lost during the last month of the experiment but data were available for 11 of the 12 months of the study.

Statistical Analysis

Statistical analyses were carried out using R version 3.3 (R Core Team, 2017) and PAST v. 3.2.2 (Hammer et al., 2001). We used mixture analysis on shell length at the end of the growing season to estimate the number of different cohorts (age classes) that had colonized our experimental panels. For this, we varied the number of putative cohorts from 1 to 8 and chose the most likely number based on changes in AIC values (Hammer et al., 2001). We used linear models to examine variation in mussel size and density using month, depth and site as predictors. To examine the influence of water parameters, we used principal component analysis using the *prcomp* function in the *factoextra* R package (Kassambara and Mundt, 2017) and used the coordinates of the first principal component as predictors of mussel size and density in a linear mixed effect model using sampling station as a random factor, as above. Model simplification was achieved by examining

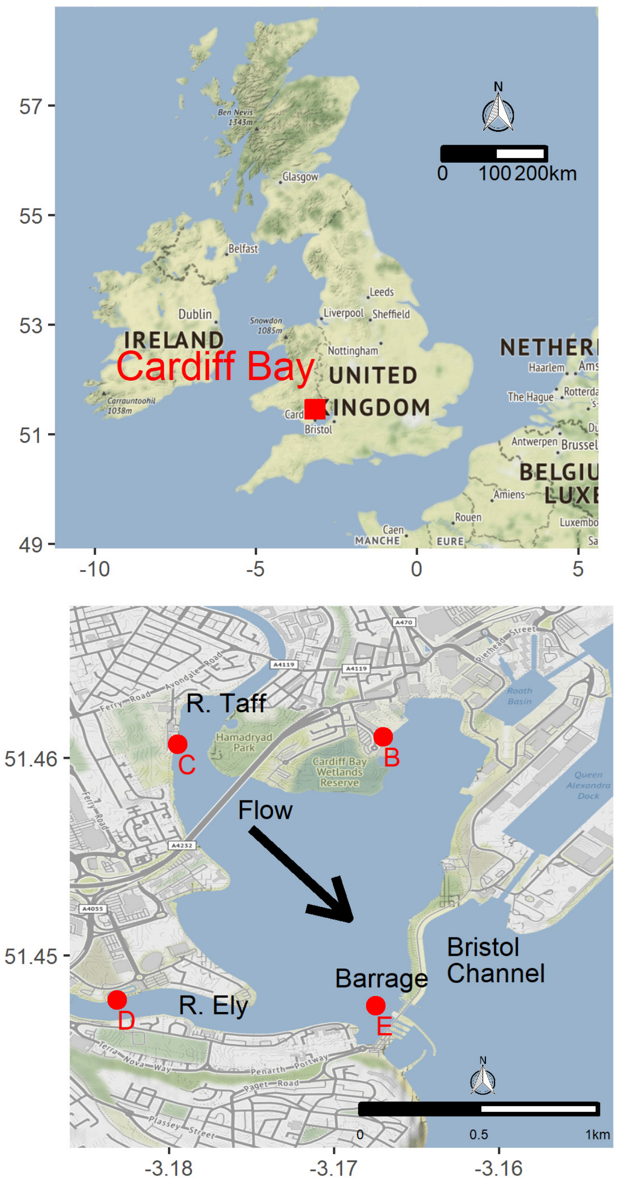

FIGURE 1 | Study site for zebra mussel population dynamics (Cardiff Bay, United Kingdom) showing location of experimental buoys (B–E) used to sample new recruits.

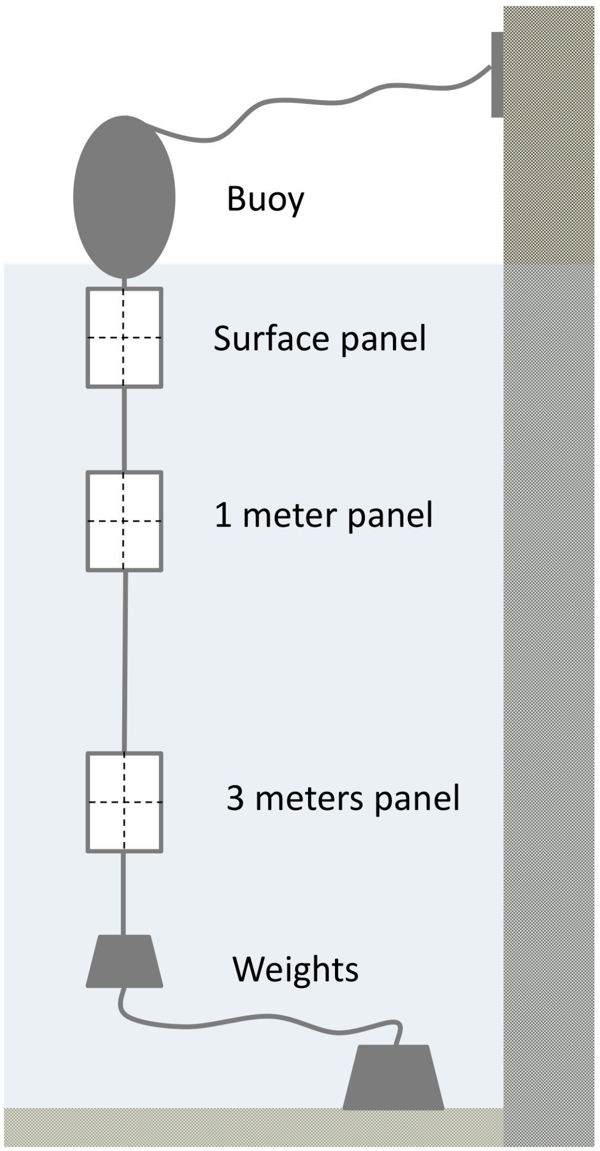

FIGURE 2 | Experimental buoys used to assess the influence of water depth and density on zebra mussel population dynamics. Each month 25% of the area of each side-panel (156 cm^2) was scrapped clean and all the attached zebra mussels were counted and measured.

changes in AIC using the *step* and *dredge* functions, followed by Maximum Likelihood comparisons of nested models with the *anova* command.

To test for evidence of density-dependence, we analyzed if density was a significant predictor of the average size of mussels in each sample, taking into account the effects of

TABLE 2 | Characteristics of the four sampling sites and presence of veligers and adults of zebra mussel reported in a previous study (Alix et al., 2016).

Station	Location	Depth (m)	Flow	Vegetation	Veligers	Adults
B	Inner Harbor	3.3	low	low	variable	variable
C	Mouth of River Taff	4.5	moderate	low	low	low
D	Docks at River Ely	5.3	low	low	low	low
E	Cardiff Barrage	7.3	low	low	high	high

season, water depth, and variation among sites. We carried out an analysis separately for 1 month old mussels (originating from our monthly scrape panels) and for mussels sampled from undisturbed panel-sections at the end of the growing season. As the relation between density and size was not linear, we employed generalized additive modeling (GAM) using a penalized regression spline fitted by REML in the *mgcv* package to account for non-linearity (Wood, 2001), and dropped non-significant terms from the final model. We excluded site B from analysis as there was no colonization of the surface panel in any month, and used the *gam.check* command to assess departures from model assumptions.

RESULTS

Variation in Water Chemistry

Water chemistry changed both seasonally (**Figure 3**) and spatially across Cardiff Bay. Water temperatures peaked in July–September (max = 20.4°C) and reached a low in March (min = 5.6°C; month $F_{11,207}$ = 1055.6, $P < 0.001$), being generally warmest at the mouth of the River Ely (site D) and the barrage (site E), and coldest at the mouth of the River Taff (site C) and the inner harbor (site B, $F_{3,207}$ = 23.1, $P < 0.001$). Dissolved oxygen reached a minimum in July (min = 6.1 mg/L), coinciding with the warmest temperature ($F_{11,207}$ = 1055.6, $P < 0.001$), and was highest at the outlet of the Bay (site E), and lowest at site D ($F_{3,207}$ = 23.1, $P < 0.001$). The four other water chemistry parameters also varied significantly from month to month, as well as from site to site (conductivity: month $F_{11,207}$ = 1055.6, $P < 0.001$, site $F_{3,207}$ = 23.1, $P < 0.001$; pH: month $F_{11,207}$ = 1055.6, $P < 0.001$, site $F_{3,207}$ = 23.1, $P < 0.001$; salinity: month $F_{11,207}$ = 1055.6, $P < 0.001$, site $F_{3,207}$ = 23.1, $P < 0.001$; turbidity: month $F_{11,207}$ = 1055.6, $P < 0.001$, site $F_{3,207}$ = 23.1, $P < 0.001$). However, no significant variation in water chemistry was found with respect to water depth, at least within the first 3 m ($P > 0.5$ in all models).

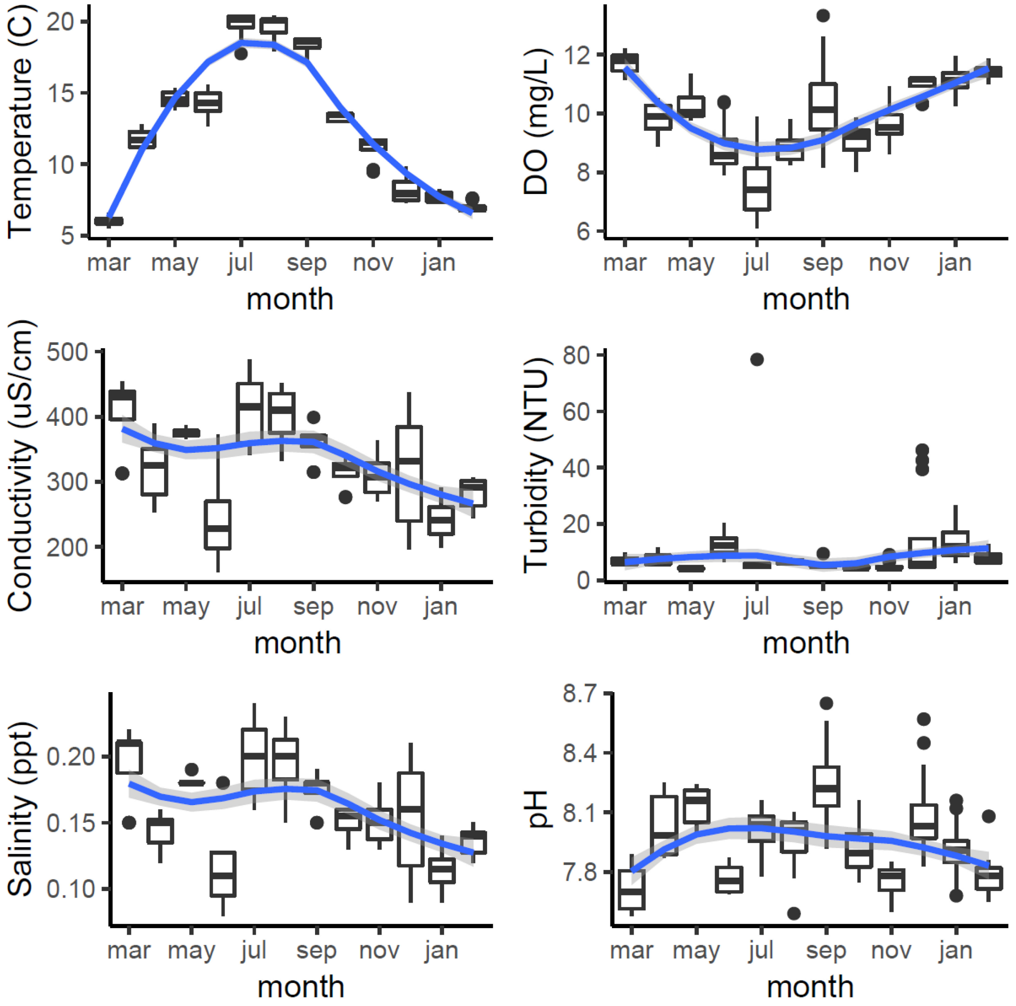

FIGURE 3 | Monthly variation in water chemistry in Cardiff Bay during the course of the study showing median values and interquartile range (boxplots) and smoothed fitted trends with 95CI envelopes (blue and gray bands).

Principal component analysis indicated that the first component (PC1) accounted for 39% of the variation in water chemistry parameters, but was not a significant predictor of either the average size ($t = 0.787$, $P = 0.434$) or density ($t = 2.46$, $P = 0.750$) of new recruits colonizing the experimental panels.

Density and Settlement Rates

Densities of 1 month old mussels sequentially sampled during the reproductive season varied between 0 in June and 1.8 individuals/cm^2 in September (**Figure 4**). Colonization of the experimental panels began in July, peaked during September and October, and then decreased rapidly, so that by November no new recruits were detected in any of the panels (**Figure 4**). Densities of 1 month old zebra mussel varied significantly between months ($F_{1,134} = 15.8$, $P < 0.001$), sampling sites ($F_{3,134} = 4.02$, $P = 0.009$), and depths ($F_{2,134} = 3.46$, $P = 0.03$). The surface panels had the lowest number of recruits, while the deepest panel had the highest.

Densities in the undisturbed panels, sampled over the winter, also varied between sampling sites ($F_{3,75} = 87.3$, $P < 0.001$)

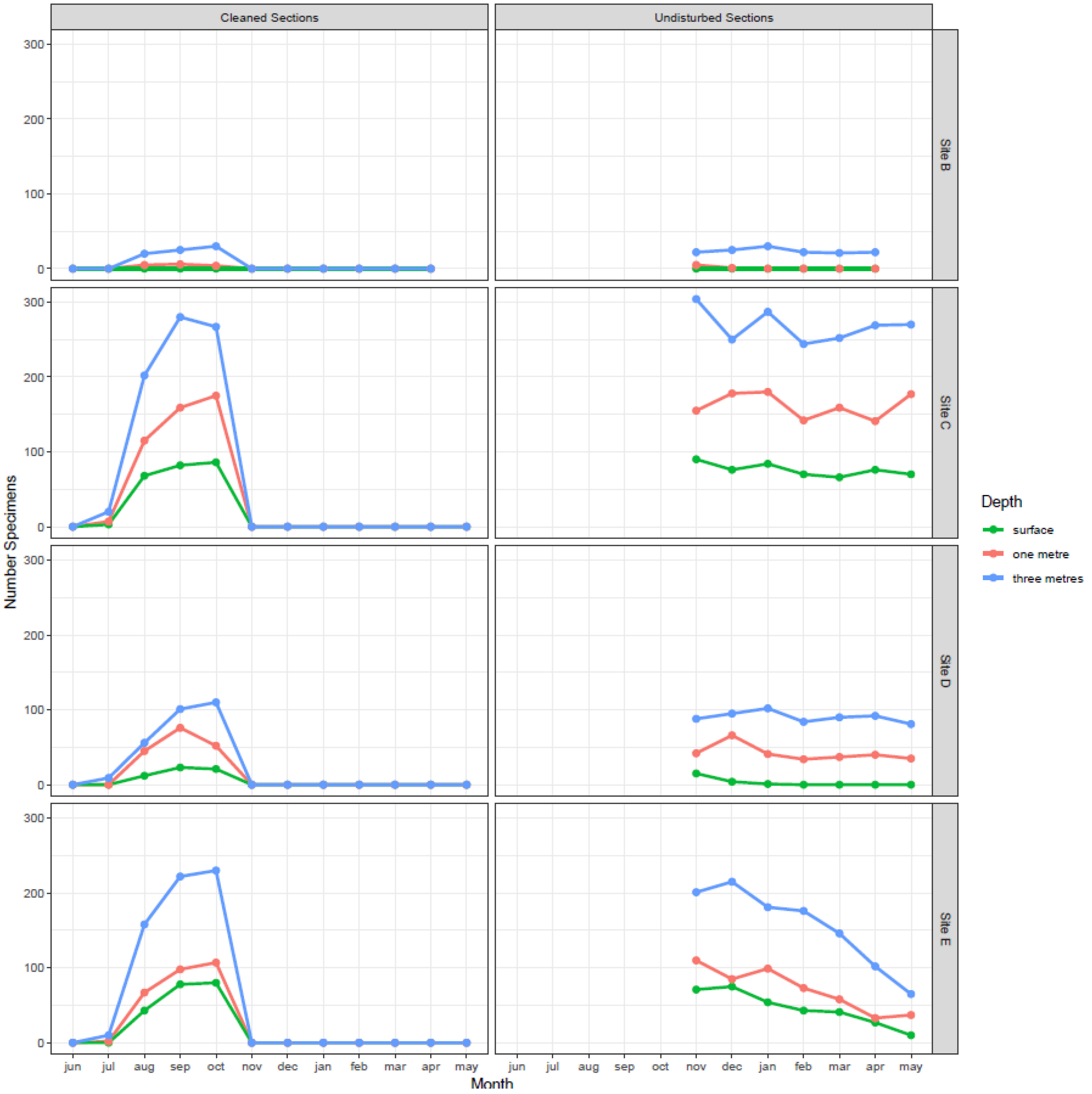

FIGURE 4 | Monthly variation in the number of zebra mussel found at different depths and at different locations in Cardiff Bay. **Left panel** shows results for panels scrapped clean every month during the growing season and **right panel** the results for panels left undisturbed and sampled after the end of the growing season.

and depths ($F_{2,75} = 71.2$, $P < 0.001$), but were stable across time (month $F_{6,69} = 1.67$, $P = 0.142$), confirming the lack of recruitment observed between November and May. In general, the highest densities and settlement rates were found at the mouth of the River Taff (site C) and at the outlet at the barrage (site E), while the lowest were found at the inner harbor (site B).

Settlement rates across the Bay followed a marked seasonal cycle (**Figure 5**), closely tracking variation in water temperature, beginning in June when temperature reached 14°C, peaking in August and September, and then ceasing when temperature dropped below 14°C in October–November. Across sampling stations, settlement rates were 0.42–0.62 individuals cm^2 month^{-1}, with peaks of 1.790 individuals cm^2 month^{-1}. This is equivalent to 4,200–6,200 mussels per m^2 (peaks of 18,000 individuals/m^2).

Determinants of Mussel Size and Growth

The average size of 1 month old mussels colonizing the clean panels during the reproductive season varied between 9 mm in July and 21 mm in October, and differed significantly between months ($F_{3,1377} = 700.7$, $P < 0.001$), sampling sites ($F_{3,1377} = 293.3$, $P < 0.001$), and also with depth ($F_{1,1377} = 385.4$, $P < 0.001$; **Figure 6**). The largest mussels were found at the mouth of the River Taff (site C) and at the outlet at the barrage (site E), while the smallest ones were found at the mouth of the River Ely (site D). Growth increased rapidly from July to October, and then plateaued for the rest of the year. The size of mussels was largest at 3 m depth (95 CI = 15.7–16.3 mm) and smallest at the surface (95CI = 14.0–14.8 mm). Such variation persisted in the undisturbed panels over the winter, after the reproductive season, as mussel size continued to vary significantly between months ($F_{6,2774} = 11.6$, $P < 0.001$), sampling sites ($F_{3,2774} = 170.9$, $P < 0.001$) and also with depth ($F_{1,2774} = 206.8$, $P < 0.001$; **Figure 6**). Thus, the average size of mussels in April 2018, 10 months after the buoys were first deployed, was still significantly smaller at the surface (mean = 17.0 mm) than at 1 m depth (mean = 18.3 mm) and at 3 m depth (mean = 18.4 mm; Tukey HSD P adj = 0.003), which were not different among themselves (Tukey HSD, P adj = 0.732).

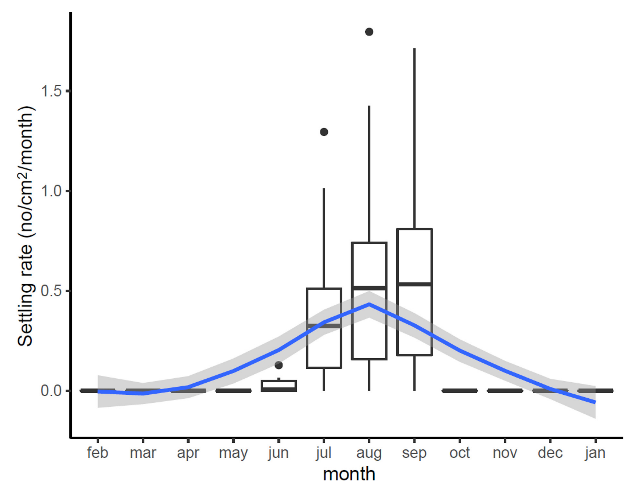

FIGURE 5 | Estimated variation in monthly settling rates (No. new recruits/cm^2/month) across Cardiff Bay showing median values and interquartile range (boxplots) and smoothed fitted trend with 95CI envelope (blue and gray bands).

Cohort Analysis

Inspection of experimental panels revealed that new recruits were only found during July–October, suggesting that the reproductive season in Cardiff Bay likely extended from May or June to September. Results from mixture analysis suggest that the most plausible number of discrete cohorts colonizing the experimental panels over the course of the study was 5 age classes (**Supplementary Figure S1**), with an estimated age of approximately 2 months for the youngest settlers (size 11–12 mm) to 330 days for the oldest ones when zebra mussels had already attained a size of 23–31 mm (**Table 3**). The distribution of cohorts varied significantly among sites (Chi-squared = 387.5, df = 12, $P < 0.001$) and there were comparatively more younger settlers at the warmest sites (sites D and E) than at the coldest ones (sites C and B, **Figure 7**).

Density Dependence

The size of 1 month old mussels (i.e., new settlers successively sampled from cleaned panels) was not affected by density, once the effects of site, water depth and month of sampling had been statistically controlled for (density $F_{1,29} = 0.427$, $P = 0.519$). However, density was a significant predictor of mussel growth in the undisturbed panels (GAM estimates for smooth terms; density, $F_{3\cdot357,4\cdot090} = 4.433$, $P = 0.004$; density × site C, $F_{5\cdot405,6\cdot099} = 14.881$, $P < 0.001$; density × site D, $F_{3\cdot154,3\cdot606} = 16.688$, $P < 0.001$; density × site E, $F_{1,1} = 3.732$, $P = 0.06$; parametric terms, depth 1 m estimate = 0.466, SE = 0.05, $t = 9.266$, $P < 0.001$; depth 3 m estimate = 0.759, SE = 0.07, $t = 10.267$, $P < 0.001$). The model explained 91.6% of deviance in mussel size, of which 39.4% was explained by density alone. Across sites, mussel size increased with density (**Figure 8**), but there were significant differences between sites. Thus, at sites with high recruitment (site C, mouth of River Taff; site E, outlet of the barrage, **Figure 5**.) mussel size decreased at high densities, whereas at the site with low recruitment (site D, mouth of River Ely) the opposite was found (**Figure 5**). This suggests the existence of positive density-dependent growth at low densities, and negative density-dependent growth at high densities.

DISCUSSION

Our study indicates that within 14 years of being invaded, Cardiff Bay has a large, established population of zebra mussel, confirming the conclusions of a previous survey of veliger density using a different sampling approach (Alix et al., 2016). Our study also suggests that zebra mussel may be spatially structured into different cohorts that grow and settle at different rates. This has implications for management because if density, settlement rates, and growth vary spatially among locations, this creates the conditions necessary for zebra mussel metapopulations to

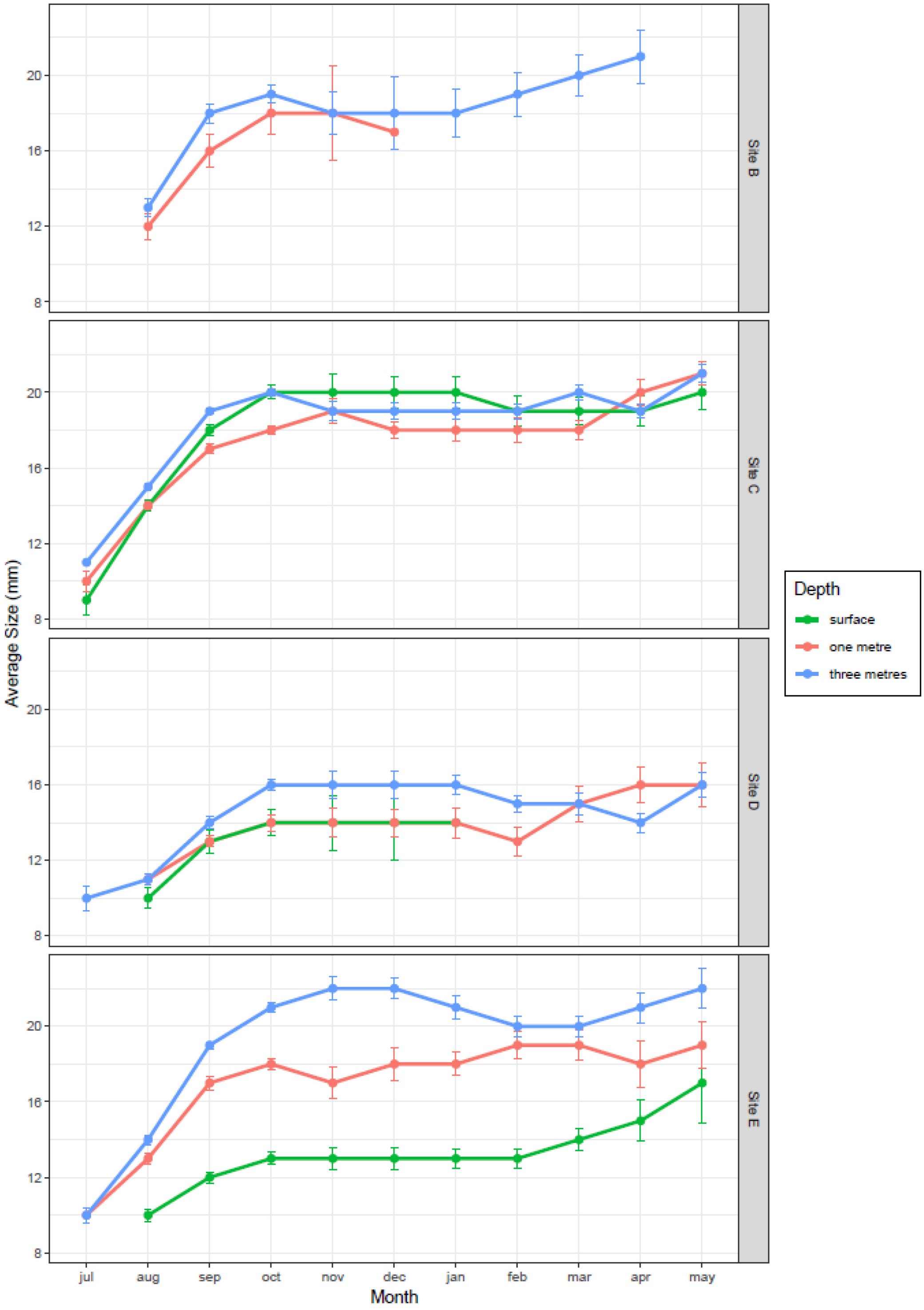

FIGURE 6 | Growth trajectories of zebra mussel at different depths and locations in Cardiff Bay (mean ± 95CI).

evolve, which would make control measures considerably more challenging (Mari et al., 2014).

Although the growth of zebra mussel on suspended substrates cannot be assumed to be representative of that on the bottom (Karatayev et al., 2006, 2018), Cardiff Bay is rather shallow (mean = 4.5 m; (Olivier, 2016) and our experimental panels extended to 41–91% of the water column (mean = 67%). Most of the zebra mussel production in Cardiff Bay takes place on vertical

TABLE 3 | Estimated cohort composition of successive waves of zebra mussels colonizing experimental panels in Cardiff Bay over a 12 month period, based on mixture analysis of shell length frequency data (*n* = 2,785).

Cohort	Approx. Age (days)	Mean size (mm)	Size range (mm)	SD	% Frequency
1	66	12.2	11–12	0.85	7.3
2	132	15.4	9–17	2.02	47.2
3	198	18.6	18–19	0.57	17.3
4	264	21.5	20–22	0.84	12.2
5	330	25.0	23–31	2.12	15.9

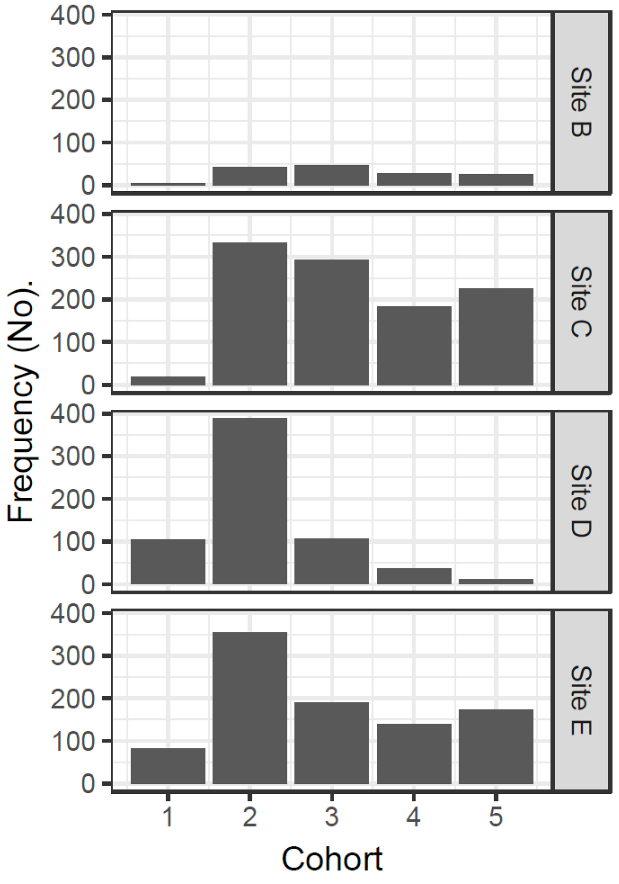

FIGURE 7 | Site variation in the relative frequency of different cohorts colonizing the experimental panels in Cardiff Bay derived from mixture analysis.

substrates, including submerged margins, piers, docks, and boat hulls that our panels tried to mimic. Zebra mussel have only been recorded on the bottom of Cardiff Bay on the aeration system or in small scattered patches (Alix et al., 2016), as the Bay has a high deposition rate of mud and silt (Vaughan et al., 2008; Olivier, 2016), which typically prevents settlement of juvenile mussels (Alix et al., 2016).

By carrying out monthly scrapes in artificial substrates, we have shown that zebra mussel began to settle in Cardiff Bay in July, 1 month after we deployed the experimental panels, and continued until October, with no evidence of new colonizations from November to May. Density and growth followed the same pattern, increasing over the summer and ceasing in October, after which no further recruitment or growth occurred. These findings are similar to those of previous studies (Fong et al., 1995; Ram et al., 1996; Alix et al., 2016), although there is large variation in the timing of reproduction and settlement of zebra mussel populations depending on temperature (Pollux et al., 2010). The fastest growth in Cardiff Bay was generally observed at the deepest waters (3 m), which also coincides with the highest settlement rates of new recruits. This strongly suggests that conditions that favor growth of zebra mussel also favor their survival. However, no significant differences in water chemistry were found within the first 3 m, despite a large variation in growth and settlement rates within the water column and our index of water chemistry (PC1) did not explain the size or abundance of mussels, despite large variation in water parameters among sites. This suggests that factors other than water chemistry control growth and colonization of zebra mussel, most likely physical disturbance, food abundance and UV exposure. Water parameters for Cardiff Bay are within the optimal values for zebra mussel (Kilgour et al., 1994; Spidle et al., 1995; Bowman and Bailey, 1998). However, the Bay is fitted with a bottom aeration system to maintain high dissolved oxygen and permit the passage of migratory Atlantic salmon and brown trout (Alix et al., 2016), and as a result, water is more mixed than would normally be, which may explain the apparent lack of stratification in water parameters (Alix, 2010).

The low settlement rate of mussels found in surface samples has been noted previously in laboratory and field studies (Kobak, 2001, 2004; Alix et al., 2016). Veligers appear to be absent from the upper 50 cm of the water column in Cardiff Bay (Alix et al., 2016). Avoidance of surface waters appears to be related to light intensity (Kobak, 2001; Seaver et al., 2009) and may confer mussels some protection against bird predators and desiccation caused by fluctuating water levels. For example, Alix (2010) reported that waterfowl fed on surface mussels in Cardiff Bay and has also been found that waves reduce settlement rates (Chase and Bailey, 1999; Kobak, 2004), which may explain the low abundance of mussels in our surface panels. Mean settlement rates varied between 4,200 and 6,200 mussels m^{-2} $month^{-1}$, which are similar to those reported for well established populations elsewhere (Cleven and Frenzel, 1993; Stanczykowska and Lewandowski, 1993; Mackie and Schloesser, 1996), and are also consistent with adult densities of 450–5,100 mussels m^{-2} estimated at Cardiff Bay during 2006–2009 (Alix, 2010; Alix et al., 2016). However, the peak of 17,960 adult mussels m^{-2} $month^{-1}$

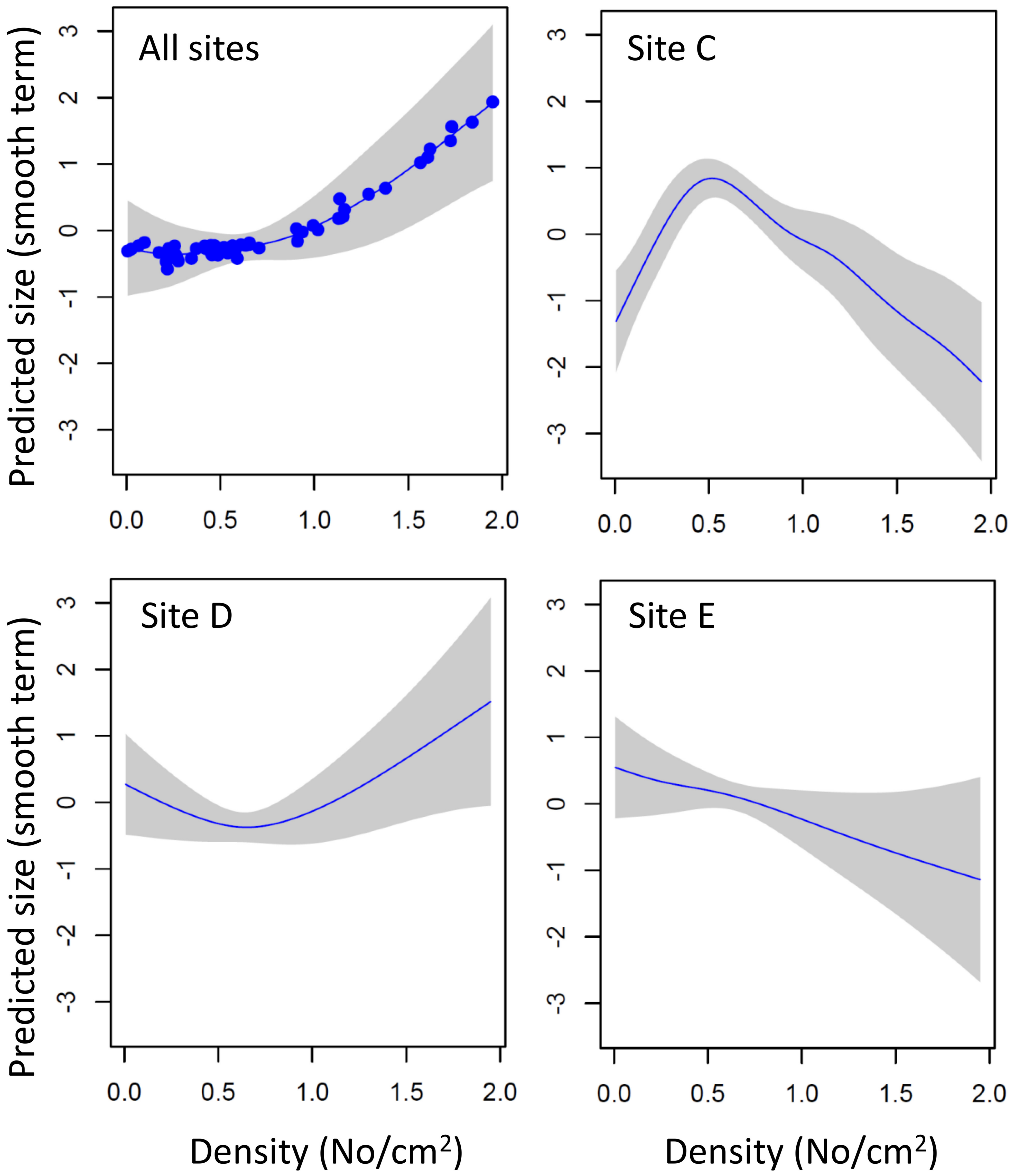

FIGURE 8 | Density-dependent changes in the growth of zebra mussel in different parts of Cardiff Bay. Shown are predicted sizes (GAM smoothed term centered around zero) and 95 CI envelopes across **All sites**, at sites with high recruitment **(Sites C,E)** and at a site of low recruitment **(site D)**.

recorded on September 2017 at a depth of 3 m at the mouth of the river Taff (site C) is over 2.3 times higher than the highest value reported previously for Cardiff Bay [7,700 individuals m^{-2} – Alix et al. (2016)], and also higher than the highest density ever recorded in Britain [11,000 individuals m^{-2}, – Aldridge et al. (2004)]. This may indicate that the zebra mussel population in Cardiff Bay is increasing despite the removal of approximately 4 tonnes of mussels every year (Alix et al., 2016).

Our results suggest the existence of five distinct cohorts resulting from the same spawning season, that our data suggest it extends from June to September (**Figure 5**). This is in agreement with results from previous surveys on veliger density that also indicated a reproductive season extending from late May/June to late September/October for Cardiff Bay (Alix et al., 2016). The presence of multiple cohorts from the same spawning season has not been reported previously but is consistent with results from the laboratory that indicate that the release of gametes occurs over 2–6 spaced events (Walz, 1978; Haag and Garton, 1992). It can also result from spatial variation in the timing of reproduction and growth across the Bay, as well as from dispersal at the post-settlement adult stage. Dispersal of zebra mussels is mostly through the planktonic veliger stage but settled adults can also disperse. Adults may choose to dislodge when conditions become unsuitable and be transported long distances attached to macrophytes and other vectors, and also drift using the byssus to gain buoyancy (Martel, 1993; Kobak, 2001). This means that colonization is not restricted to the veliger stage immediately after reproduction, but that it can be extended beyond this phase. Post-settlement dispersal, therefore, coupled with variation in timing of reproduction among locations and multiple releases of gamete could give rise to multiple cohorts and different waves of settlers, as observed in our study. More generally, variation in habitat quality, timing of reproduction and demography creates the conditions necessary for source and sink metapopulations to develop (Stoeckel et al., 1997) which may need to be taken into account in the management of zebra mussel. For example, our results suggest that different density dependent mechanisms may operate in different habitats, once the effects of depth and month of sampling have been taken into account.

In general, mussel size increased with density, but there were marked differences between sites. There was positive density-dependent growth at sites with low densities, and negative density-dependent growth at sites with high densities, suggesting some form of population regulation, presumably caused by competition. Zebra mussel settlement rates have been reported to be lower on substrate densely populated by adults (Nalepa et al., 1995) which is consistent with negative density dependence. Although river studies may not be extrapolated to lake populations, Strayer and Malcom (2006) found recruitment to be negatively related to the size of an adult river population, and showed how very rapid somatic growth could make some year-classes dominant and suppress recruitment of the next year class. Likewise, Mörtl and Rothhaupt (2003) reported a lower abundance of juveniles on substrates already colonized by adults, and suggested that adults were preying on veligers. These findings are consistent with negative density-dependence, as observed in our study. However, examples of more complex negative and positive density-dependence mechanisms have also been reported. For example, Chase and Bailey (1996) observed that veligers were chemically attracted to adult conspecifics and preferred to settle in high density areas, but only within certain densities. When densities were too high, veligers chose the lower densities, suggesting the existence of density thresholds and different density-dependent relationships. These results could have implications for management because removal could be beneficial or counter-productive depending on density thresholds and conditions.

The cost of controlling zebra mussel populations is generally high (Adams and Lee, 2012) and while different eradication methods have been tested in the laboratory (Costa et al., 2011; Watters et al., 2013; Claudi et al., 2014; Luoma et al., 2015) these are not always successful in the field (**Table 1**). The development of more efficient eradication methods should benefit from insights into natural factors regulating population growth (Lund et al., 2018). Currently, control measures against zebra mussel are limited to the use of various chemicals (Glomski, 2015), ultraviolet radiation (Lewis and Whitby, 1997) and mechanical removal and drying (Durán et al., 2010). These aim to reduce populations size, but our results may help explain why removal may not always work. Zebra mussels often follow boom and bust population dynamics (Stanczykowska, 1977; Burla and Ribi, 1998; Casagrandi et al., 2007; Strayer et al., 2017), but the underlying drivers of population regulation are not clear. Several predators feed on zebra mussel (Wisniewski, 1974; Pedroli, 1977; Petrie and Knapton, 1999; Werner et al., 2005; Naddafi and Rudstam, 2014), but "natural enemies" alone are unlikely to regulate zebra mussel populations (Molloy et al., 1997). Density-dependent drivers like disease outbreaks (Strayer and Malcom, 2006), or food depletion caused by mussel filtration (Karatayev et al., 2018) are thought to play a much greater role in regulating the abundance of zebra mussel. Whatever the reasons, we suggest that since complete eradication will seldom be possible, local zebra mussel populations dynamics should be considered before embarking on partial removal as this may prove expensive, ineffectual, and may in some cases enhance production and aggravate the problem.

In conclusion, our results indicate that the zebra mussel is a well established aquatic invader in Cardiff Bay and despite periodic removal, its numbers appear to be growing as evidenced by having the highest densities recorded in Britain to date, and also higher than previous estimates for this artificial water body. Given the overriding effect of water temperature on reproduction, under current predictions of climate change the spawning period of zebra mussel in Cardiff Bay will likely extend, which may result in even higher production. This makes the search for more effective control measures paramount. Based on the observed seasonal pattern of growth and recruitment, we suggest that control measures might benefit from targeting the summer and early autumn for removal of zebra mussel, as this appears to be the most critical period for the colonization and dispersal of this invasive species. We also suggest that control actions should target suitable structures located 3 m deep or deeper, as this appears to be the zone where most of the zebra mussel production occurs. However, the existence of contrasting patterns of density dependent growth (positive at sites of low recruitment and negative at sites of high recruitment) suggests that removal of adult mussels may help curtail biomass production at some sites but may enhance it at other sites. The existence of significant spatial variation in growth and settlement rates across the Bay, coupled with multiple cohorts, suggest that zebra mussel might be structured as a metapopulation governed by source and sink dynamics (Stoeckel et al., 1997). Although our study cannot resolve this possibility, this merits further attention and could be addressed by using molecular markers to determine patterns of gene flow (e.g., Therriault et al., 2005).

DATA AVAILABILITY STATEMENT

The datasets generated by this study are available on Figshare https://doi.org/10.6084/m9.figshare.8216279.v1.

ETHICS STATEMENT

The animal study was reviewed and approved by the Swansea University College of Science Ethics Committee Permit Student 210519/1557.

AUTHOR CONTRIBUTIONS

CG and SC designed the study and secured the funding. MR collected the data and carried out the analyses with advice from CG. MR and CG wrote the manuscript with contributions from SC and DH.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00159/full#supplementary-material

FIGURE S1 | Determination by mixture analysis of the most likely number of zebra mussel cohorts colonizing the experimental panels in Cardiff Bay (undisturbed panels) showing **(A)** changes in AIC values depending on number of putative cohorts, and **(B)** mean size (± SD) of the smallest cohort (cohort 1).

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Trophic Plasticity of the Highly Invasive Topmouth Gudgeon (*Pseudorasbora parva*) Inferred From Stable Isotope Analysis

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A wide trophic niche and high trophic plasticity are often invoked to explain the successful establishment of many aquatic invaders, but there is little information regarding the diet of most invasive fish in European waters. We combined stomach content and stable isotope analysis (SIA) of ^{13}C and ^{15}N to examine the trophic niche of the highly invasive topmouth gudgeon (*Pseudorasbora parva*) in three contrasting ponds and a reservoir in South Wales. Marked differences in diet and trophic position were found among neighboring systems only a few kilometers apart. The most diverse diet was found in ponds with the fewest number of inter-specific competitors, and resulted in topmouth gudgeon having the highest condition factor, the widest variation in δ^{13}C and δ^{15}N values, and the highest carbon content, typical of generalist feeders. In contrast, topmouth gudgeon that coexisted with other cyprinids, were much more likely to have an empty stomach and relied almost exclusively on plant seeds, resulting in fish having a poor condition factor and low trophic position. Comparisons with other trophic studies indicate that variation in isotope values among neighboring sites can exceed variation across continents, making it difficult to predict the diet or trophic impacts of the invasive topmouth gudgeon. Given the importance of obtaining reliable data on trophic position for risk assessment, our study shows that the use of SIA could be used to prioritize control and eradication measures that take into account trophic plasticity.

Keywords: aquatic invasive species, diet breadth, generalist feeder, foraging, stomach content analysis, isotopes

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INTRODUCTION

The topmouth gudgeon (TMG, *Pseudorasbora parva*) is a highly invasive fish native of Asia that has become one of the worst aquatic invaders in Europe due its potential impact on native biodiversity (Pinder et al., 2005; Britton et al., 2007). TMG possesses many ecological traits that make it a successful invader, including short generation time, high fecundity, and substantial phenotypic plasticity (Gozlan et al., 2010). The species can also grow rapidly (Ye et al., 2006; Adrović and Skenderović, 2007; Kapusta et al., 2008) and mature fast (Pinder et al., 2005), especially when living at high density (Britton et al., 2007, 2008).

During the first few years of the invasion, TMG populations appear to be composed mainly of young-of-the-year, up to 50 mm in total length, although the species can reach up to 120 mm (Ye et al., 2006; Adrović and Skenderović, 2007; Kapusta et al., 2008). The species displays substantial variation in the timing of spawning, depending on water temperature (Yan and Chen, 2009). Its diet can be wide and, although previous studies had described it as mostly omnivorous (Weber, 1984; Xie et al., 2001), it can also adopt a strict planktivorous diet in some cases (Priyadarshana et al., 2001; Asaeda et al., 2007). Depending on prey availability, TMG can feed on chironomid larvae (Wolfram-Wais et al., 1999), but also on the eggs and larvae of other fish, which may pose a threat to native species (Stein and Herl, 1986; Xie et al., 2000). The preferred prey size appears to be size dependent, as observed in a comparative pond study where the smaller size class (20–25 mm) fed exclusively on cladoceran zooplankton, the bigger size classes (35–60 mm) fed mainly on chironomid larvae, and the intermediate size classes fed on a mixture of the two preys (Declerck et al., 2002). The diet of topmouth gudgeon can also change seasonally, shifting from chironomid larvae in spring and summer to ostracods in winter (Xie et al., 2000). Where introduced, topmouth gudgeon has been found to compete for food with native species in Great Britain (Britton et al., 2010), Belgium (Declerck et al., 2002), and Poland (Witkowski, 2002) which can lead to depressed growth rates, reduced reproduction outputs and shifts in the trophic position of native fish (Britton et al., 2010). When other invasive species are present, the topmouth gudgeon's plasticity in food selection allows it to shift its trophic niche and co-exist with other invaders (Jackson and Britton, 2014).

TMG can negatively impact native fish through competition for food and space, resulting in depressed growth rates (and also in production), and also a shift in trophic levels (Britton et al., 2010). Another relevant threat posed by TMG is its ability to carry non-native pathogens such as the intracellular parasite *Sphaerothecum destruens* that can become lethal for salmonids (Gozlan et al., 2005; Andreou et al., 2011). For these reasons, it is feared that the spread of TMG could result not just in a significant loss of native river biodiversity, but also on substantial economic losses caused by damage to sport fisheries and salmon farming alike (Gozlan et al., 2005).

TMG was first recorded in Great Britain in 1996, in a pond in Southern England (Domaniewski and Wheeler, 1996). It is thought that the first introduction may have occurred a decade earlier, in the mid 1980's, when TMG originating from Germany were delivered to an ornamental aquaculture facility in Hampshire, and subsequently became established in Lake Tadburn (Gozlan et al., 2002). Within few years, TMG had spread rapidly and has now been reported in 32 locations across England and Wales, most of which are lentic systems, and 10 of which are connected to major catchments (Britton et al., 2007, 2010). Recent eDNA data show that more sites are infested than previously thought (Robinson et al., 2019), probably because traditional sampling techniques do not detect the species at low densities (Britton et al., 2011). Due to its speed of colonization, and the potential for major ecological and economic impacts, TMG has been classified as highly impactive by the UK Technical Advisory Group on the Water Framework Directive (Panov et al., 2009) and is the subject of costly eradication programs (Robinson et al., 2019).

Stable isotope analysis (SIA) can be used to study food webs (Vander Zanden et al., 1999), based on the fact that the ratios of heavy to light isotopes in animal tissues are often affected by diet (DeNiro and Epstein, 1978, 1981). Carbon and nitrogen isotopes (δ^{13}C and δ^{15}N, respectively) are the ones most commonly used in freshwater biology, as they are related to the sources of primary productivity (δ^{13}C) and to trophic level (δ^{15}N). As trophic markers, SIA can be used at different ecological levels, to study variation among individuals, species, and also among communities (Whitledge and Rabeni, 1997; Newsome et al., 2009). They can be used to examine trophic position (Roth et al., 2006; Cherel et al., 2008), animal migrations (Cherel et al., 2007; McClellan et al., 2010), impacts of invasive species (Vander Zanden et al., 1999; Nilsson et al., 2012), trophic subsidies (Izquierdo-Gomez et al., 2015), and the contribution of allochthonous vs. autochthonous food resources in aquatic ecosystems (Solomon et al., 2011; Venarsky et al., 2014).

Here we employed SIA of nitrogen and carbon, complemented with analysis of stomach contents, to investigate variation in the trophic ecology of topmouth gudgeon in four contrasting freshwater habitats in South Wales. Our aims were twofold: (1) to describe the trophic niche of this highly invasive species to gain a better understanding of the potential for interference competition with native fish, and (2) to assess the value of SIA for monitoring diet plasticity of a rapidly expanding invasive species in different colonized habitats.

MATERIALS AND METHODS

Origin of Samples

We analyzed 118 topmouth gudgeon originating from four contrasting water bodies in South Wales (**Figure 1** and **Table 1**) that differed greatly in size, use, and fish community composition: two small decorative ponds with few or no fish species other than eel (Turbine Pond, TUR, $n = 27$; Dyfatty pond, DYF, $n = 29$), a eutrophic pond used for recreational fishing regularly stocked with eight species of coarse fish (Sylen Lake, SYL, $n = 30$), and a larger, cooler reservoir used for water supply and stocked with salmonids (Upper Lliedi Reservoir, LLI, $n = 32$). Sylen Lake, Turbine pond, and Dyfatty pond were fished between November 2012 and February 2013 while the Upper Lliedi Reservoir (LLI) was fished in June 2018 as no fish could be caught during the winter of 2017/2108. Specimens were kept frozen until analysis. We obtained biometry data (total length, weight, condition factor) and stable isotope data (δ^{13}C, δ^{15}N, C:N) for the four ponds, and stomach contents for three of them (no stomach data was available for LLI).

Biometry Data

We took paired measurements of total length and weight of TMG from one site (LLI) before (fresh) and after they had been frozen to derive a regression equation to estimate fresh body size from

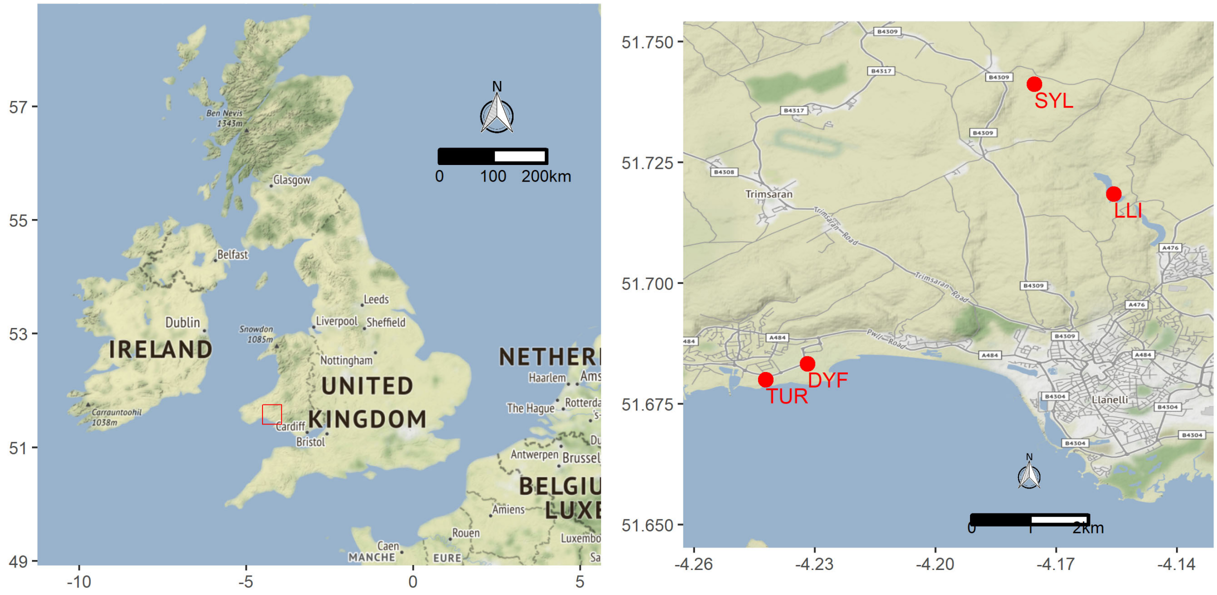

FIGURE 1 | Location of study sites sampled for topmouth gudgeon in Wales (UK): Upper Lliedi Reservoir – LLI, Sylen pond – SYL, Turbine pond – TUR, and Dyfatty pond – DYF.

TABLE 1 | Characteristics of the four study sites examined for trophic position of topmouth gudgeon (*Pseudorasbora parva*) in South Wales.

Site	UK National Grid Map Reference (SN)	Area (km^2)	Alt (m)	Sampling period	No. TMG sampled	Use	Other fish present	1st TMG record
Upper Lliedi (LLI)	5109 0459	0.130	86	June 2018	32	Public water reservoir Recreational angling	Brown trout (*Salmo trutta*) Rainbow trout (*Oncorhynchus mykiss*) Others	Unknown
Sylen (SYL)	49914 07018	0.016	144	November 2012	30	Recreational angling	Carp (*Cyprinus carpio*) Tench (*Tinca tinca*) Bream (*Abramis brama*) Roach (*Rutilus rutilus*) Chub (*Squalius cephalus*) Rudd (*Scardinius erythrophthalmus*) Goldfish (*Carassius auratus*)	2005
Turbine (TUR)	45021 00334	0.011	7	February 2013	27	Decorative pond	Eel (*Anguilla anguilla*)	2001
Dyfatty (DYF)	45829 00627	0.004	4	February 2013	29	Decorative pond	Eel (*Anguilla anguilla*) Perch (*Perca fluviatilis*) Rudd (*Scardinius erythrophthalmus*) Roach (*Rutilus rutilus*)	2001

frozen specimens collected from the other three populations. The regression equations were:

$$\text{Length}_{(fresh)} = 1.025013 \times \text{Length}_{(frozen)}{}^{0.99633324}$$

$$R^2 = 0.9744, P < 0.001$$

$$\text{Weight}_{(fresh)} = 1.029898 \times \text{Weight}_{(frozen)}{}^{1.01114309}$$

$$R^2 = 0.9979, P < 0.001$$

We calculated Fulton's condition factor (K) from total length (TL) and weight (WT) measurements [K = (WT * 100) / TL^3] to derive an index of body condition (Bolger and Connolly, 1989).

Stomach Content Analysis

For stomach content analysis, frozen samples were slowly thawed and then transferred to 30, 50, and 70% ethanol solutions. We analyzed stomach contents of fish in three ponds (TUR, DYF, SYL) with a stereo microscope (Nikon SMZ1270) at 12.7× (0.63–8×) magnification. The vast majority of prey consisted of zooplankton (67%) that we classified at family level. Insect parts (1%), seeds (24.1%) and plant material were also present, but these could not be identified. We counted every food item and measured the size (longest axis) of a subsample of food items to obtain an average size for each prey category.

Stable Isotope Analyses

We obtained 1–2 mg of white muscle from the caudal part of the fish, above the lateral line, and freeze dried the samples for 24 h before analysis. Analyses of %C, %N, δ^{13}C, and δ^{15}N were conducted by Isotope Ratio Mass Spectrometry (EA-IRMS) at Iso-Analytical Ltd (Marshfield Bank, Crewe CW2 8UY, United Kingdom) using a Europa Scientific 20–20 IRMS Elemental Analyzer, samples being combusted at 1,000°C in the presence of oxygen using IA-R068 as reference material (soy protein, $\delta^{13}C_{V-PDB}$ = -25.22‰, $\delta^{15}N_{AIR}$ = 0.99‰). The following standards were run as quality controls: IA-R068, IA-R038 (L-alanine, $\delta^{13}C_{V-PDB}$ = -24.99‰, $\delta^{15}N_{AIR}$ = -0.65‰), IA-R069 (tuna protein, $\delta^{13}C_{V-PDB}$ = -18.88‰, $\delta^{15}N_{AIR}$ = 11.60‰) and a mixture of IAEA-C7 (oxalic acid, $\delta^{13}C_{V-PDB}$ = -14.48‰) and IA-R046 (ammonium sulfate, $\delta^{15}N_{AIR}$ = 22.04‰) was run as quality control samples during analysis and were calibrated against inter-laboratory standards distributed by the International Atomic Energy Agency, Vienna. Repeatability of isotope values in samples analyzed in duplicate (20%) was high (Cohen's weighted kappa coefficient: δ^{13}C = 0.99; δ^{15}N = 0.98; C:N = 0.94) indicating that SIA results were precise. Duplicates were then averaged before numerical analysis. The carbon to nitrogen ratio (C:N) ranged between 3.13 and 3.81, but was significantly below 3.5 (U95CI = 3.35), so lipid correction normalization was not deemed necessary (Post et al., 2007). In total we analyzed 118 specimens for SIA (DYF, n = 29; LLI, n = 32; SYL, n = 30; TUR, n = 27).

Statistical Analysis

We used R 3.3 (R Core Team, 2017) for analyses. We transformed % carbon and % nitrogen content with the arcsine transformation to make the data suitable for linear model analysis. We analyzed nitrogen and carbon content %, δ^{13}C, δ^{15}N and C:N ratio as function of fish size, condition factor and pond (location), and used the *dredge* and *anova* functions in the MuMIn R package for model selection. We calculated the Shannon diversity index (H) to estimate the taxonomical diversity of the food items present in fish stomachs. Quadratic weights were applied to give greatest emphasis to large differences between scores. GLM binary logistic regression was used to model the occurrence of an empty stomach (yes or no) as a function of fish size, condition factor and pond type. We performed an analysis of similarities (ANOSIM) with the *vegan* R package (Oksanen et al., 2013) to test for differences in diet composition among locations, merging together the less frequent food classes (unidentified, insect parts and *Cyclops*). Differences in the Shannon index of diversity (H) were assessed by bootstrapping (1,000 permutations) to obtain means and 95 confidence intervals (Gardener, 2014), we then used the Hutcheson t-test to compare the diversity among sites (Hutcheson, 1970). Trophic breadth for each pond was calculated using Levin's measure of niche breadth (Krebs, 1999):

$$B = \frac{Y^2}{\sum N_j^2}$$

where, B = Levin's measure of niche breadth.
N_j = Number of individuals using prey$_j$.
Y = ΣN_j = Total number of individuals sampled.

Ethics Statement

This study was reviewed and approved by Swansea University, College of Science Ethics Committee, Permit Student Project 210519/1544.

RESULTS

Variation in Body Size and Condition Factor

The body size (total length) of topmouth gudgeon varied from 33 to 100 mm, and the calculated wet weight from 0.44 to 5.02 g. There were no significant differences between ponds in the length or weight after Tukey's HSD correction (**Figures 2A,B**) but there was a significant difference in condition factor [$F_{(3,114)}$ = 5.05, P = 0.003; **Figure 2C**] with fish in Turbine Pond having a better condition than those in Dyfatty pond (P = 0.006) or the Upper Lliedi Reservoir (P = 0.003).

Diet Analysis

The proportion of fish with an empty stomach differed significantly among sites (GLM binary logistic regression: deviance = 22.09, $P < 0.001$), but was unrelated to body size (deviance = 2.57, P = 0.417), or condition factor (deviance = 0.14, P = 0.70). The pond with the greatest incidence of empty stomachs was DYF (21/25 or 84%), followed by SYL (19/29 or 65.5%), while TUR had the lowest incidence (6/27 or 22.2%). Tukey multiple comparisons at 95% family wise confidence level show that the incidence of empty stomachs was significantly lower at TUR than at DYF ($P < 0.001$) or SYL ($P < 0.005$), while no significant difference was observed between DYF and SYL (P = 0.237).

The majority of stomach contents (756 food items or 67% of the total) was composed of zooplankton, including *Daphniidae* (268 specimens or 23.8%), *Bosminidae* (136 specimens or 12%), *Chydoridae* (348 specimens or 30.9%), and *Cyclopidae* (4 specimens or 0.3%). The remaining was composed of plant seeds (272 items or 24.1%), unidentified insect parts (12 items or 1%), and unidentified organic and plant material (88 items or 7.8%; **Figure 3**). Mean prey size varied considerably, being largest in insect parts (2.14 mm), followed by *Cyclopidae* (1.51 mm), *Daphniidae* (1.28 mm), *Bosminidae* (0.67 mm), plant seeds (0.62 mm), and *Chydoridae* (0.48 mm).

Analysis of similarities (ANOSIM) indicated that the diet of TMG differed markedly among sites (R = 0.28, P = 0.005), and this was reflected in different prey diversity estimates.

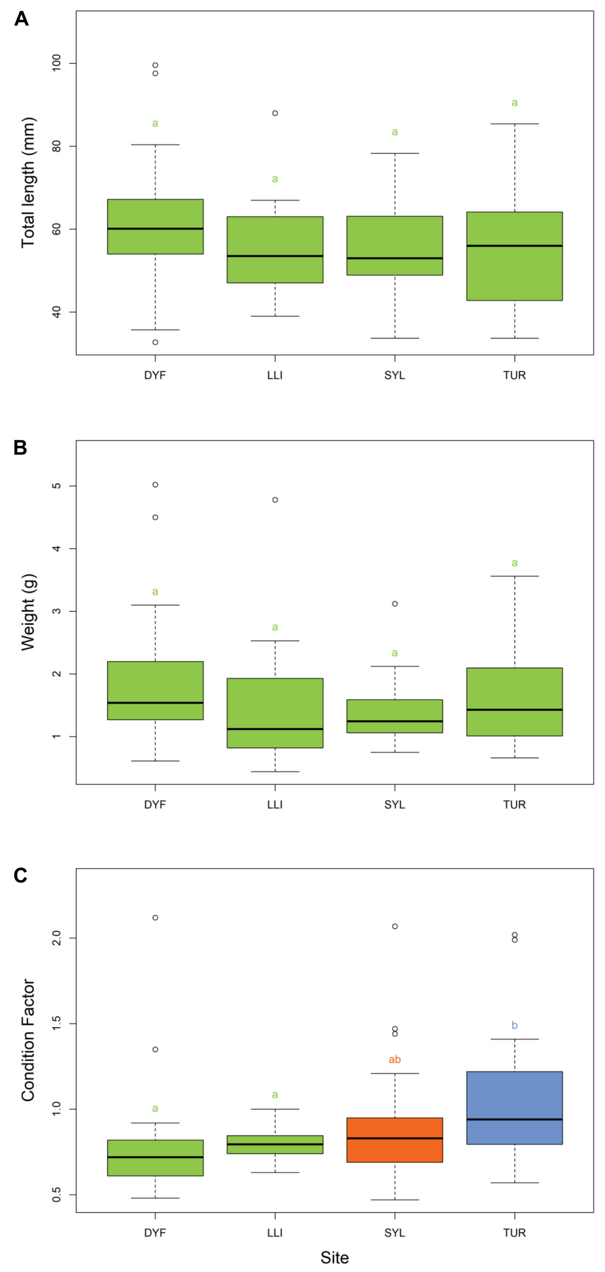

FIGURE 2 | Variation in **(A)** total length, **(B)** weight, and **(C)** Fulton's body condition factor of topmouth gudgeon across study sites. Sites that have different colors and letter subscripts are statistically different from each other following Tukey's HSD *post-hoc* pairwise tests.

Thus, the diet of fish at DYF had a much lower prey diversity (mean bootstrapping estimate H = 0.148, 95CI = 0.089–0.218) than those at TUR (H = 1.270, 95CI = 1.223–1.317) or SYL (H = 1.244, 95CI = 1.173–1.318). Pairwise Hutcheson t-test comparisons indicated that there were statistical differences between TUR and DYF ($P < 0.001$) and between SYL an DYF ($P < 0.001$), but not between TUR and SYL ($P = 0.392$).

Differences in diet were reflected in measures of diet breadth (**Table 2**). Thus, while fish at TUR and SYL had, similarly, wide diets (bootstrapping estimates B, TUR = 3.286, 95CI = 1.666–5.005; SYL = 3.270, 95CI = 1.611–4.998), the diet breadth at DYF was much narrower (B = 1.522, CI = 1.058–2.000), consistent with greater diet specialization, but also with lack of prey choice.

Stable Isotope Analysis

The carbon content in the muscle of topmouth gudgeon ranged between 41 and 60% among individuals, and differed significantly among ponds (**Figure 4A**, $F_{(3,113)} = 11.54$, $P < 0.001$), being lowest in the LLI reservoir and highest in the eutrophic SYL pond (*post-hoc* pairwise HSD, SYL-LLI, $P < 0.001$; TUR-LLI, $P < 0.001$). Carbon content did not depend on body length (t = -0.385, P = 0.701), condition factor (t = -0.035, P = 0.972) or the presence of food items in the stomach (t = -0.69, P = 0.490). The nitrogen content varied from 12 to 18% among individuals, and as with %C, it also varied significantly among ponds (**Figure 4B**, $F_{(3,113)} = 6.77$, $P < 0.001$), but did not depend on body length (t = 0.51, P = 0.610), condition factor (t = 0.31, P = 0.754), or presence of food items in the stomach (t = -1.51, P = 0.133). Nitrogen content was lowest in the reservoir and highest in the eutrophic angling pond (*post-hoc* pairwise HSD, SYL-LLI $P < 0.001$; SYL- DYF $P = 0.004$).

C:N ratios (a measure of lipid reserves) varied between 3.13 and 3.81 among individuals, but were unaffected by body length (t = -1.22, P = 0.224), condition factor (t = 0.2, P = 0.835), or presence of food items in the stomach (t = -0.96, P = 0.335). They varied significantly among sites (**Figure 4C**, $F_{(3,113)} = 4.15$, $P = 0.007$), being highest in one of the small ornamental ponds, and lowest in the reservoir (DYF-LLI, $P = 0.016$).

δ^{13}C varied markedly among individuals (range = –30.95‰ to –16.43‰) and differed significantly among all sites (**Figure 5**; $F_{(3,113)} = 24.77$, $P < 0.001$), being highest in the small ornamental ponds and lowest in the eutrophic angling pond (pairwise comparisons, LLI-DYF, $P < 0.001$; TUR-DYF, $P < 0.001$; SYL-LLI, $P < 0.001$; TUR-SYL, $P < 0.001$). δ^{13}C was unaffected by body length (t = -0.09, P = 0.927) or condition factor (t = -1.14, P = 0.909), but increased (i.e., it was more enriched) among fish that had food in their stomach (t = 2.90, P = 0.004).

δ^{15}N varied from 5.17 to 15.10‰ among individuals, and also varied significantly among all sites (**Figure 5**; $F_{(3,113)} = 574.27$, $P < 0.001$), all ponds being significantly different from each other ($P < 0.001$), being highest in the reservoir and lowest in the small ornamental ponds. As for δ^{13}C, δ^{15}N was unaffected by body length (t = -0.567, P = 0.572) or condition factor (t = -0.156, P = 0.876), but increased (i.e., it became more enriched) among fish that had food in their stomach (t = -3.23, P = 0.001).

Examination of the isotopic δ^{13}C vs. δ^{15}N plot (**Figure 5**) indicate a substantial trophic discrimination among study sites, particularly with respect to δ^{15}N, with the large, deep reservoir (LLI) being nitrogen enriched and the small ornamental pond (DYF) being nitrogen depleted compared to the other sites.

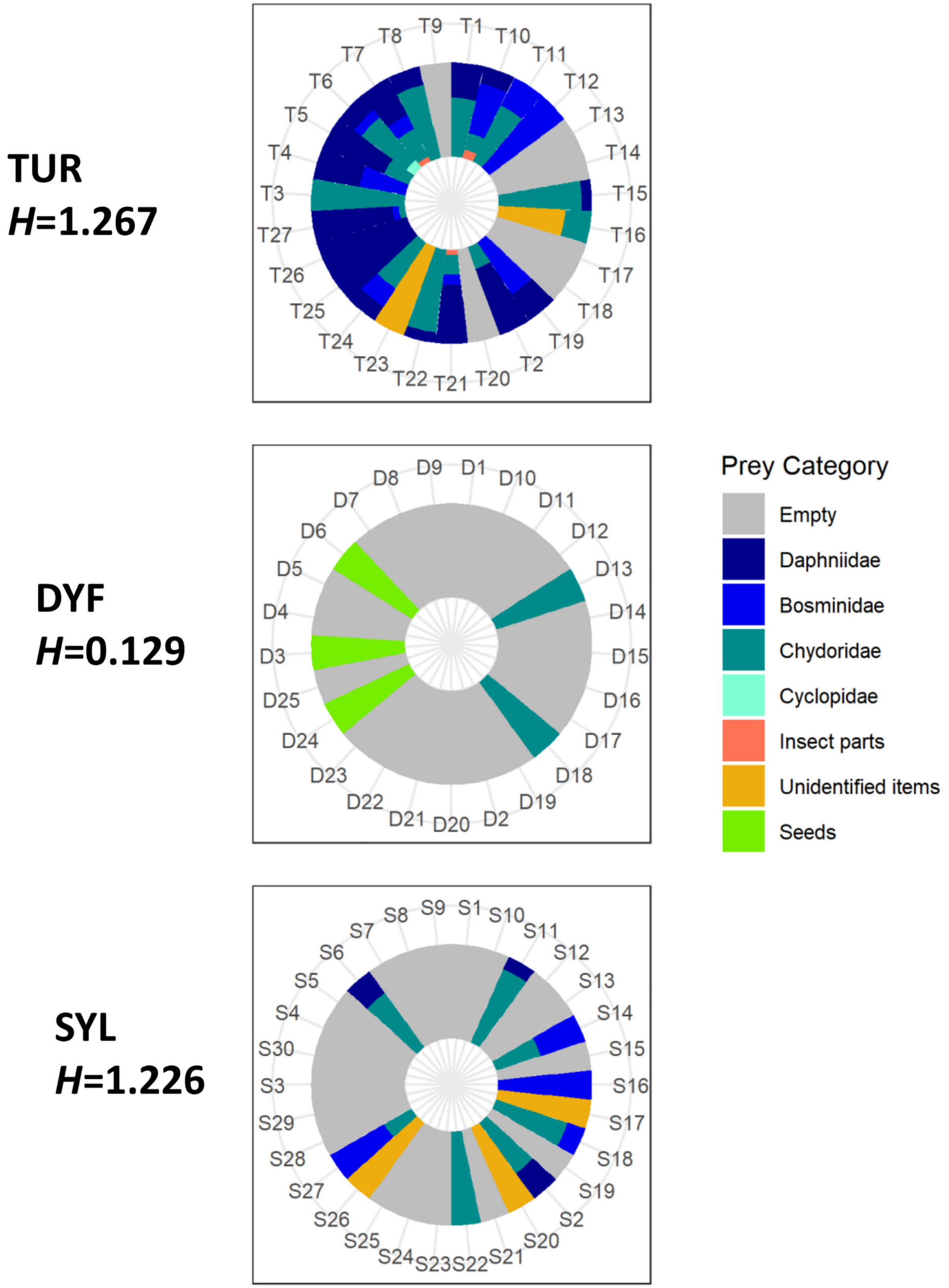

FIGURE 3 | Individual variation in the diet of topmouth gudgeon (% items) across study sites showing Shannon diversity index (*H*). Each numbered sector represents one fish.

TABLE 2 | Diet of topmouth gudgeon based on stomach content analysis (% number).

Site	*Daphniidae*	*Bosminidae*	*Chydoridae*	*Cyclopidae*	Insect parts	Unidentified items	Seeds	No. prey items	Percentage empty stomach (%)	Levins's *B* (niche breadth)	Lower CI	Upper CI
Turbine TUR	0.38	0.16	0.39	0.01	0.02	0.04	0.00	664	22.2	3.07	1.666	5.005
Sylen SYL	0.09	0.15	0.43	0.00	0.00	0.33	0.00	184	65.5	3.07	1.611	4.998
Dyffatty DYF	0.00	0.00	0.03	0.00	0.00	0.00	0.97	280	84.0	1.06	1.058	2.000

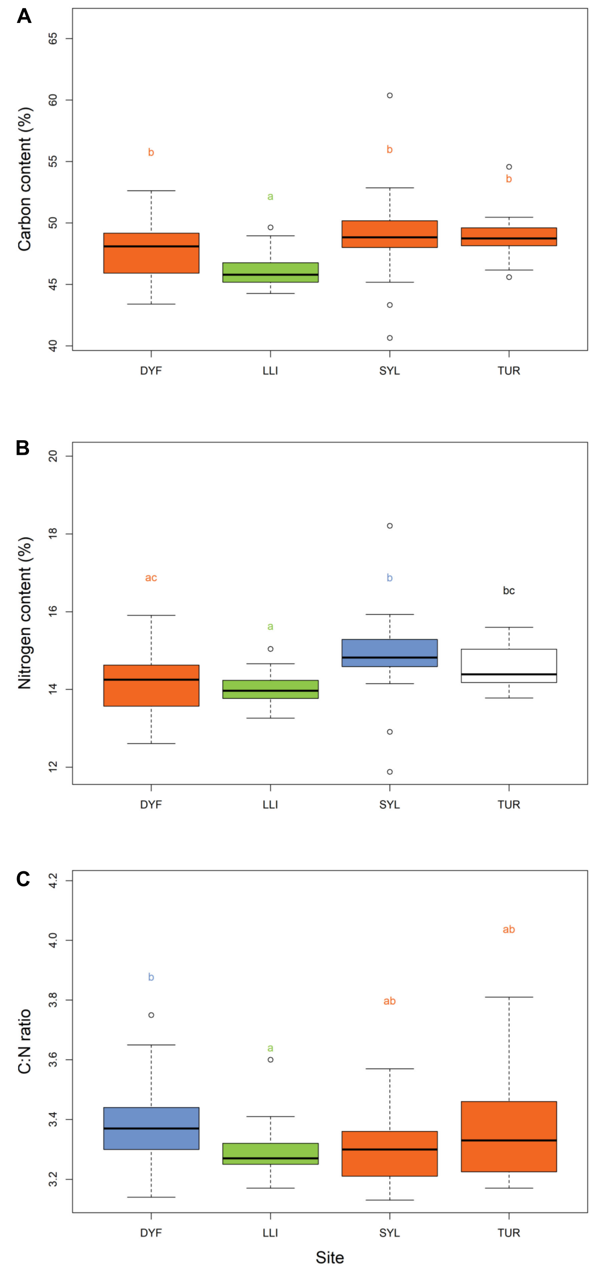

FIGURE 4 | Variation in **(A)** % carbon content, **(B)** % nitrogen content, and **(C)** C:N ratios of topmouth gudgeon across study sites. Sites that have different colors and letter subscripts are statistically different from each other following Tukey's HSD *post-hoc* pairwise tests.

DISCUSSION

Our study sheds light on the topmouth gudgeon's trophic plasticity and shows that its diet varies markedly among sites, even between neighboring ponds only a few kilometers away from each other. We acknowledge that differences in the timing of sampling must have introduced some unwanted variation in our study, but marked differences in stomach contents, δ^{13}C, δ^{15}N, and condition factor were also found between sites DYF and TUR which were sampled at the same time in February. Thus, season alone cannot explain our results. Isotopic turnover in TMG is not known, but data on other cyprinids indicate that isotopic equilibrium may take several months in muscle tissue. Half-life muscle turnover rate for δ^{15}N was 84 days for barbel *Barbus barbus* (Busst and Britton, 2018) and 68 days for grass carp *Ctenopharyngodon idellus* (Xia et al., 2013); equilibrium in barbel was not reached even after 125 days, with 95% turnover taken 371 days (Busst and Britton, 2018). This indicates that SIA signals, unlike stomach contents, are relatively stable over time. Furthermore, as muscle isotopic turnover is mostly influenced by body growth (Busst and Britton, 2018), we would not expect substantial changes in isotopic signatures during the colder parts of the year, when three of our four ponds were sampled. Isotopic signatures of δ^{13}C and δ^{15}N showed little seasonal variation in the muscle of whitefish in Lake Geneva during autumn and winter, and only reflected the food consumed during spring and summer (Perga and Gerdeaux, 2005).

Trophic plasticity, i.e., the ability to switch diets, is expected to facilitate establishment success among invasive species (Schröder and Garcia de Leaniz, 2011; Pettitt-Wade et al., 2015), and may have allowed the topmouth gudgeon to colonize different environments, exploit novel food resources, and outcompete many native fish species (Záhorská et al., 2009, 2010). However, trophic plasticity also carries costs (Shea and Chesson, 2002). Trophic shifts are common in response to changes in habitat and food availability (Werner and Hall, 1977), but can also represent a strategy to avoid potential trophic competition (Jackson et al., 2012). In our study, topmouth gudgeon with a mean size of 57 mm were feeding on relatively small food items, most of which were within the 0.7–1.3 mm size range, and hence less than 2% of their body size. The consumption of small zooplankton, shown at all our sites, has been reported previously (Arnold, 1990; Rosecchi et al., 1993), along with a diet composed of benthic crustaceans (Rosecchi et al., 1993; Xie et al., 2000), chironomids (Declerck et al., 2002), phytoplankton and algae (Muchacheva, 1950). However, the reliance on plant seeds found at one of our

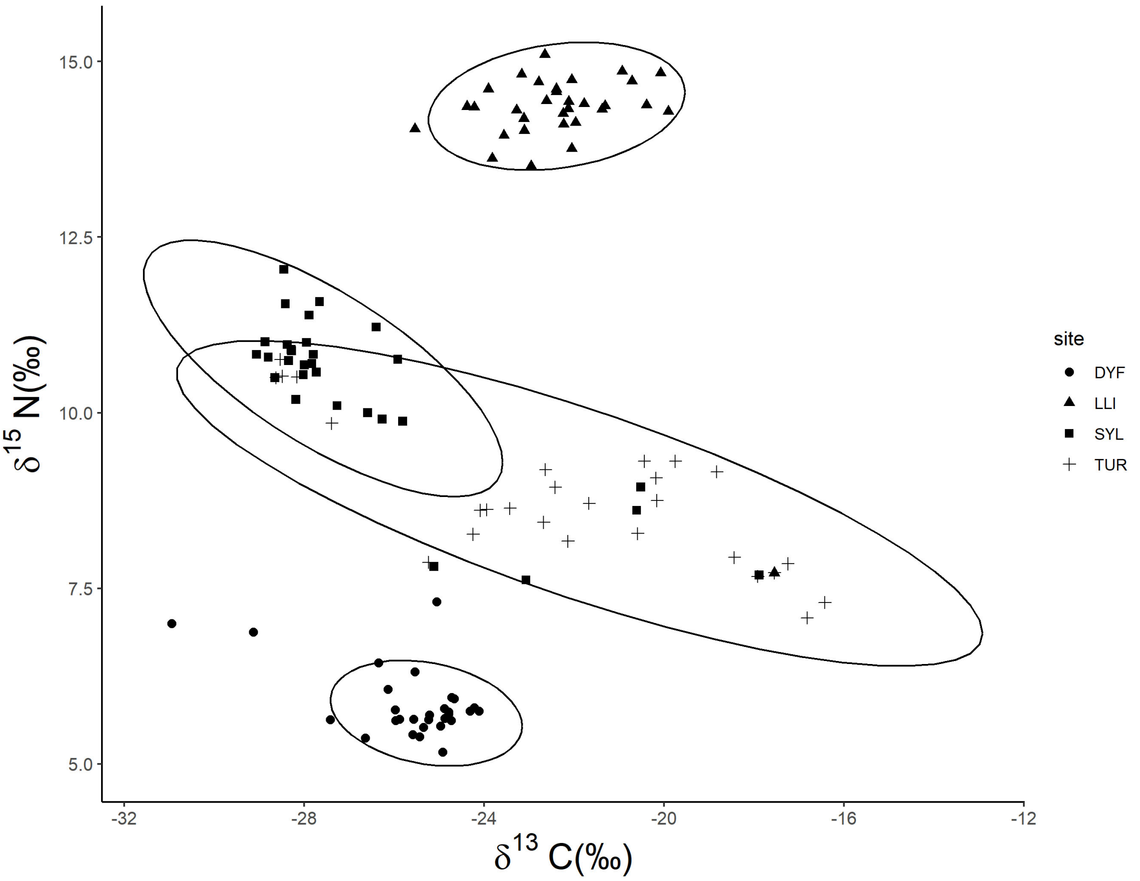

FIGURE 5 | Biplot of δ^{13}C vs. δ^{15}N of topmouth gudgeon (95% confidence ellipses) across study sites.

TABLE 3 | Comparison of isotope values for topmouth gudgeon across locations.

Mean length (±SE) or range (mm)	Habitat	δ^{13}C	δ^{15}N	Country	Status	References
19–118	Pond (3,250 m^2)	−23.3 ± 0.7	8.4 ± 0.7	England	Invasive	Britton et al., 2010
20–108	Lake Izunuma (3.69 km^2)	−29.5 to −25.2	12.2–15.9	Japan	Native	Yasuno et al., 2012
60–90	Lake Taihu (2,338 km^2)	−24.0 ± 0.2	14.2 ± 0.2	China	Native	Mao et al., 2012
74 ± 2.2	Pond (600 m^2)	−38.6 ± 0.1	–	Belgium	Invasive	Tran et al., 2015
72 ± 2.3	Pond (1,900 m^2)	−35.8 ± 0.4	–	Belgium	Invasive	Tran et al., 2015
63 ± 3.9	Pond (3,000 m^2)	−25.5 ± 0.1	–	Wales	Invasive	Tran et al., 2015
–	8 ponds (240 m^2–400 m^2)	−32.8 ± 0.5	–	England	Invasive	Tran et al., 2015
90–110	3 polyculture ponds (6,000 m^2)	−21.9 ± 0.6	9.8 ± 0.2	China	Native	Mao et al., 2016
–	Lake Huizhou West (1.8 km^2)	−24.9 ± 0.2	17.5 ± 0.3	China	Native	Gao et al., 2017
63 ± 13.7	Dyfatty pond (3,800 m^2)	−25.6 ± 1.4	5.8 ± 0.5	Wales	Invasive	This study
54 ± 10.2	Lliedi reservoir (0.13 km^2)	−22.3 ± 1.5	14.1 ± 1.2	Wales	Invasive	This study
55 ± 10.1	Sylen pond (16,200 m^2)	−26.7 ± 2.7	10.3 ± 1.1	Wales	Invasive	This study
55 ± 14.3	Turbine pond (10,600 m^2)	−22.1 ± 3.8	8.8 ± 1.0	Wales	Invasive	This study

sites was unexpected, and may have been the consequence of strong inter-specific competition.

Results from stomach content analysis were largely confirmed by results from SIA and seemed to reflect the combined effects of food availability and competition. However, both techniques are needed to properly characterize individual variation in diet, as found for other lentic fish, for example the pumpkinseed sunfish (Locke et al., 2013). Thus, the widest variation in δ^{13}C and δ^{15}N was observed at one of the small recreational ponds (TUR), which is consistent with a generalist diet largely unaffected by inter-specific competition as only one other fish was recorded (European eel, *Anguilla anguilla*). In contrast, at the other recreational pond (DYF) topmouth gudgeon coexisted with four other species (eel, perch *Perca fluviatilis*, rudd *Scardinius erythrophthalmus*, and roach *Rutilus rutilus*), displayed a very high occurrence of individuals with an empty stomach (84%)

and relied almost exclusively on plant seeds. This may explain why they attained a much lower condition factor, and displayed a lower trophic level, suggesting that plant seeds are nutritionally poor and not a preferred food resource. Britton et al. (2010) have shown that roach occupies a similar trophic position as topmouth gudgeon and this can result in competition for food, while rudd occupies a higher trophic level and may prey on topmouth gudgeon. Therefore, the presence of competitors and predators may have forced the topmouth gudgeon to adopt a more specialized feeding behavior in this pond. In a similar way, the narrow trophic position of TMG at the reservoir could be explained by high predatory pressure, since this reservoir is managed by an angling association which regularly stocks it with trout for recreational fishing.

We found that the body condition factor (a measure of growth performance – Froese, 2006) and the C:N ratio (a proxy for stored lipids – Jardine et al., 2008) was highest in the pond with the most diverse diet, widest diet breadth, and fewest competitors (Turbin pond where only eel was reported). This was also reflected in high carbon and nitrogen contents, as well as in high $\delta^{13}C$ values. In aquatic food webs, high $\delta^{13}C$ values tend to correlate with better feeding conditions and less competition (Hinz et al., 2017), which is consistent with our results. In contrast, TMG sampled in the much larger reservoir (LLI) where predatory salmonids abound, had the lowest C:N ratios, indicative of low lipid reserves, as well as low carbon and nitrogen contents, which are also consistent with poor feeding conditions (Vander Zanden et al., 1999).

Comparisons of SIA values from our study and other studies (**Table 3**) indicate that TMG in Wales generally have higher $\delta^{13}C$ and $\delta^{15}N$ values (i.e., more enriched) than those reported in another study in Great Britain (Britton et al., 2010), indicating a higher trophic level ($\delta^{15}N$) and a different primary source of carbon ($\delta^{13}C$). Our $\delta^{13}C$ values are relatively high and closer to the ones reported in China – where topmouth gudgeon is a native species (Mao et al., 2012, 2016; Gao et al., 2017) than to values observed in Belgium where the species is invasive (Tran et al., 2015). High $\delta^{13}C$ values are more typical of benthic food pathways, compared to pelagic food pathways which tend to be more ^{13}C depleted (Pinnegar and Polunin, 1999). Variation in $\delta^{15}N$ was also greater in our study than those reported in the literature, and one of our sites (DYF) had the lowest $\delta^{15}N$ value reported for the species; this coincides with a diet dominated by plant seeds. In general, the variability among sites is so high that for example $\delta^{15}N$ values obtained from fish at one of our sites (LLI) are more similar to those found in fish from Lake Taihu in China (Mao et al., 2012) than to those at neighboring sites in Wales. This indicates that it is difficult to predict the diet or trophic position of TMG even within small geographical areas. Substantial spatial variation in stable isotope signatures have also been reported for other lentic fish (Locke et al., 2013).

CONCLUSION

Our study indicates that when the topmouth gudgeon colonizes a new area it may be difficult to predict its trophic impacts from local information, since differences in diet are largely independent of geographical proximity. Populations living in neighboring water bodies can have diets more dissimilar than those from populations thousands of kilometers away. Given the importance diet information has on models that attempt to predict the risks posed by invasive fish, such as FISK (Copp et al., 2009; Mastitsky et al., 2010; Copp, 2013; Simonovic et al., 2013), uncertainty on the expected diet of the topmouth gudgeon could result in incorrect risk assessments. To improve the precision of risk assessments, information on trophic level seems to be important for predicting the diet, and therefore the likely feeding impacts, of topmouth gudgeon on native communities. As trophic impacts will likely vary widely from site to site, our study shows that SIA could be used, perhaps in combination with eDNA (Robinson et al., 2019), for species detection to prioritize control and eradication measures that consider variation in diet.

DATA AVAILABILITY STATEMENT

The datasets generated by this study are available on Figshare: 10.6084/m9.figshare.1197447.

ETHICS STATEMENT

The animal study was reviewed and approved by Swansea University College of Science Ethics Committee, Permit Student 210519/1544.

AUTHOR CONTRIBUTIONS

CG and SC designed the study and secured the samples and funding. MR collected the data and carried out the analyses with advice from CG. MR and CG wrote the manuscript with contributions from SC. All authors contributed to the article and approved the submitted version.

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Alternative Life-History in Native Trout (*Salmo* spp.) Suppresses the Invasive Effect of Alien Trout Strains Introduced Into Streams in the Western Part of the Balkans

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The diversity of native trout fish *Salmo* spp. comprises a variety of nominal taxa in Serbia, Montenegro, and Bosnia and Herzegovina. Recent mapping of the resident trout populations detected the presence of brown trout *Salmo trutta* (*sensu stricto*) of the Atlantic (AT) mtDNA lineage introduced into populations of both tentative Danubian trout *Salmo labrax* and of tentative Adriatic trout *Salmo farioides* belonging to the Danubian (DA) and Adriatic (AD) mtDNA lineages, respectively. Introduction of the tentative Macedonian trout *Salmo macedonicus* of the AD lineage was also detected in a native population of the tentative *S. labrax*. In almost all recipient nonmigratory trout populations, a cross-breeding between native and introduced trout was detected by heterozygosity in either only the *LDH-C* nuclear locus or the *LDH-C* and specific microsatellite loci. The only exception was a population where both resident and migratory, lake-dwelling individuals of the tentative Adriatic trout spawned in a downstream section of a stream in Montenegro, as no microsatellite alleles of Atlantic brown trout that had been introduced upstream were detected. The occurrence of cross-breeding between Adriatic and brown trout was evident in the isolated, upstream section. It appears that migrating, lake-dwelling Adriatic trout in combination with their resident, stream-dwelling conspecifics suppress the introgression of genes from those situated upstream. In this regard, consideration should be given to the occurrence of the migratory brown trout in the Danube River at the broader Iron Gate Gorge area. They migrate in late summer and early fall from the Iron Gate One reservoir to the lower sections of tributaries devoid of any trout fish. However, some of these streams house very special native trout of the DA lineage in their short-extending upper sections. These native trout populations are, so far, still out of contact with the reservoir-dwelling trout. However, given the resilience of trout and their migratory life history, the outcome of this introduction could be deleterious for those native fish that are very precious in the conservation sense.

Keywords: migratory trout, resident trout, non-indigenous strains, invasiveness, threats, conservation

INTRODUCTION

Some 25 nominal trout *Salmo* spp. taxa have been described throughout the dispersal area of this complex species (Kottelat, 1997). This illustrates the great variety in their traits, but also introduces confusion in their taxonomy from the conceptual point of view. There are taxonomic studies and reviews (Bernatchez, 2001; Simonović et al., 2007; LoBrutto et al., 2010; Vera et al., 2011; Meraner et al., 2013) that consider *Salmo trutta* L., 1758, to be a species complex comprising phylogenetically closely related species, while others (Abolhasan et al., 2017; Kalayci et al., 2018; Sanz, 2018; Rezaei et al., 2019; Whiteley et al., 2019) consider this to be a single highly polymorphic species. Whiteley et al. (2019) stated that the current taxonomic richness within the *S. trutta* was promoted by Kottelat (1997), who followed the evolutionary species concept of Simpson (1961) to assign local brown trout subspecies originally described on the basis of slight differences in a limited number of traits between local populations as nominal species (Kottelat and Freyhof, 2007).

The Western Balkans is a region known for its prominent trout diversity, as seen in the many trout taxa described in all three main drainage areas found here. Marble trout *Salmo marmoratus* (Cuvier, 1829), soft-muzzled trout *Salmo obtusirostris* (Heckel, 1851), Adriatic trout *Salmo farioides* (Karaman, 1938), and *Salmo montenegrinus* (Karaman, 1933) are species endemic to the eastern Adriatic Basin in Montenegro, Dalmatia, and Herzegovina, whereas the Lake Ohrid trout *Salmo letnica* (Karaman, 1924) (together with the tentative *Salmo lumi* (Poljakov et al., 1958), and *Salmo aphelios* (Kottelat, 1997), belvica trout *Salmo ohridanus* (Steindachner, 1892), and the Lake Prespa trout *Salmo peristericus* (Karaman, 1938), are endemic to the Adriatic Basin in Northern Macedonia. Taler's trout *Salmo taleri* (Karaman, 1933) and Danubian salmon *Salmo labrax* (Pallas, 1814), tentatively occur in the Black Sea Basin, while the Macedonian trout *Salmo macedonicus* (Karaman, 1924) and Pelagonian trout *Salmo pelagonicus* (Karaman, 1938), are found in the Vardar River (Axios) and Struma River (Strymon) in the Aegean Sea Basin.

The nominal taxonomy of *Salmo* spp. presented here was recently supplemented with a prominent insight into their diversity, assessed using molecular markers, both mitochondrial, [e.g., cytochrome *b* and control region (CR), i.e., the D-loop (Bernatchez et al., 1992; Bernatchez, 2001), and nuclear, e.g., *LDH-C** and several microsatellite loci (Hamilton et al., 1989; Richard and Thorpe, 2001)]. The basic mapping of indigenous populations of nominal trout taxa accomplished using CR as a marker in the Adriatic Sea Basin assigned them to the Adriatic (AD), *marmoratus* (MA), and Danubian (DA) mtDNA lineages (Marić et al., 2006; Mrdak, 2011; Mrdak et al., 2012; Simonović et al., 2017b; Škraba Jurlina et al., 2018); those native to the Black Sea Basin belonged to the DA lineage (Marić et al., 2006; Mrdak, 2011; Tošić et al., 2014, 2016; Simonović et al., 2017b; Škraba et al., 2017), whereas in the Aegean Sea Basin, they belonged to the AD lineage (Marić et al., 2006, 2017; LoBrutto et al., 2010).

Every introduction poses a risk to the loss of original genetic structure in the recipient local brown trout population (Templeton, 1986; Laikre and Ryman, 1996; Laikre et al., 2010; Hansen, 2002; Ferguson, 2006a). The effect of introductions of domesticated, hatchery-reared brown trout of the DA haplogroup is difficult to detect using diploid genetic markers. This is an especially sensitive matter, given the fact that very few stocks at brown trout hatcheries in the Western Balkans have been genotyped. Though some countries have prescribed mandatory marking and registration of brood fish, this is poorly enforced. It is more feasible to detect the effects of introduction of brown trout of the AT haplogroup into the dispersal range of trout of either DA or AD haplogroups by seeking out the *LDH-C*90* allele they transfer to their offspring by admixture with trout of DA and AD haplogroups. Subsequent analysis of microsatellite alleles can provide insight into the changes in the genetic structure of the recipient brown trout population by detecting private alleles specific to the introduced brown trout strain.

Since the introduction of brown trout worldwide (MacCrimmon and Marshall, 1968), it has become one of the most widely introduced fish species (Welcome, 1992; Fausch, 2007). Its invasiveness has been noticed everywhere, resulting in its proclamation as one of the World's 100 worst invasive alien species (Lowe et al., 2000). The most invasive impact of brown trout on the recipient fish fauna was recorded in New Zealand (Townsend, 1996; McIntosh et al., 2010; Jones and Closs, 2018), where the local native galaxiids approached extirpation after the introduction of the brown trout into their native streams. Behnke (2007) reported a similar effect in the Yellowstone National Park ecosystem, where introduction of the brown trout strongly suppressed the native West slope cutthroat trout *Oncorhynchus clarkii* (Richardson, 1836).

The economic impact of their invasive character is closely tied to conservational issues and trout fisheries, where fishery management is the main driver of the increased risk of introduction of non-native trout strains. Recently, activities to build small hydropower plants as renewable energy sources have been focused primarily on trout streams, which threatens very fragile mountain stream ecosystems and their native trout (Simonović, 2019). The adverse effects of small hydropower plants onto native trout populations in the sections of streams along the derivations are compensated through planned stocking, which perpetuates and augments the risk of introducing alien brown trout. This widely adopted stocking practice in trout stream management accompanied by the flow of money among fishery managers, trout fish farmers, and trout fishermen is the primary obstacle to effective conservation of indigenous and often unique native brown trout stocks (Simonović et al., 2017b), both in common fisheries (Simonović, 2019) and even in those situated in protected areas (Simonović et al., 2014).

The life history plasticity of brown trout makes the species highly resilient and able to adapt to abrupt alterations in habitat and to long-term harsh conditions when resources are scarce, which sustains adverse effects and maintains the genetic variability inherent to local populations (Ferguson et al., 2019). Three life forms of trout known as morphae (i.e., sea trout, m. *marinus*; lake trout, m. *lacustris*; and river trout, m. *fario*) generally depict that plasticity, which extends also to the life history in sea and lake morphs featuring migratory behavior-anadromy and lymnodromy, respectively. In the Balkans, migratory behavior in native trout is found in only a few populations. In the Eastern Balkans, [e.g., sea trout *S. labrax* migrate to spawn in the small tributaries on the Bulgarian coast of the Black Sea (Kohout et al., 2013)]. Migratory trout in the Western Balkans include lake-dwelling forms belonging to the Adriatic trout *Salmo farioides* (Karaman, 1938) in Montenegro, [e.g., the Lake Skadar "strun" (Mrdak et al., 2006) and to the marble trout *S. marmoratus* in Herzegovina, e.g., the tentative "zubatak" nominally described as *Salmo dentex* Heckel and Kner (1958)]. Snoj et al. (2002) reported that sea trout recorded in the Northern Adriatic Sea are brown trout of the AT lineage, as escapees from trout farms in streams draining to the sea. They considered them distinct from the sea trout, reported by Chiereghini (1818), which Kolombatović (1890) described as the tentative *Trutta adriatica*. Contrary to the mapping of brown trout of the AT lineage introduced into streams of the Danube River catchment (Simonović et al., 2017a), there are no reliable records on the introduction of the hatchery-reared brown trout of the Atlantic origin into streams at the Adriatic Sea watershed in Montenegro in the last 50 years at least. In the inland waters of the Danube River catchment, reservoir-dwelling migrating trout were recorded soon after the construction of dams, though it is not yet known whether those individuals belong to native trout of the DA lineage or to introduced trout of the AT lineage or whether they are an admixture of both of them. The discovery of brown trout individuals in the Djerdap (Iron Gate) One reservoir and in the lowermost sections of streams draining into the Danube River there (**Figure 1**, localities 1–7) confirmed they belong to the AT lineage or are admixed with it (Marić et al., 2012; Simonović et al., 2015, 2017a; Tošić et al., 2016). There are no records of these trout in the upstream, headwater sections, where pure brown trout of the very specific Da23c haplotype still occur (Tošić et al., 2016).

The aims of this study were: (1) to consider how alternative life histories and occurrence of migratory behavior in native brown trout of the AD lineage responded to the introduction of an alien strain of brown trout of the AT lineage into their home streams; (2) how introduced alien brown trout of the AT lineage affected the genetic structure of the stream-dwelling native individuals by introgressing into their gene pools; and (3) how migratory behavior in alien brown trout of the AT lineage acted in their favor when invading the recipient streams with resident, native, stream-dwelling brown trout of the DA lineage in the Western Balkans, regarding either the loss of original genetic diversity, or increased migratory behavior in the native populations. The results should allow scientists to anticipate the risks faced by the native brown trout population and the expected effects in real circumstances occurring in the area.

MATERIALS AND METHODS

Sampling

Trout sampling was conducted by fly fishing and by electrofishing using either an engine-powered electrofishing gear Suzuki-Bosch^{TM} (220 V DC, $I_{max} = 6$ A) or battery-powered portable electrofishing gear AquaTech^{TM} IG200/1$^{®}$ (380/600 V DC, $I_{max} = 15$A).

Sampling was conducted in the Svetinja and Krušnica Rivers, two tributaries of the middle reach of the Una River in the Black Sea Basin (**Figure 1**, localities 11 and 12), in autumn of 2012 and 2013. All individuals were small [of the standard length (SL) <25 cm] resident, stream-dwelling, with coloration common for brown trout in the Black Sea Basin: dark brown back and golden-yellow flanks, with the moderately large black spots scattered along the back and upper, dark flanks, and with uniformly sized red spots aligned in two almost regular rows along the golden mid-flanks of the body. Sampling in the lower Mrtvica River in Montenegro in the Adriatic Sea Basin (**Figure 1**, locality 13) was conducted by angling, from 2004 to 2007, in the peak of the spawning season in late autumn, when both life history forms co-occur in the spawning grounds. The sample consisted of both small, stream-dwelling fish of both sexes, but predominantly males, up to 20 cm in SL, with dark blue back and light bluish flanks scattered with tiny black and reddish spots, and large (some individuals exceeded 40 cm SL), smoltified (i.e., silvery in color, with only tiny, black spots on the back and flanks), lake-dwelling, exclusively female individuals, as seen by freely leaking roe. Samples from the upper Mrtvica River (**Figure 1**, locality 14) were collected in the spring of 2014 by electrofishing and consisted of only small, stream-dwelling fish, not exceeding 15 cm in SL. Samplings in the streams draining into the Danube River at the Djerdap Gorge (Iron Gate) area (**Figure 1**, localities 1–7) were conducted during the late summer–early autumn period from 2011 to 2015, and most samples in all streams consisted of typical stream-dwelling, small (of SL ≤15 cm) brown trout that resembled those from Svetinja and Krušnica Rivers. Only a couple of brown trout from the Brnjica River were slightly larger (of SL >25 cm SL) and silvery in color, with numerous, tiny black spots on the back and flanks, resembling the smoltified brown trout. After sampling, all fish were released into their home streams. Therefore, the determination of sex (except for individuals from the lower Mrtvica River) was not possible.

From each fish, an anal fin clip was taken and stored in microtubes filled with 96% ethanol. Fin clips were collected from 10 individuals from the Svetinja River, from 11 caught in the Krušnica River, from 11 individuals caught in the lower reach, and 12 from the upper reach of the Mrtvica River, where the size of samples was limited most likely by extensive poaching occurring there (Škraba Jurlina et al., 2018). As for the Danube River tributaries in the Iron Gate Gorge area, fin clips were collected from each of a very small number of individuals due to their small size and the scarcity of water and brown trout in those streams (Tošić et al., 2016), [e.g., two samples from the Brnjica River, 18 from the KoŽica River, six from the Mala Boljetinska River, 12 from the Zlatica River, 11 from the north fork of the Rečka River, 10 from the Vratna River, and seven from the Zamna River (**Figure 1**, localities 7, 4, 5, 6, 3, 2, and 1, respectively) (**Supplementary Table**)].

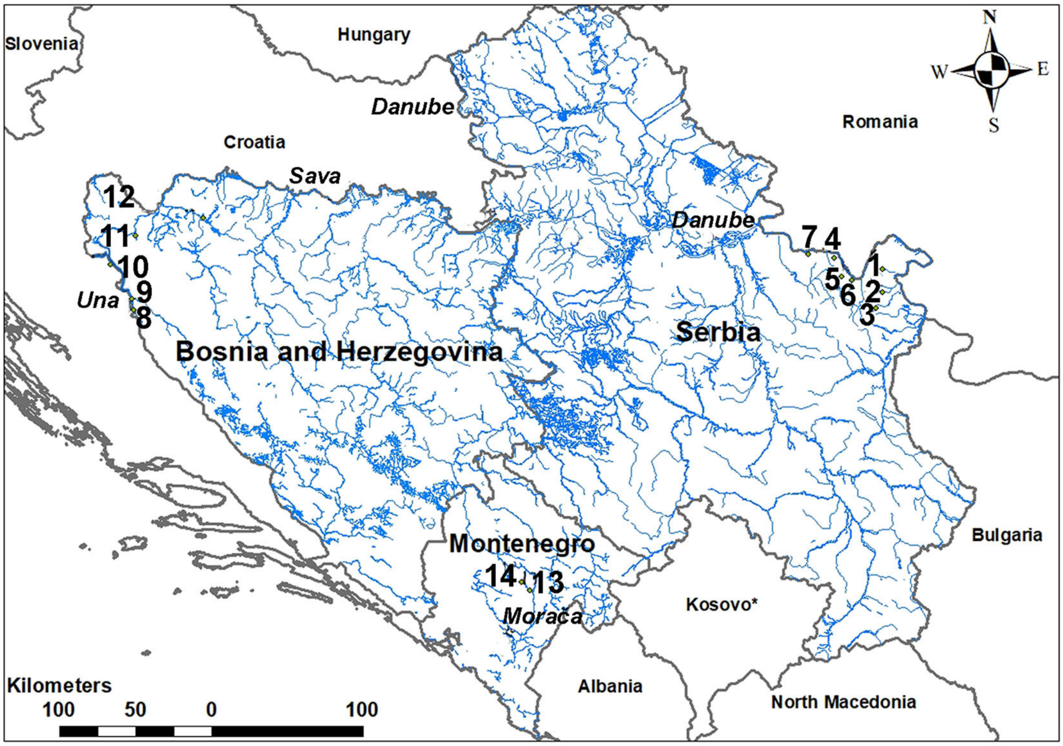

FIGURE 1 | Map of the study area in the Western Balkans where brown trout populations were sampled (1. Zamna River; 2. Rečka River; 3. Vratna River; 4. KoŽica River; 5. Mala Boljetinska River; 6. Zlatica River; 7. Brnjica River; 8. Krka River; 9. Upper Una River at Martinbrod; 10. Upper Una River at Loskun; 11. Svetinja River; 12. Krušnica River; 13. lower Mrtvica River; 14. upper Mrtvica River).

DNA Extraction and Analysis

DNA was extracted using either the High-Salt Extraction technique of Miller et al. (1988) or Quick-gDNA^{TM} MiniPrep extraction kit following the manufacturer's instructions (Zymo Research Corporation, Irvine, CA).

Amplification and Analysis of the mtDNA

To identify introductions of non-native brown trout stocks, we amplified and analyzed the CR or D-loop that has been proven to be a good phylogeographic and taxonomic marker (Bernatchez et al., 1992; Cortey and García-Marín, 2002; Tougard et al., 2018). It is used for a range of purposes in combination with other molecular markers, including determining implication for conservation and management (Vera et al., 2011), migrating strains (Habibi et al., 2013), and analyzing the consequences of stocking (Škraba et al., 2017).

Amplification of the CR of mtDNA was carried out using the forward primers 28Riba (Snoj et al., 2000) and Trutta_mt_F (5′-TGAATGAACCTGCCCTAGTAGC-3′, designed by M. Brkušanin), and the reverse primer HN20 (Bernatchez and Danzmann, 1993), following the protocol from Tošić et al. (2014). PCR products were purified and sequenced at Macrogen Europe. Sequencing reactions were performed in a DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD) using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), following the protocols supplied by the manufacturer by single-pass sequencing on each template using the forward (Trutta_mt_F) primer. Sequences were aligned with those from the GenBank using program Mega 7.0.21 (Larkin et al., 2007).

LDH Gene Amplification and Analysis

In addition to mtDNA showing maternal inheritance, other nuclear markers that complement with paternal inheritance in brown trout were used to infer the relationships between their populations. The lactate dehydrogenase (*LDH*) gene (Hamilton et al., 1989), specifically its eye-specific locus (*LDH-C1**), was mostly used for this purpose. It is found only within the genus *Salmo* with two codominant alleles: *LDH-C* 100* and *LDH-C* 90*. The first has an ancestral character and features a DA haplogroup, while the second features exclusively in northwestern European populations of the AT mtDNA haplogroup (McMeel et al., 2001). This was used to analyze introgression of alien individuals in the native populations.

Part of the *LDH* gene was amplified using Ldhxon3F and Ldhxon4R primers (McMeel et al., 2001). The final concentrations of PCR components were: 1× PCR buffer (Invitrogen^{TM}, SAD), 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.5 μM for both primers (Metabion, DE), 1.5 U of *Taq* polymerase (Invitrogen^{TM}, USA), and ∼100 ng of DNA. PCR amplification started with initial denaturation (95°C, 5 min), continued with 30 cycles of denaturation (94°C, 1 min), followed by primer annealing (62°C, 1 min) and DNA extension (72°C, 1 min), and final elongation (72°C, 10 min) in the ProFlex^{TM} PCR System (Applied Biosystems$^{®}$, USA). A 2.5% gel was used for DNA electrophoresis. Restriction fragment length polymorphism (RFLP) was analyzed using *BselI* endonuclease that cuts DNA at the CCCNNNNN/NNGGG position. PCR products were incubated for 16 h with *BselI* endonuclease (Thermo Fisher Scientific, USA) at 55°C according to instructions. The mixture contained ∼100 ng of DNA, 10 U of enzyme, 10× Tango Buffer and molecular water. A 2.5% gel was used for DNA electrophoresis with $^{®}$ SYBR Green^{TM} for visualization.

Microsatellites Amplification and Analysis

Microsatellite markers are often used for conservational and population genetic purposes, specifically to estimate population genetic diversity, influences of stocking on native populations (Hansen et al., 2000, 2001), effective population size (Serbezov et al., 2012), and broodstock formation (Hansen et al., 2000).

Analysis of the samples from both the Svetinja and Krušnica Rivers and from streams in the broader Iron Gate Gorge area was based on eight microsatellite loci—SsaD190, SsaD71 (King et al., 2005), Str73INRA (Estoup et al., 1998), Ssa410Uos (Cairney et al., 2000), Ssa85 (O'Reilly et al., 1996), SSsp2216 (Paterson et al., 2004), OMM1064 (Rexroad et al., 2002), and SsoSL438 (Slettan et al., 1995). All were amplified in four duplex reactions. The last microsatellite locus, SsoSL438, was excluded from the analysis of specimens from the Mrtvica River, while the remaining seven loci were combined in one single and three duplex reactions, with the forward primer labeled with a fluorescent dye (FAM or NED) (Škraba Jurlina et al., 2018). Fragment analysis was performed using GeneScan 500 LIZ Size Standard (Applied Biosystems, USA) on an ABI-3130 Genetic Analyzer (Applied Biosystems, USA). Analysis was performed using GeneMapper ID v3.2.1 (Applied Biosystems, USA).

Statistical analysis was performed on the microsatellite data to determine the differences in genetic structure between populations whose members revealed an alternative life history by partial migration and populations consisting exclusively of resident, stream-dwelling brown trout. Genetic structure from microsatellite data was determined as reported in Škraba Jurlina et al. (2018). Factorial correspondence analysis (FCA) was carried out using the program GENETIX 4.05 (Belkhir et al., 2004). Program FSTAT 2.9.3.2 (Goudet, 2002) was used to calculate allelic richness and values for Fisher's F statistics per locus (F_{IS}, F_{IT}, F_{ST}) and to test Hardy–Weinberg equilibrium within populations. Population structure was analyzed using the STRUCTURE 2.3.4 (Pritchard et al., 2000), with the proposed number of clusters $K = 10$. The length of burn in periods was set to 20,000, with the number of Markov Chain Monte Carlo (MCMC) repeats of 10 for each K depending on convergence after burning was set to 500,000. Structure Harvester software (Earl and VonHoldt, 2012) was used to estimate the most probable K according to Evanno et al. (2005).

RESULTS

Mitochondrial Control Region

Sequencing of the mtDNA CR revealed the introduction of brown trout belonging to the AT haplogroup in all three areas. In the streams feeding the middle course of the Una River, where the native brown trout belongs to the Da22 haplotype (GenBank accession number AF321993; Duftner et al., 2003), three out of 10 brown trout from the Krušnica River (33%) and three out of 10 from the Svetinja River (33%) belonged to the Atcs1 haplotype (#AF321990; Weiss et al., 2001; Škraba et al., 2017).

In the upper course of the Mrtvica River, where Adriatic trout is the native brown trout taxon bearing the Adcs11 haplotype (#AY836340; Cortey et al., 2004), three of 12 (25%) fish were of the A17 haplotype (#HQ848368; Kohout et al., 2012), and all of 11 fish from the lower course were native Adriatic trout (Škraba Jurlina et al., 2018).

In all streams in the Iron Gate Gorge area, brown trout of the AT haplogroup were recorded in the Brnjica River, Dobra River, and Porečka River and in the Danube River near the confluence with the Dobra River, while in the Vratna River, they were found in sympatry with the brown trout of the Da23c haplotype (#KC630984; Tošić et al., 2014), [i.e., three of 10 (33%) brown trout in total were of this haplotype (Tošić et al., 2016)].

Partial *LDH-C** Locus

RFLP analysis of the partial *LDH-C* gene in brown trout samples from the middle course of the Una River showed that two fishes of the AT haplogroup were homozygotes for the slower allele (*LDH-C* 90/90*), and the remaining brown trout of the AT haplogroup were heterozygotes *LDH-C* 100/90*. The slower allele was also dominant in the DA haplogroup, where only four brown trout from the Krušnica River were homozygotes for the faster allele (*LDH-C* 100/100*). That high proportion of slow allele occurrence in 75% of brown trout implied strong introgression of brown trout of the AT haplogroup into the gene pool of the native DA haplogroup.

In the upper course of the Mrtvica River, two of three fishes (66%) with the A17 haplotype were heterozygous (*LDH-C* 90/100*), as were two of the remaining of nine fishes (22%) with the Adcs11 haplotype, indicating their paternal origin from the AT lineage. Other fishes in the upper course and all fishes in the lower course were homozygous for the *LDH-C* 100* allele. The remaining individuals belonging either to the ADcs11 or to the A17 haplotypes were homozygotic for the faster allele (*LDH-C* 100/100*).

Of the three brown trout of the AT haplogroup from the Vratna River, only one (33%) was heterozygous (*LDH-C* 90/100*), while two were homozygous for the faster allele (*LDH-C* 100/100*) characteristic to brown trout of the DA haplogroup. This implies a paternal DA origin for those fishes and a long-lasting introgression with the recurrent cross-breeding between brown trout of the DA and AT lineages.

Microsatellites

In the (sub)populations from the Svetinja and Krušnica Rivers in the middle course of the Una River catchment, genetic diversity

TABLE 1 | Population genetics of brown trout populations from streams situated in the Una River catchment (Svetinja, Krušnica, and Krka Rivers, upper Una River at the sites Martinbrod and Loskun sites), Mrtvica River (upper and lower), and in the Iron Gate Gorge area (Mala Boljetinska, Zlatica, KoŽica, Vratna, and Zamna Rivers), as assessed from microsatellite loci ($H_{e.}$, expected heterozygozity; $H_{o.}$, observed heterozygosity; F_{IS}, intrapopulation fixation index; $\bar{A}n$, mean allelic diversity per population; *Ar*, allelic richness; *H-W*, deviation from the Hardy–Weinberg equilibrium).

	$H_{e.}$	*Ho*	F_{IS}	$\bar{A}n$	*Ar*
Svetinja	0.76	0.73	0.125	7.45	4.71
Krušnica	0.74	0.64	0.193	7.45	4.63
Krka	0.63	0.63	0.043	8.50	3.86
Una Martinbrod	0.49	0.52	−0.019	4.12	4.00
Una Loskun	0.65	0.62	−0.017	7.75	4.03
Upper Mrtvica	0.61	0.51	0.006	4.14	4.28
Lower Mrtvica	0.59	0.58	0.061	4.57	4.57
Mala Boljetinska	0.41	0.48	−0.074	2.87	2.58
Zlatica	0.48	0.58	−0.118	4.62	3.00
KoŽica	0.43	0.52	−0.121	3.00	2.32
Vratna	0.51	0.55	0.020	4.25	3.13
Zamna	0.39	0.32	0.196	2.75	2.40
Rečka	0.25	0.28	−0.100	0.26	1.75

TABLE 2 | Microsatellite loci revealing private alleles (in bold letters) for the AT haplogroup in brown trout from four populations: in the middle course of the Una River (Svetinja and Krušnica Rivers), Mrtvica River (upper Mrtvica River), and middle Danube River at the Iron Gate Gorge area (Vratna River).

River		Svetinja	Krušnica	Upper Mrtvica	Vratna
Microsatellite locus	SsaD190		**107**/119 **107**/119	**151**/163 **151**/163	**115**/119
	SsaD71				
	Ssa410Uos	**208**/256		**200**/284	**210**/222
	Ssa85	**129**/**129**			
	SSsp2216		181/**189**		
	OMM1064	**175**/179 **175**/199 **195**/283 **195**/239 179/**195**	**219**/255 259/**291**		207/**223**
	SsoSL438				**103**/107 107/**109**

TABLE 3 | Pairwise F_{ST} distance values (above and right of the diagonal) and their significance (*p*-values) levels between samples from **(A)** Una River catchment and **(B)** Iron Gate Gorge area (the pairwise F_{ST} distance value between the upper and lower Mrtvica River populations is 0.22060, $p < 0.05$).

Samples	Sv	Kš	Kr	UMb	Ul	
(A) SAMPLES FROM R IN THE UNA RIVER CATCHMENT						
Svetinja (Sv)		0.02902	0.09324	0.08725	0.09476	
Krušnica (Kš)	0.07		0.06389	0.05510	0.03920	
Krka (Kr)	<0.05	<0.05		0.04246	0.00977	
Una Martinbrod (Umb)	<0.05	0.06	<0.05		0.01808	
Una Loskun (Ul)	<0.05	<0.05	0.09	0.14		
Samples	**MB**	**Zl**	**KŽ**	**Vr**	**Zm**	**Re**
(B) SAMPLES FROM RIVERS IN THE IRON GATE GORGE AREA						
Mala Boljetinska (MB)		0.09289	0.15593	0.23336	0.30469	0.44589
Zlatica (Zl)	<0.05		0.10157	0.15493	0.28107	0.40949
KoŽica (KŽ)	<0.05	<0.05		0.19178	0.31471	0.40112
Vratna (Vr)	<0.05	<0.05	<0.05		0.11171	0.42276
Zamna (Zm)	<0.05	<0.05	<0.05	<0.05		0.54541
Rečka (Re)	<0.05	<0.05	<0.05	<0.05	<0.05	

in brown trout individuals was greater, but with the lower genetic differentitation, as revealed by their fixation indices $F_{IS} = 0.1250$ ($p < 0.02$) and 0.1926 ($p < 0.001$), respectively (**Table 1**), in comparison with F_{IS} values ranging between−0.01695 and 0.04296 ($p > 0.1$) in brown trout from the (sub)populations in the upper Una River, consisting exclusively of native fishes. AMOVA results showed the greatest proportion of genetic variation to be in individuals (71.0%, $F_{ST} = 0.705$, $p < 0.01$), followed by the component of variability inherent to populations (22.5%, $F_{IT} = 0.290$). Several private alleles were found in fish belonging to the AT haplogroup. Private alleles found in samples from the Svetinja River were mostly heterozygous for two loci, Ssa410UoS and OMM1064, and one sample was in a homozygous state for the locus Ssa85 (**Table 2**). All private alleles from the Krušnica River were heterozygous. Pairwise distance F_{ST} values between the populations gave clear depiction of their position in the Una River catchment; the only deviation was for the sample from the Una River at Martinbrod (**Table 3A**). In the FCA, the first four correspondent factors explained 18.35% of the total genetic variability. Correspondent factors 1 and 2 explained 6.22 and 4.36% of the variability, respectively, and clustered the brown trout from the Svetinja and Krušnica Rivers separately from all others (**Figure 2**, top). STRUCTURE analysis revealed two (with greatest value of Delta $K = 31.759468$) distinct subpopulations (**Figure 3**, top): the first one consisting of brown trout from the Svetinja and Krušnica Rivers, and the second one of brown trout from the Krka River and upper Una River (localities Martinbrod and Loskun).

In the upper Mrtvica River where brown trout of the AT haplogroup were admixed with the native AD trout, the observed heterozygosity $H_o = 0.16667$ was significantly lower than the expected $He = 0.72826$ for the locus SsaD71, demonstrating a significant ($p < 0.001$) deviation from the Hardy–Weinberg equilibrium, with the greater genetic diversity, [i.e., a weak differentiation within that population ($F_{IS} = 0.779$), compared with the lower Mrtvica River population ($F_{IS} = 0.135$) (**Table 1**)]. Pairwise distance between the upper and lower Mrtvica populations ($F_{ST} = 0.221$, $p < 0.05$) showed their significant differentiation. AMOVA showed that the majority of genetic variability to be that of individuals in the two populations (77.9 %, $F_{ST} = 0.221$, $p < 0.01$), whereas the within-population component was lower (22.1%, $F_{IT} = 0.247$, $p < 0.01$). Private alleles specific for the AT haplogroup were found in brown trout of the AT haplogroup at two microsatellite loci and were heterozygous for the loci SsaD190 (151) and Ssa410Uos (200), whereas a heterozygous private allele for the locus OMM1064 (215) was recorded in one fish of the AD haplogroup, also in the heterozygous state. None of these private alleles for the AT haplogroup were recorded in any of the fish of the AD haplogroup in the lower Mrtvica River population (**Table 2**). In the FCA, the first four correspondent factors explained 36.02% of the total genetic variability. The first and second correspondent factors explained 14.40 and 8.10% of the variability, respectively, and clearly separated the two populations (**Figure 2**, middle). STRUCTURE analysis demonstrated that brown trout in the upper and lower Mrtvica River are two distinct subpopulations (with greatest value of Delta $K = 39.321459$) (**Figure 3**, middle).

In the brown trout population from the Vratna River in the Iron Gate Gorge area, the expected average heterozygosity for all microsatellites' loci was $H_{exp} = 0.51$, the highest among all populations in the area (**Table 1**). Several private alleles were found in individuals belonging both to the AT and DA haplogroups. AMOVA revealed that the majority of genetic variability in samples from the Iron Gate Gorge area was inherent to individuals (62.1%, $F_{IT} = 0.621$, $p < 0.01$), and then to samples, [i.e., the populations (37.9%, $F_{ST} = 0.379$, $p < 0.05$)]. The allelic, [i.e., genetic diversity was highest in the brown trout population from the Vratna River, e.g., that obtained for the loci Ssa410Uos ($A_R = 5.348$, $F_{IS} = 0.875$), SsaD190 ($A_R = 2.860$, $F_{IS} = -0.213$), SSsp2216 ($A_R = 4.528$, $F_{IS} = 0.034$), and SsoSL438 ($A_R = 2.698$, $F_{IS} = 0.191$)]. One brown trout from the Vratna River was of the AT haplogroup and was homozygous for the *LDH-C** allele characteristic for the DA haplogroup (100/100) and had private microsatellite alleles of the AT haplogroup at the loci SsaD190 (115) and SsoSL438 (109), which were likely of maternal origin (**Table 2**). In two brown trout of the DA haplogroup, several other private alleles were found to occur exclusively in these fish and no others of that haplogroup from any population in the area. These alleles were recorded at three loci (**Table 2**) and were probably of paternal origin. Private alleles found at all microsatellite loci were in heterozygous state. In the FCA, the first four correspondent factors explained 34.51% of the total genetic variability of brown trout in the Iron Gate Gorge area. Correspondent factor 1 explained 12.55% of the total variability and clearly separated the Rečka River population from all others, while correspondent factor 2 explained 8.14% of the variability and separated populations from the Mala Boljetinska, Zlatica, and KoŽica Rivers from populations in the Vratna and Zamna Rivers (**Figure 2**, bottom), which was supported by pairwise F_{ST} distances between populations (**Table 3B**). STRUCTURE analysis revealed four (with greatest value of Delta $K = 85.869231$) distinct brown trout subpopulations (**Figure 3**, bottom): one in the Mala Boljetinska and Zlatica Rivers, the second in the KoŽica River, the third in the Zamna and Vratna Rivers, and the fourth in the Rečka River.

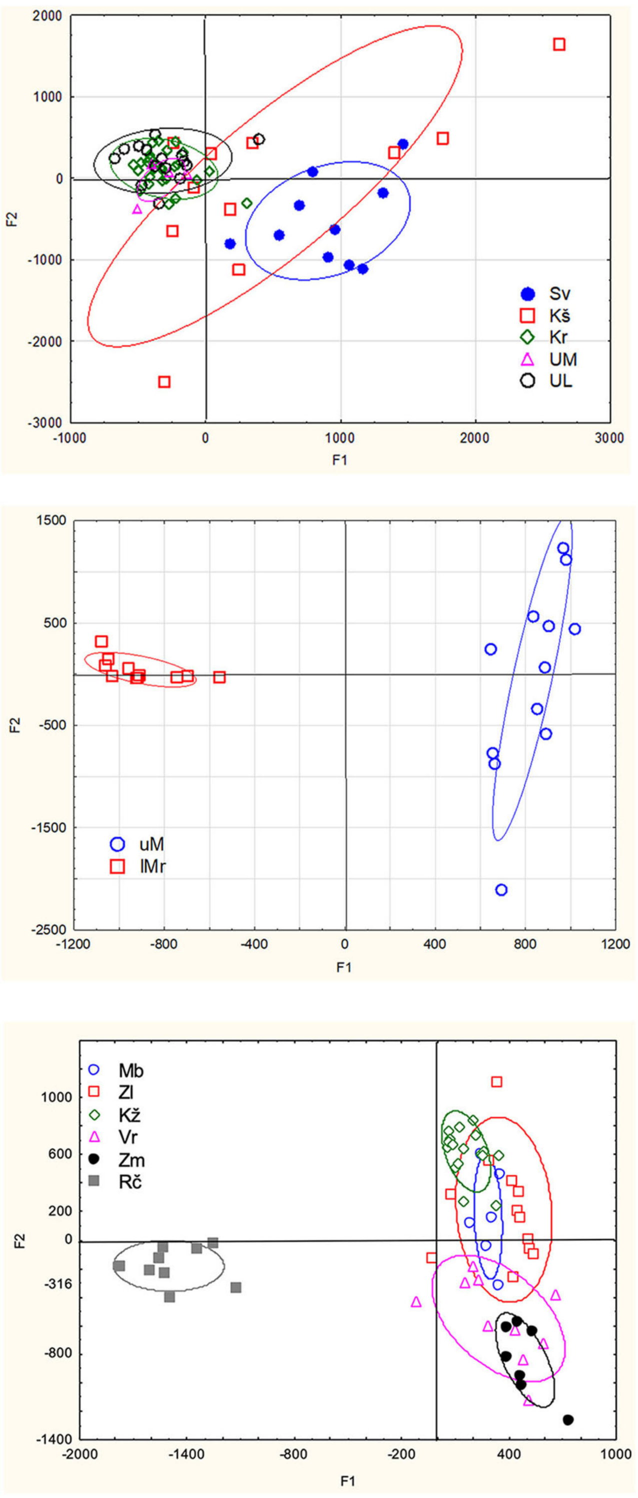

FIGURE 2 | Correspondence analysis of brown trout populations from the Una River catchment (top), Mrtvica River (in the middle), and streams in the Iron Gate Gorge area (bottom) (Sv, Svetinja River; Kš, Krušnica River; Kr, Krka River; UM, Una River at Martinbrod; UL, Una River at Loskun; uM, upper Mrtvica River; lM, lower Mrtvica River; MB, Mala Boljetinska River; Zl, Zlatica River; KŽ, KoŽica River; Vr, Vratna River; Zm, Zamna River; Rč, Rečka River).

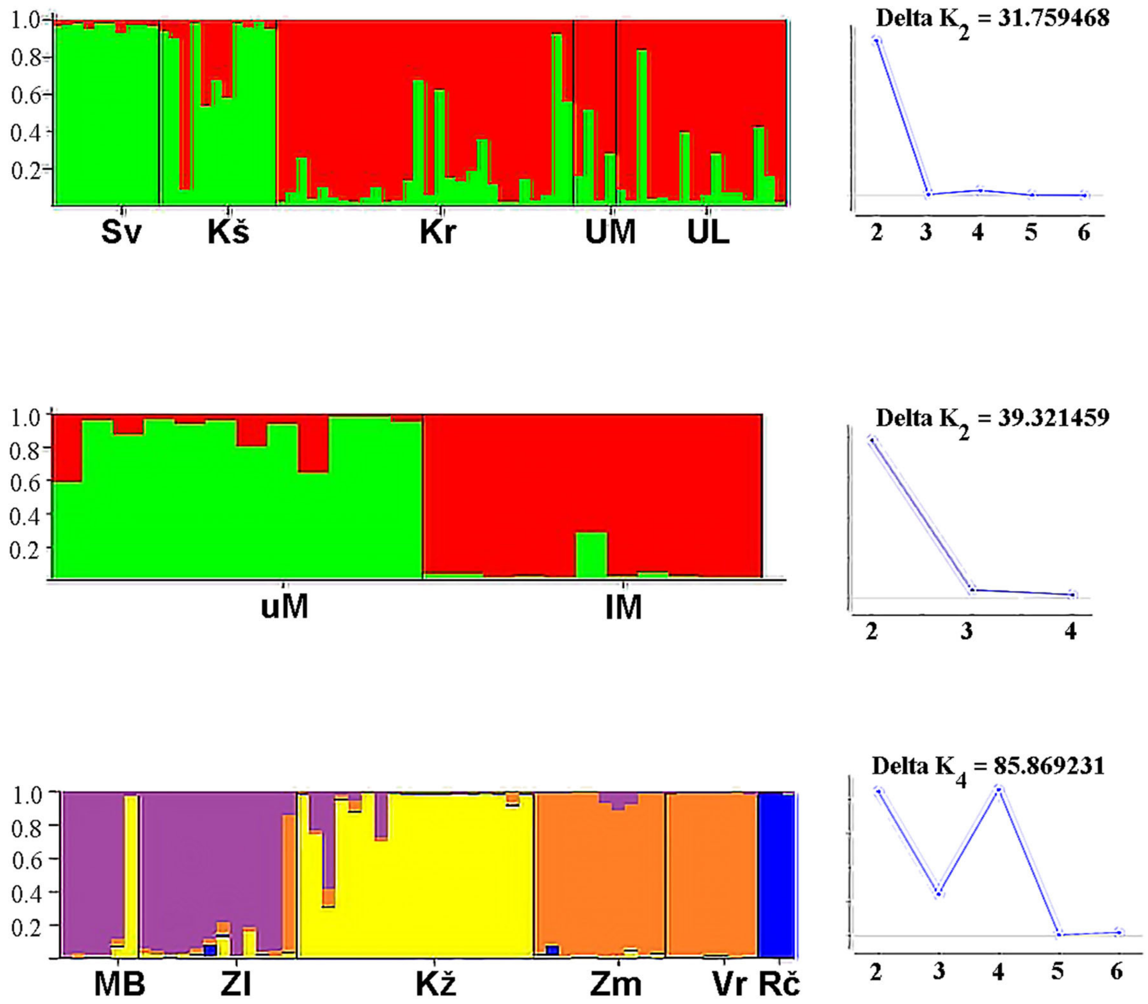

FIGURE 3 | STRUCTURE analysis of brown trout populations from the Una River catchment (top), Mrtvica River (in the middle), and streams in the Iron Gate Gorge area (bottom) (Sv, Svetinja River; Kš, Krušnica River; Kr, Krka River; UM, Una River at Martinbrod; UL, Una River at Loskun; uM, upper Mrtvica River; lM, lower Mrtvica River; MB, Mala Boljetinska River; Zl, Zlatica River; KŽ, KoŽica River; Vr, Vratna River; Zm, Zamna River; Rč, Rečka River).

DISCUSSION

The relatively small sample sizes from certain localities due either to extensive poaching (e.g., in the Mrtvica River) or very small size of streams and extreme scarcity of water during sampling (e.g., the Svetinja River and all streams in the Iron Gate Gorge area) can in general weaken the conclusions of their population-genetic features. Interpretation of results for population genetics features (**Table 1**) should include caution, as a stronger effect of admixing with brown trout of the AT haplogroup is expected on the H_o. The high inbreeding coefficient F_{IS} (**Table 1**) in line with the reduction of heterozygosity that depicts their fixation for particular alleles, e.g., in the populations from the Svetinja, Krušnica. and Zamna Rivers could be a consequence of the small sample size. However, the use of three molecular markers and occurrence of heterozygous alleles specific for brown trout of the AT haplogroup (**Table 2**) unequivocally demonstrated admixing with the resident, native brown trout. This showed the invasive risk that non-native, migrating brown trout of the AT haplogroup pose on the resident brown trout populations in their native dispersal areas and demonstrated the resilience in native Adriatic trout population which have partial migratory behavior.

In contrast to the positive effects that sustainable management in trout fisheries have [e.g., the conditional and unconditional catch-and-release (Simonović et al., 2018)], both fishery managers and trout farmers fostered stocking of farmed brown trout fry and even spent brood fish (Simonović et al., 2014) to quickly enhance brown trout fisheries and accelerate return of investments (Simonović et al., 2017b). Based on recent knowledge of the native diversity of brown trout in the Western Balkans (Marić et al., 2006; Simonović et al., 2017a) and the risks posed to them by the introduction of non-native strains of brown trout and related species might pose to them (Simonović et al., 2013, 2015), recent surveys have revealed the occurrence of permanent, long-lasting introduction caused by stocking streams attractive for trout fisheries (Simonović et al., 2014;

Škraba Jurlina et al., 2018), streams originally devoid of trout prior to stocking (Jadan et al., 2007; Simonović et al., 2018), and even streams that are not at all suitable for brown trout fisheries (Tošić et al., 2016; Škraba et al., 2017). Introduction of trout fish of the non-native haplogroups and of the non-native haplotypes in the dispersal area of brown trout of the DA haplogroup in the Western Balkans has been a long-standing practice since the mid-nineteenth century at least, when Slovenia, Croatia, and Bosnia and Herzegovina were parts of the Austro–Hungarian Empire (Razpet et al., 2007; Simonović et al., 2017b). The nominal genetic diversity assessed from microsatellite loci and the F_{IS} and A_R values in the populations of stream-dwelling, resident trout where brown trout of the AT lineage introgressed suggests that alien brown trout of the AT lineage have impacted brown trout populations, which could lead to a loss of native haplotypes and co-adapted gene complexes created through the long-term adaptation of aboriginal brown trout populations in their native habitats (Templeton, 1986). In that way, non-native brown trout of the AT haplogroup unequivocally reveal the invasive effect as defined by Kolar and Lodge (2002) and Copp et al. (2005), justifying their assessment as having high *sensu stricto* invasive potential (Simonović et al., 2015).

Salmonids commonly possess a migratory instinct. In many species, populations partition between resident, stream-dwelling, predominantly male individuals, and migrating, lake- or sea-dwelling, predominantly female individuals (Fleming and Gross, 1990; Ferguson, 2006a) that admix (Hansen, 2002). This is known for many brown trout populations in the Atlantic Ocean basin. Unlike the differences in morphological, demographical, and ecological characteristics (Klemetsen et al., 2003) between resident and migratory fish, no genetic differences have been found between them (Hindar et al., 1991; Hansen, 2002; Charles et al., 2005), demonstrating their common population status. The common genetic structure and gender proportion in the stream- and lake-dwelling Adriatic trout in the lower Mrtvica River are in agreement with population features reported for other migratory salmonid stocks. The low value of gene flow between two (sub-)populations supports the view that brown trout of the AT haplogroup from the upper Mrtvica River failed to admix with native Adriatic trout (Škraba Jurlina et al., 2018), despite a lack of any physical or reproductive obstacles. The stocked fish of the AT haplogroup have certainly not bred and have hence vanished from the lower Mrtvica River, accessible to the spawning migratory, lake-dwelling Adriatic trout ("strun"). In contrast, brown trout of the AT haplogroup still remain in the upper Mrtvica River and spawn with the native, stream-dwelling, resident Adriatic trout, producing hybrids of reduced heterozygosity (**Table 1**), a feature also reported by Ryman et al. (1995), Laikre and Ryman (1996), and Ferguson (2006b). Snoj et al. (2010) reported an occurrence of brown trout of the Atlantic haplogroup (At-s1, #M97969, Bernatchez et al., 1992) in the lower Neretva River, where the lake-dwelling form of marble trout *S. marmoratus* (locally known as "dentex") occurs together with the resident marble trout and Adriatic trout. Regardless of the similarity of Adriatic trout and the tentative "dentex" for the commonly shared AdN haplotype (#DQ297172), the genetic structure of "dentex" was more similar to marble than to Adriatic trout, suggesting that "dentex" is the lake-dwelling, migratory form of marble trout, and not a form of Adriatic trout. As the AT haplogroup was not detected in the phenotypic, resident marble trout (Snoj et al., 2010), it seems that their life history plasticity helped to preserve their genetic structure, whereas the lack of a lake-dwelling form of Adriatic trout in the lower Neretva River made them susceptible to hybridization with the stocked brown trout of the AT haplogroup.

In the native, stream-dwelling brown trout of the two haplotypes belonging to the DA haplogroup (Da23c in the Vratna River in Eastern Serbia, as well as Da22 in the Svetinja and Krušnica Rivers in Bosnia and Herzegovina), all diploid genetic markers revealed an undisturbed admixture with stocked brown trout of the AT haplogroup and their persistence, as in the upper Mrtvica River. Moreover, it seems that brown trout of the narrowly dispersed and very specific modern haplotype Da23c found in several streams in the Iron Gate Gorge area are strongly threatened by the occurrence of the lake- (actually, a reservoir-) dwelling brown trout of the AT haplogroup (Marić et al., 2012) that were detected in the lowermost section of those streams in the late summer and early autumn periods (Tošić et al., 2016). Their silver body coloration and numerous black dots on the back and flanks suggest they smoltified prior to descending downstream to the reservoir. If they have already started returning to the closest available spawning grounds upon achieving maturity, there is a high risk of admixing with the native brown trout of the DA haplogroup and introgression into their gene pools in those Iron Gate streams where there are no physical obstacles for upstream migration, e.g., in the Brnjica, KoŽica, Mala Boljetinska, and Zlatica Rivers. In time, they might produce hybrid offspring which could begin to alter their life history and produce both stream- and reservoir-dwelling individuals and to strongly impact the original brown trout of the native DA haplogroup. Makhov et al. (2018) reported the strong capability of the recently resident, stream-dwelling Black Sea trout in the Mzymta River population, which they assigned *S. labrax*, to retain migratory behavior, despite being cut off from the sea by damming. The lack of reproductive isolation increases the invasive risk from brown trout strains that have retained a capacity to alter their life history. In addition to the restoration measures outlined in Simonović et al. (2015) that are applicable for already affected native trout stocks, constructing physical barriers (insurmountable cascades) may be a reasonable precautionary measure to preserve the highly valuable stocks in the conservation sense from the upstream migrating, reservoir-dwelling brown trout of the AT haplogroup.

In conclusion, it seems that migratory behavior fortunately provides native trout stocks a mechanism to cope with the alien strains and/or species introduced into their home streams, but also enables non-native brown trout to intrude into the recipient streams and introgress into their resident trout stocks successfully. This feature supports the evaluation of brown trout of the AT haplogroup as of the high risk *sensu stricto* in the FISK evaluation performed by Simonović et al. (2015) for non-native trout fish species and strains in the rivers of Serbia as a recipient area. The migratory instinct and life history plasticity inherent to various nominal trout taxa facilitate them in overcoming the scarcity of resources in their native, home streams (Gross et al., 1988), thereby preserving the original genetic structure of locally adapted populations. Current knowledge about brown trout stocks in the Western Balkans suggests that these features act as a stabilizing population mechanism that can also facilitate them to deal with alien trout strains that intrude into streams in their native area of dispersal either naturally or through human intervention.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GenBank: KC630984.1, https://www.ncbi.nlm.nih.gov/nuccore/506485009.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because According to Serbian legal acts, such approval isn't needed. Each year, the sampling license for scientific purposes was issued by Ministry for Environment Protection that is in charge for Fisheries. All fish were returned alive to their home streams after taking the fin clip for DNA analyses.

AUTHOR CONTRIBUTIONS

The authors participated in fieldwork at the following localities: DŠ, AM, VN, MP, and PS at the Una River catchment. DŠ, DM, and PS at the Morača River catchment. AM, TK, VN, and PS at streams in the Iron Gate Gorge broader area. VN organized the database. DNA extraction and amplification of particular loci were accomplished by DŠ, AM, DM, TK, and PS. Statistical analyses of laboratory results were performed by DŠ, AM, TK, and PS, and their preparation for publication by VN. Manuscript design and its first draft were by PS, while DŠ and AM contributed suggestions during drafting. DM, IŠ, and MP reviewed the first draft. All authors read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00188/full#supplementary-material

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REVIEW
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Can Invasiveness in Freshwater Fishes Be Predicted From Life-History Traits?

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We review a model we developed in 2007 to predict the invasion potential of an introduced population, based on the relationship between juvenile growth (mean total length at age 2) and mean age-at-maturity, and test the model with data from 75 non-native populations of pumpkinseed *Lepomis gibbosus* in nine countries of Europe and western Asia. The database used to construct the model was derived from a mix of primary and secondary data collected between 1981 and 2016. Based on number of specimens collected or catch-per-unit-effort data, and/or comments by the original authors, populations were classified *a priori* as "invasive" or "non-invasive." The plot of the proposed model placed populations in three invasiveness categories (non-invasive, transitional, invasive). Of those predicted by the model to be invasive, only 57% were correct with regard to their *a priori* classification, a result that was not statistically significant. For populations predicted by the model to be non-invasive, 70% were correct with regard to their *a priori* classification when "transitional" were grouped with non-invasive ($P = 0.0024$), and 64% were correct when "transitional" were excluded from the test ($P = 0.12$). Applications of the model to two other non-native freshwater fishes (black bullhead *Ameiurus melas* and crucian carp *Carassius carassius*) are also discussed. The lack of stronger statistical support for the model may have been the result of using life-history traits from the populations after they were introduced, as the source populations were unknown, as well as to shortcomings in the dataset that could affect *a priori* and modeled classifications. We conclude that such life-history models can be useful for predicting invasiveness status in non-native freshwater fishes, though the basis of the predictions, statistical or heuristic, will depend on the size and quality of the underlying life-history database.

Keywords: age-at-maturity, juvenile growth, non-native species, risk analysis, horizon scanning, alien species, predictive modelling

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INTRODUCTION

A major challenge confronted by environmental managers and policy makers in the development and implementation of strategies to avoid or mitigate bioinvasions is the identification of non-native species likely to become invasive and exert adverse impacts on native species and ecosystem services (Williams and Grosholz, 2008; Zengeya et al., 2017). To examine a species' "invasiveness," one must first acknowledge that there are numerous definitions of the term "invasive" (for a review, see Copp et al., 2005). These can be grouped into those that refer to a species that reproduces prolifically, spreads and may (or may not) exert an adverse impact (i.e., equivalent to a "weed") and those that restrict the definition to species that exert an adverse impact. For the purposes of this paper, we rely loosely on the definition given on page 244 of the aforementioned article: "These are native or alien species that spread, with or without the aid of humans, in natural or semi-natural habitats, producing a significant change in composition, structure, or ecosystem processes, or cause severe economic losses to human activities." However, studies that actually assess adverse impacts by freshwater fish species are in general limited to a few prominent species, e.g., common carp *Cyprinus carpio* Linnaeus, 1758 (reviewed in Vilizzi et al., 2015), so risk analysis considers a non-native species' attributes, which include invasion history, the propagule pressure associated with the species' introductions, population traits, and life-history traits.

The underlying purpose of risk analysis is to inform decision makers as to the threats posed by non-native species so that measures may be taken to avoid the introduction of potential pest species, or to control the spread as well as to limit (mitigate) the impacts of existing non-native species. The first step in this process is to identify which species are likely to be invasive, and the tools used in this non-native screening process range from purely qualitative (e.g., Kohler and Stanley, 1984) to the highly quantitative (e.g., Kolar and Lodge, 2001). The use of models to horizon scan for future invasives can aid the risk assessor in evaluating a species; perhaps the most common are climate matching models. Much more rare are models based primarily on life-history traits for predicting invasiveness (Ricciardi, 2007; see also Ricciardi and Kipp, 2008), such as the life-history-trait-based models for predicting invasiveness we proposed (Copp and Fox, 2007) for the North American centrarchid, pumpkinseed *Lepomis gibbosus* (Linnaeus, 1758). This model is based on the inverse relationship in female pumpkinseed between mean age-at-maturity and juvenile growth, i.e., mean total length (TL) at age 2, to identify which introduced populations in European inland waters are (or can be expected to be) invasive. We chose pumpkinseed as a test (or "model") species because: (a) the relationship between juvenile growth and mean age-at-maturity had already been assessed in populations of this species in part of its native range (Fox, 1994); (b) an increasing amount of growth and life-history data was being acquired for pumpkinseed populations introduced to European waters with which to evaluate the use of these traits in a model to predict invasiveness; (c) many of the populations for which these data are available have been evaluated for invasiveness by one or more of the criteria listed, though with regard to definitions of the term "invasive," the most commonly reported aspects were high density and/or prolific reproduction; and (d) population expansion is facilitated in environments with a species initially not present with "*r*-selected traits" like early maturity. Based on the relationship between juvenile growth and mean age-at-maturity elaborated by Fox (1994) for native pumpkinseed populations in Ontario, Canada (**Figure 1A**), the "model" proposed by Copp and Fox (2007) was applied to non-native pumpkinseed populations in Europe (e.g., Cucherousset et al., 2009; Ağdamar et al., 2015; Masson et al., 2015).

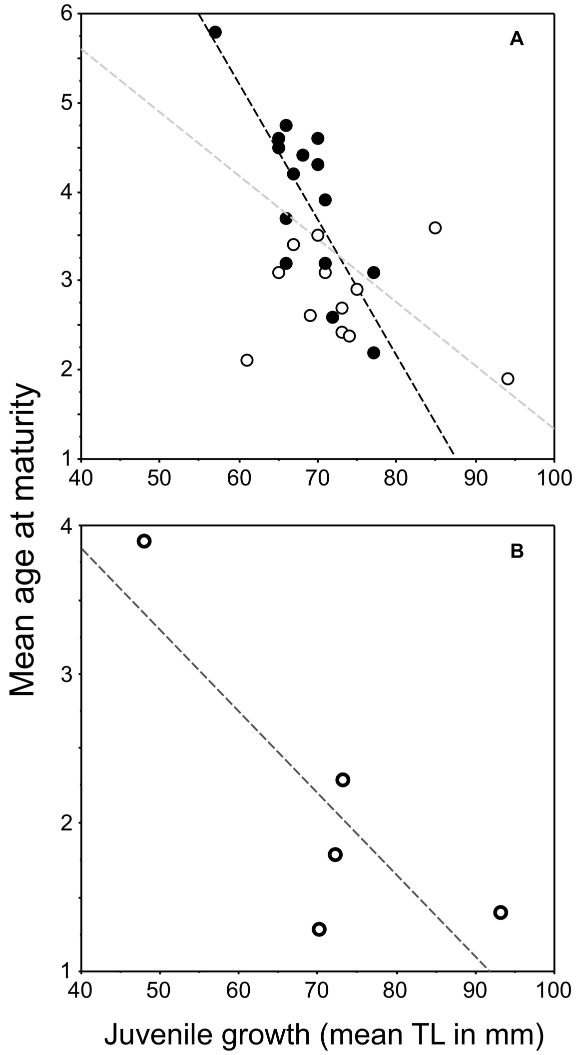

FIGURE 1 | Plots of the relationship between juvenile growth [mean back-calculated total length (TL) at age 2] and mean age-at-maturity of female pumpkinseed: **(A)** redrafted values from Fig. 6 in Fox (1994) for native populations in Ontario, Canada, and **(B)** plot of values from Table 6 in Copp et al. (2002) for introduced populations in Europe. The regression lines in "**(A)**" are significant for populations from lakes containing bluegill *Lepomis macrochirus* (Rafinesque, 1819) (filled dots, black dashed line: $y = -0.153x + 14.382$, $r^2 = 0.646$, $P = 0.0002$), for all lakes in Ontario (filled and open dots, gray dashed line: $y = -0.071x + 8.449$, $r^2 = 0.283$; $P = 0.0036$), but not for the Ontario lakes where bluegill was absent (open dots, no line: $y = -0.011x + 3.582$, $r^2 = 0.029$, $P = 0.5977$). In "**(B)**," the relationship for the non-native populations in Europe prior to 2000 (data from Table 6 in Copp et al., 2002) was not significant ($y = -0.055x + 6.049$, $r^2 = 0.684$, df = 3, $P = 0.0842$) despite as similar trend that that in native populations.

However, despite there being considerable knowledge of the underlying biological response of species quantified by the model, what remains to be determined is how good this combination of traits is as a predictor of actual invasiveness in an introduced fish species or population. Thus, the aim of this review is to evaluate the application of the Copp and Fox (2007) model with regard to its potential for predicting the potential invasiveness of non-native freshwater fish populations. Also briefly discussed are two applications of this model to predict the invasiveness of the North American ictalurid catfish, black bullhead *Ameiurus melas* (Rafinesque, 1820) in European waters and that of the European cyprinid *Carassius carassius* (Linnaeus, 1758), which is not native to Great Britain (Copp et al., 2016; Tarkan et al., 2016).

BACKGROUND TO THE MODEL

Model Development

With regard to pumpkinseed introduced to Europe, the first review and analysis of existing data (Copp et al., 2002) revealed that mean age-at-maturity appeared to be inversely related to the juvenile growth rate of the population (**Figure 1B**). This relationship, which is predicted from some life-history models (e.g., Stearns and Koella, 1986) and observable at the population level in many fish species (reviewed in Fox, 1994), was not quite significant but resembled that described by Fox (1994). This initial plot for introduced European populations comprised a single population in England (upper left of the graph, **Figure 1B**) and a few, mainly southern and central, European populations (Copp et al., 2002). When new data points from England (Villeneuve et al., 2005) were included in the regression, we found the relationship for introduced European pumpkinseed populations to be highly significant (**Figure 2**). This relationship between juvenile growth and maturity appeared to explain some of the inter-population variation in mean age-at-maturity not explained by latitude alone (Copp and Fox, 2007). And because virtually all of the populations considered to be "invasive," i.e., those located in Southern Europe, were in the lower right of the graph, and the non-invasive English populations (Villeneuve et al., 2005; Fox et al., 2011) were situated in the upper left of the graph, we proposed the use of this relationship (**Figure 2**) as a predictor of the potential invasiveness of pumpkinseed populations in Europe (Copp and Fox, 2007).

To delineate the upper extent of the invasive-non-invasive transition zone in this model (**Figure 2**), we identified "i" the mean TL at age 1 year (i.e., the minimum age at which maturity is achieved in pumpkinseed) of the oldest-maturing population and traced a 45° line from the origin through that point on the *x*-axis. To delineate the lower extent of the transition zone, two points were used: "ii" the mean minimum size (in TL) of mature age-2 females on the *x*-axis, representing their juvenile growth rate in the first year of life, and "iii" the mean size (TL) of females at the end of juvenile growth, i.e., at age 2 years. The designated zone effectively spans the second-year growth phase during which juveniles are allocating differential amounts of energy to somatic and gonadal growth as they mature into adults.

Data Sources and Model Predictions

The first evaluation of this model was undertaken by the addition of new data points (**Figure 3A**) acquired through the sampling of pumpkinseed populations in northerly parts of Europe (Cucherousset et al., 2009). Three further applications of the model, as presented in Cucherousset et al. (2009), include new data from England (**Figure 3B**), Turkey (**Figure 3C**), and France (**Figure 3D**). In England, a study of pumpkinseed dispersal and life-history traits in a small stream system examined the applicability of the model with respect to a long-established (source) population in an upstream mill-pond reservoir and a new downstream population (**Figure 3B**), which gained access to and established in an adjacent ornamental pond that connected temporarily to the stream during an extreme flood event during July 2007 (Fobert et al., 2013). By 2010, the new population had undergone an increase in juvenile growth and a corresponding decrease in mean age-at-maturity, relative to the source population, since gaining access to the ornamental pond (**Figure 3B**). This newly-established population was re-sampled during the summer of 2016 (same methods as Villeneuve et al., 2005), and these previously unpublished data comprise 130 specimens. During the 6-year period, the mean age-at-maturity of this relatively-new population decreased further by >1 year, but with a nominal increase in juvenile growth (**Figure 3B**), which within the model represented a transitional shift toward invasiveness.

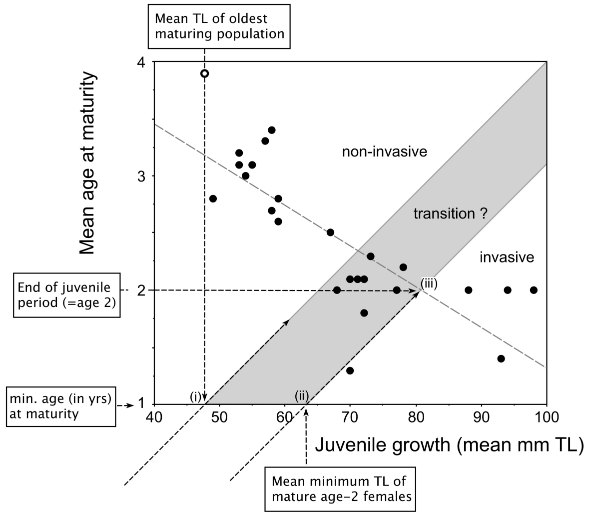

FIGURE 2 | Redrafted plot of invasiveness prediction model proposed by Copp and Fox (2007, Fig. 6) based on the relationship between juvenile growth (in mm of back-calculated TL at age 2) and mean age-at-maturity of female pumpkinseed in the species' non-native European range. Labeled arrows indicate the key coordinates for designation of the grayed area, which represents the populations transitioning between non-invasive populations (upper left-hand) and invasive populations (lower right-hand). See text for detailed explanation.

A separate application of the model in Turkey, described by Ağdamar et al. (2015), resulted in new data points for female pumpkinseed from five populations in artificial still waters (four reservoirs and one canal) revealed relatively similar mean ages-at-maturity across an approximate 20-mm span of juvenile growth (**Figure 3C**). And in France, a study of growth

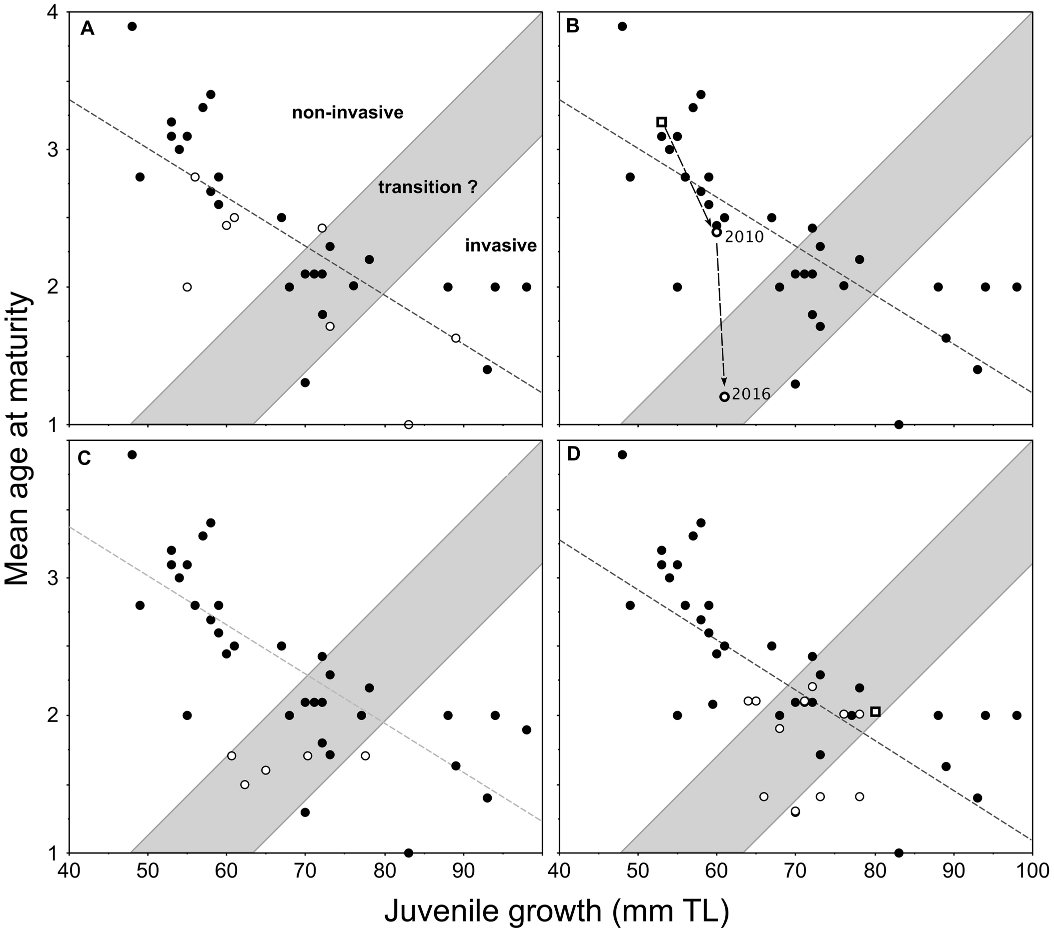

FIGURE 3 | Redrafted plots of the applications of invasiveness prediction model proposed for female pumpkinseed in Fig. 6 of Copp and Fox (2007) with the addition of data (open circles) from populations in **(A)** northerly locations of Europe (Cucherousset et al., 2009); and **(B)** the same populations as in **(A)** but with the source population (from a mill-pond reservoir) and a downstream ornamental pond population, which gained access to and established in the pond during an extreme flood in July 2007 (Fobert et al., 2013); **(C)** the same populations as in **(A)** but with five established populations in Turkey (Ağdamar et al., 2015); and **(D)** the same populations as in **(A)** with various sites along the longitudinal course of the River Moselle basin, France (Masson et al., 2015) as well as a recently-established population (open square) in a small angling lake in England (Copp et al., 2017).

and reproduction in populations at various sites along the longitudinal course of the River Moselle basin showed that the life-history response of Moselle pumpkinseed populations to warmer temperatures occurred primarily in age-1 individuals (Masson et al., 2015). Three of the 11 Moselle populations, including both of those inhabiting heated waters, were predicted to be invasive, with six populations in the transition zone and only two just inside of the "non-invasive" group of populations (**Figure 3D**).

TEST OF THE MODEL

In the various examinations of the relationship between juvenile growth and mean age-at-maturity (as per Fox, 1994) in non-native European fishes (Masson et al., 2015; Copp et al., 2016; Tarkan et al., 2016), the data points tended to further strengthen the underlying growth–maturity relationship that was the basis of the model that Copp and Fox (2007) proposed to predict pumpkinseed invasiveness. However, the ability of this model to predict invasiveness has yet to be tested. To test the model, we focused on pumpkinseed for two reasons: (1) a largest data set exists for non-native populations (Fox and Copp, 2014), and (2) a large amount of bibliographic evidence exists with which to categorise *a priori* the populations as either "invasive" or "non-invasive." However, this data set was constructed from sources that date back to studies carried out in the early 1980s (Constantinescu, 1981) when bioinvasion terminology, such as invasive, pest, alien species, etc., was not used in those publications. Therefore, to determine whether or not pumpkinseed was considered to be invasive in water body, we examined available documents for evidence of the species being referred to as very abundant and widespread, or simply very abundant. If no such evidence was provided in the source paper, then we relied on information we could obtain for water bodies of the same type in the same river basin.

In the more recent studies, the published articles tended to label the study population as being "invasive" and/or the article provides more detailed information on the numbers of pumpkinseed captured from, or processed for, each population. If the total number of fish captured was available, such as studies carried out by us and our colleagues, then our databases and field notes were used to classify them *a priori* as invasive or non-invasive. In some cases (e.g., Spain), the source publications for pumpkinseed life-history and growth data used in our study (e.g., Gutiérrez-Estrada et al., 2000a,b) did not mention invasiveness or high abundance despite reporting large numbers of pumpkinseed captured. However, other articles on fish assemblages in the same river or lake basins refer to pumpkinseed as invasive or very abundant (e.g., Vila-Gispert et al., 2002; Prenda et al., 2006; Almeida et al., 2009; Ribeiro and Collares-Pereira, 2010; Almeida and Grossman, 2014; De Miguel et al., 2014; Rooke and Fox, 2018). For pumpkinseed in ponds and streams in England, we know from our extensive studies (e.g., Copp et al., 2002, 2010; Klaar et al., 2004; Villeneuve et al., 2005; Cucherousset et al., 2009; Fobert et al., 2013) that the species does not reproduce in streams, and that pumpkinseed densities in ponds occupied by a characteristic assemblage of native fish species are not invasive. However, in "disturbed" waters, such as newly-created (dug out) ponds, e.g., water hazards on a golf course, pumpkinseed densities were high (Fox et al., 2011) and therefore the population was classed as "invasive."

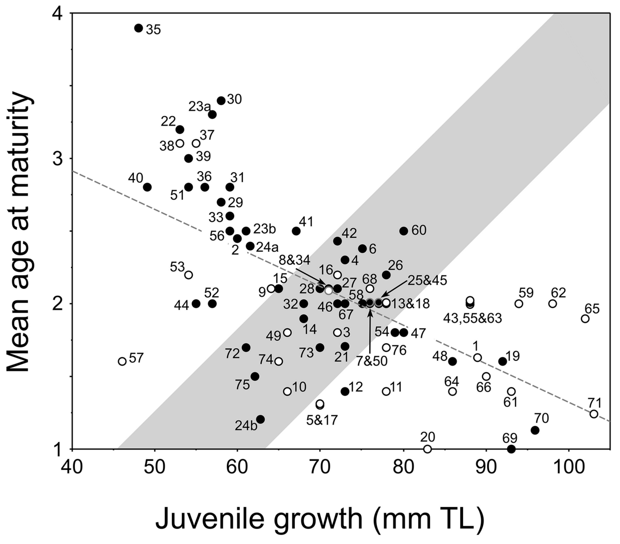

FIGURE 4 | New plot of the relationship between juvenile growth [mean back-calculated total length (TL) at age 2] and mean age-at-maturity of female pumpkinseed in non-native European populations (i.e., Fox, 1994): $y = -0.027x + 3.981$, $r^2 = 0.39$, $F = 47.291$, df = 74, $P = 0.0001$. Plot presents re-delineated transition zone due to new data (from Fox and Copp, 2014), with populations considered *a priori* as invasive distinguished (open dots) from those considered non-invasive (filled dots) as described and numbered (1–76) in **Supplementary Table 1**. In cases of overlapping values, the dots are slightly separated vertically.

In cases where the total number of pumpkinseed captured was not recorded, then the number of fish processed was used as an estimator of fish density, with populations for which the numbers of fish processed was ≥100 specimens, that population was classified *a priori* as invasive and those of fish processed <100 classified as non-invasive. In some cases, a population was known (from raw data for that population or from our colleague's knowledge of the pumpkinseed populations) to occur in high (invasive) densities despite the number of fish processed being <100 fish. This was the case for one of the ten pumpkinseed populations examined in the River Moselle, France (Masson et al., 2015) – that the population was classified accordingly and half of the populations were classified *a priori* as invasive and half as non-invasive. In some cases, detailed data on pumpkinseed densities were reported. For example, a study in Netherlands of growth and life-history traits of pumpkinseed invasiveness in ponds (Van Kleef and Jongejans, 2014) provided, in the article's supplementary information, density estimates using a standardise sampling protocol. The ponds with elevated density estimates were used to classify the populations as invasive (109–1288 fish per 100 m transect; $n = 3$) and non-invasive (1–69 fish per 100 m; $n = 10$).

True and false predictions from the model for the pumpkinseed database were tested against their classification with the aforementioned criteria. For this analysis, two separate binomial tests were conducted, one for populations predicted to be invasive and one for populations predicted to be non-invasive. Two versions of the test were conducted for the non-invasive prediction: one in which populations predicted to be transitional were grouped with those predicted to be non-invasive, and one where the transitional populations were excluded from the analysis. The rationale for including the predicted transitional populations with the predicted non-invasive populations is based on two related sources. Firstly, the implementation of non-native species policy in the management of invasive species prioritises high-risk species ("need to act" species), with low- and medium-risk species dealt with when circumstances and/or resources permit ("nice to act" species). Secondly, and inspired by the above, the calibration of assessment scores generated by risk screening tools, such as the freshwater Fish Invasiveness Screening Kit (Vilizzi et al., 2019) and the Aquatic Species Invasiveness Screening Kit (see applications cited in Li et al., 2020), has focused on the identification of the threshold between species that pose a high risk of being invasive in a given risk-assessment area and those that pose as a low or medium risk of being invasive (Vilizzi et al., 2019).

The results of our binomial test with the European pumpkinseed database do not strongly support the utility of the model for predicting invasive and non-invasive populations. For populations predicted to be invasive, only 57% (12 of 21) were correct by our classification criteria for invasive, a result that was not significant (binomial test, $P = 0.33$). On the other hand, 70% (39 of 56) of populations predicted to be non-invasive or transitional were correct by our criteria and significant ($P = 0.0024$), but not when transitional populations were excluded (64% correct, $P = 0.12$).

DISCUSSION

An essential question in the use of a life-history model to predict invasiveness is whether the underlying biological state reflected in that model facilitates a species acclimation to, and invasion of, novel environments. In the present case, the basic interpretation of our model is that fast pre-maturational growth and early maturity predisposes a population to be successful in a situation when it is newly introduced, and ultimately allows it to expand not only its population, but also its distribution. Early maturity, which works with fast growth to predict invasion success in the model, is what one would expect in "*r*-selected" populations of MacArthur and Wilson (1967), i.e., those that may be favoured during the initial stages of a species' establishment and spread in a novel environment because of its low density (Fox and Copp, 2014). Since fecundity is related to body size in fishes, fast juvenile growth combined with early maturity would potentially result in a large number of propagules, which is a strong predictor of invasion success (Duggan et al., 2006; Copp et al., 2007; Simberloff, 2009).

Despite these theoretical reasons why the life-history model should be a good predictor of invasion success, the model was not strongly supported in our tests with the pumpkinseed. This could be simply due to shortcomings in the dataset, which include the variety of sampling methods used (i.e., non-uniformity of sampling), the absence of density estimates for some of the populations, the extensive time interval over which the data emanates (from the 1970s onward), and differences in environmental factors that could influence the growth–maturity relationship.

Furthermore, the life-history traits used in the tests are based on samples taken in the recipient water bodies, and not the source populations that were originally introduced. This was out of necessity because, although we now have an indication of the general geographic origin of non-native European pumpkinseed populations (Yavno et al., 2020), the actual source populations were unknown. Given the likelihood that the expression of these traits was influenced by conditions in the recipient water body, the assumption that the trait mean values are reflective of the source populations is questionable, and the differences between these trait mean values in the originally introduced fish and those sampled an unknown number of years later may differ in magnitude among the water bodies sampled.

Another consideration is that life-history traits that are useful during the early stages of invasion may not be at a later phase, when population density has increased (García-Berthou, 2007; Gutowsky and Fox, 2012; Masson et al., 2016). Indeed, a preliminary assessment of changes in mean reproductive effort in non-native pumpkinseed populations found that those thought to have existed for <50 years exerted a significantly higher effort than those established prior to 1950 (Copp and Fox, 2007). As such, invasion success may depend, not only on the initial life-history traits of the introduced species or population, but also on the species' ability to adapt its life-history traits in a later phase of the invasion. The pumpkinseed has demonstrated a high degree of life-history plasticity when newly introduced in both North

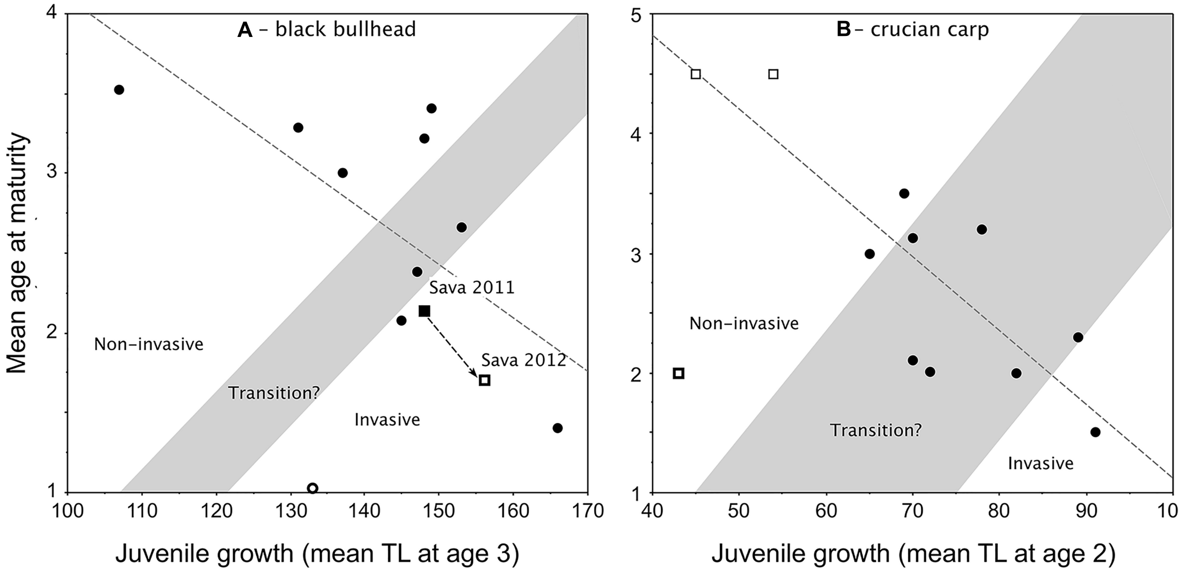

FIGURE 5 | Redrafted plots of the Copp and Fox (2007) model applied to: **(A)** non-native black bullhead *Ameiurus melas* populations in Europe (Copp et al., 2016), with retraced transition zone as per the criteria in **Figure 2** and retraced regression line ($y = -0.03x + 7.441$, $r^2 = 0.499$, $F = 8.956$, df = 9, $P = 0.0151$) that includes two data points for a population in Lake Sava (Jaćimović et al., 2019), which underwent a boom (2011) and bust (2012) sequence, but ignores the extreme (female-dominated) outlier (thick-outlined open circle; Canal de Jouy, France; Copp et al., 2016); and **(B)** crucian carp *Carassius carassius* populations (Tarkan et al., 2016) from the native continental Europe (open squares) and the non-native range in England (filled dots), with the transition zone traced for the first time based on the criteria in **Figure 2**. The regression line ($y = -0.062x + 7.296$; $F = 22.28$, df = 9, $r^2 = 0.712$, $P = 0.0011$) ignores the extreme (stunted) outlier population (thick-outlined open square; Olsztyn pond, Poland; Copp et al., 2016).

America (Fox, 1994) and Europe (Fox et al., 2007). In European waters, where there are fewer pumpkinseed competitors and predators than in its native range, the life-history traits that are predicted by our model to favour invasion success appear to have been maintained over the course of these invasions (Fox and Copp, 2014). This may explain why our model worked somewhat to predict invasion potential, but it may not work at all for other species or even for pumpkinseed in other geographic regions where more potential predators and competitors are present or where thermal regimes are less favourable.

It must be highlighted that our invasion model is population-based rather than species-based, and this raises the question of whether populations of a given species, especially a highly plastic species, are likely to vary substantially from one another in invasion success. The answer to this may depend on the degree to which adaptation in different regions, or environments within regions, has genetically altered the life-history reaction norms of populations inhabiting these areas (see Hutchings et al., 2007; Fletcher et al., 2016). In the case of pumpkinseed, the wide variety of freshwater habitats it occupies over a broad native geographic range in North America and Europe suggests that its broad inter-population variation in mean age-at-maturity and growth rate (Fox, 1994; Fox and Copp, 2014) are due to adaptation and phenotypic plasticity (see Yavno et al., 2020). If that is the case and if life-history reaction norms differ across populations as a result of these adaptations, then it follows that populations will vary in performance when introduced into novel environments. Therefore, it is appropriate to make invasion success predictions for a species at the population level.

Applications of the Copp and Fox (2007) model to black bullhead (Copp et al., 2016; Jaćimović et al., 2019) and crucian carp (Tarkan et al., 2016), despite their small data sets, certainly demonstrate the same growth–maturity relationship observed in pumpkinseed (**Figure 4**). However, the ability of these models to predict invasiveness of the species remains to be tested. The model for non-native black bullhead was elaborated using TL at age 3 as the estimator of juvenile growth (**Figure 5A**), being that females in most black bullhead populations do not achieve maturity until age 3. In an application of this model to assess the impact of a viral infection on the invasiveness status of a "boom" phase population of black bullhead in a floodplain water body of the River Sava, Belgrade, Serbia (Jaćimović et al., 2019), the model should predict a sharp increase in juvenile growth with corresponding sharp decrease in mean age-at-maturity.

However, the observed shift reported by Jaćimović et al. (2019) between 2011 and 2012 was a slight (8 mm) increase in juvenile growth (mean TL at age 3) and a decrease in mean age-at-maturity of 0.43 years (**Figure 5A**). A similar chronology in a pumpkinseed population's life-history traits was observed in England (**Figure 3B**), where pumpkinseed washed out from an upstream (former) mill reservoir during an extreme flood event in 2007 gained entry to a garden pond located within the stream's flood plain (Fobert et al., 2013). Relative to the source population (thick-lined open square in **Figure 3B**), the new garden pond population demonstrated an increase in juvenile growth (mean = 7 mm TL) by 2010 with a corresponding decrease in mean age-at-maturity of 0.83 years (0.3 years per year). Then, after 6 years in the pond, juvenile growth had increased another 7 mm TL and mean age-at-maturity decreased 1.2 years (0.2 years per annum). This is half the annual rate of shift in the life-history traits of the River Sava black bullhead population, which could be interpreted to suggest that an established fish population responds faster to a sudden availability in resources in its existing habitat (due to a sharp drop in population density) than does a species that has just entered a new water body. For example, two native pond populations of pumpkinseed populations subjected to major winterkills (in Ontario, Canada) matured earlier and at smaller size (due to continued high mortality rates) than in years without winterkills (Fox and Keast, 1990, 1991). As such, one might expect that the removal of pumpkinseed from Meeuwven Pond (Netherlands) during sampling in 2006 (Cucherousset et al., 2009) would have led to an increase in juvenile growth and decrease in mean age-at-maturity in that population when re-sampled in 2009 (Van Kleef and Jongejans, 2014). But, over that 3-year interval, juvenile growth hardly changed (an increase of 1 mm TL) and mean age-at-maturity increased by almost a year (data points 44 and 51 in **Figure 4**, respectively). Experimental work with a stocked predator (northern pike *Esox lucius* Linnaeus, 1758) reported that pumpkinseed achieve maturity earlier and at larger size in the presence of the predator than in its absence (Beaune et al., 2019).

The application of the Copp and Fox (2007) model to crucian carp (Tarkan et al., 2016) differs from those described here (**Figures 4**, **5A**) in that data from native continental European populations were included in the plot of non-native crucian populations in England (**Figure 5B**). At the time that Fig. 2 in Tarkan et al. (2016) was plotted, crucian carp were assumed to be native to England (reviewed in Copp and Sayer, 2020), though this was subsequently refuted by genetic evidence (Jeffries et al., 2017). As such, the initial plot of this model lacked delineation of a transition zone (Fig. 2 in Tarkan et al., 2016), which we have plotted here (**Figure 5B**) following the procedure described here above for pumpkinseed (**Figure 3**). Similar to pumpkinseed (**Figure 3**), female crucian carp in the majority of studied populations achieved maturity by age 2, and the relationship reveals a single population in England to be demonstrating the fast juvenile growth and precocious maturity that could be referred to as invasive (**Figure 5B**). The remaining English and native range populations (Finland and Russia) were spread across the statistically significant regression line except for one outlier (a stunted pond population at Olsztyn, Poland). Nevertheless, the relationship revealed that crucian carp populations respond well, both in terms of growth and reproduction, to the favourable environmental conditions in England. Despite the crucian carp's non-native status in Great Britain, and in view of its decline in its native range, England is considered ideal for promoting the species' conservation (Tarkan et al., 2016; Copp and Sayer, 2020), with at worst moderate risk to native species and ecosystems (Dodd et al., 2019).

The maturity–growth relationships in both black bullhead and crucian carp were statistically significant despite the relatively low number of sample populations (**Figure 5**). Adequate density estimates are not available with which to carry out tests to validate either of these two models with the same level of confidence as for pumpkinseed, but the statistical nature of this relationship suggests that both of these models offer a useful starting point for future application and testing once more populations and better density data are available for *a priori* classification of each population with regard its invasiveness status. That said, a key issue we have to address is "what does the relationship predict?" In the underlying relationship, mean age-at-maturity depends on, and therefore can be predicted from, juvenile growth, which is a population's response to available resources. As previously mentioned, resource availability could potentially be greater following a sharp drop in population density, either disease or intensive predation (including removal by humans), or when introduced (human-assisted or natural dispersal) into a novel water body. The same population response is observed in the native and introduced ranges (**Figure 1**), which suggests that a native population of fish could be referred to as "invasive" in the same way that native terrestrial weeds are referred to as "weedy" (Copp et al., 2005). Regardless, these applications of the growth–maturity relationship as predictive models of invasiveness, can provide a heuristic means of predicting invasiveness even if their predictive power is not yet statistically significant.

In summary, our life-history model has been shown to apply to three fishes that are successful invaders in some parts, if not all, of Europe and in the case of pumpkinseed also in the extreme western extent of Asia (Ağdamar et al., 2015). However, the application of the model to predict invasion potential of pumpkinseed was only weakly supported when tested against the *a priori* invasiveness classification of the various populations. That said, the tests were hampered by lack of knowledge of the actual source populations for these European introductions, as well as shortcomings in the dataset that affected our classification of invasiveness. We conclude that such life-history models could potentially provide a useful means for predicting the invasiveness status of non-native freshwater fishes, though the basis of the predictions, statistical or heuristic, will depend on the size and quality of the underlying life-history database.

AUTHOR CONTRIBUTIONS

GHC and MGF conceived the manuscript structure and content, and shared equally composition of the text. GHC prepared the draft Figures and supplementary information documents using information provided by GHC and MGF. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.605287/full#supplementary-material

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Threespine Stickleback in Lake Constance: The Ecology and Genomic Substrate of a Recent Invasion

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Invasive species can be powerful models for studying contemporary evolution in natural environments. As invading organisms often encounter new habitats during colonization, they will experience novel selection pressures. Threespine stickleback (*Gasterosteus aculeatus* complex) have recently colonized large parts of Switzerland and are invasive in Lake Constance. Introduced to several watersheds roughly 150 years ago, they spread across the Swiss Plateau (400–800 m a.s.l.), bringing three divergent hitherto allopatric lineages into secondary contact. As stickleback have colonized a variety of different habitat types during this recent range expansion, the Swiss system is a useful model for studying contemporary evolution with and without secondary contact. For example, in the Lake Constance region there has been rapid phenotypic and genetic divergence between a lake population and some stream populations. There is considerable phenotypic variation within the lake population, with individuals foraging in and occupying littoral, offshore pelagic, and profundal waters, the latter of which is a very unusual habitat for stickleback. Furthermore, adults from the lake population can reach up to three times the size of adults from the surrounding stream populations, and are large by comparison to populations globally. Here, we review the historical origins of the threespine stickleback in Switzerland, and the ecomorphological variation and genomic basis of its invasion in Lake Constance. We also outline the potential ecological impacts of this invasion, and highlight the interest for contemporary evolution studies.

Keywords: adaptive radiation, contemporary evolution, lake constance, invasive species, stickleback

INTRODUCTION

Colonizing species that invade new environments may experience novel selection pressures and adapt rapidly to local conditions, potentially culminating in divergent phenotypes between distinct habitats in the invaded range (Schluter, 2000; Reznick and Ghalambor, 2001; Sakai et al., 2001; Shine, 2012). In this way, invasive species provide a powerful opportunity to study evolution in action during colonization, population growth, and range expansion. When the invaded range includes unique environments that allow for niche expansion beyond what is observed in the native range, we may also learn about how populations respond to novel ecologically based divergent selection and witness processes akin to the earliest stage of adaptive radiation. Contemporary phenotypic evolution following anthropogenically facilitated invasions often results from selection on standing genetic variation rather than *de novo* mutations (Hendry et al., 2007; Barrett and Schluter, 2008; Prentis et al., 2008). Therefore, the nature of standing genetic variation in the invading population may have a strong influence on the dynamics of the invasion process.

The ability of a species to invade, colonize, adapt, and diversify can depend on whether the invasion derives from a single introduction, repeated introductions from the same source population, or from multiple distinct source populations (Ehrlich, 1989; Barrett and Husband, 1990; Sakai et al., 2001; Kolbe et al., 2004; Frankham, 2005; Lavergne and Molofsky, 2007; Lucek et al., 2010). Secondary contact between distinct lineages can generate potentially adaptive allelic variation through admixture (Anderson and Stebbins, 1954; Barton, 2001; Seehausen, 2004; Mallet, 2007; Prentis et al., 2008; Abbott et al., 2013; Seehausen and Wagner, 2014; Williams et al., 2014; Roy et al., 2015; Marques et al., 2019b), and this can even result in speciation through recombination of old genetic variants (Marques et al., 2019b). Such speciation can be driven by ecological (Marques et al., 2019b) or non-ecological (Schumer et al., 2015) processes. While single-source invasions are useful to investigate how a population responds to novel selection pressures during colonization of new environments, invasions with multiple genetic origins allow us to study the role of secondary contact and hybridization (or lack thereof) in ecological expansion and diversification.

The threespine stickleback superspecies (*Gasterosteus aculeatus* species complex, Linnaeus, 1758) is a popular model taxon in ecology and evolutionary biology research (Foster and Bell, 1994; McKinnon and Rundle, 2002; Hendry et al., 2013). Stickleback are known for their propensity to rapidly diversify through habitat dependent divergent selection, and this makes them a particularly useful model for addressing questions about how ecological divergence occurs in invasive species. There is strong evidence for repeated events of adaptive population divergence by stickleback during postglacial colonization of freshwater habitats (McKinnon and Rundle, 2002; Hendry et al., 2009). For such cases, the populations closely resembling the presumed ancestors of derived freshwater stickleback are still extant in the form of marine and anadromous populations with a Holarctic distribution (Baker et al., 2015; Fang et al., 2018). This allows for phenotypic comparisons between freshwater populations and their putative ancestral state. Such comparisons must be made with caution however, as marine populations are also diverging, and can be grouped into several genetically distinct clusters as well (DeFaveri et al., 2012; deFaveri and Merilä, 2014; Fang et al., 2018, 2020a; Morris et al., 2018). Furthermore, geographically adjacent marine and freshwater populations and species do not necessarily share the same common ancestor (Dean et al., 2019; Marques et al., 2019a). Within freshwater, ecotypic diversification occurs frequently along a lake-stream axis of divergence and rarely along a benthic-limnetic axis within lakes, where the latter has been found exclusively in coastal sectors of British Columbia, Canada (Bentzen and McPhail, 1984; Schluter and McPhail, 1992; Foster and Bell, 1994; McPhail, 1994; McKinnon and Rundle, 2002; Gow et al., 2008; Willacker et al., 2010; Østbye et al., 2016). Both the rapid adaptation of stickleback to freshwater, and subsequent ecotypic differentiation within freshwater habitats, has often occurred through selection on standing genetic variation present in oceanic populations (Colosimo et al., 2005; Barrett and Schluter, 2008; Schluter and Conte, 2009; Jones et al., 2012a,b; Terekhanova et al., 2014; Marques et al., 2017b; Bassham et al., 2018; Haenel et al., 2019; Fang et al., 2020a; Rennison et al., 2020). With regards to defensive morphology, freshwater stickleback typically show a reduction in plate numbers compared to the marine form (Foster and Bell, 1994; Barrett et al., 2008; Wootton, 2009), and occasional loss of pelvic spines (Morris et al., 1956; Bell, 1974; Reimchen, 1983; Campbell, 1985; Shapiro et al., 2004; Chan et al., 2010; Lescak and von Hippel, 2011), potentially in response to differences in predation regime between freshwater and the Ocean (Barrett, 2010). Alleles for low plated phenotypes, for example, are at low frequencies in oceanic populations but have increased in frequency in freshwater populations multiple times, independently (Colosimo et al., 2005; Barrett and Schluter, 2008). Other adaptations to freshwater habitats, such as the loss of the pelvic girdle (Chan et al., 2010; Xie et al., 2019) and the increased capacity to synthesize essential fatty acids (i.e., Docosahexaenoic acid) through duplications of the *FADS2* gene (Ishikawa et al., 2019) have arisen from *de novo* mutations.

European threespine stickleback populations in general (Fang et al., 2020a), and Swiss populations in particular (Kottelat and Freyhof, 2007; Lucek et al., 2010; Marques et al., 2019a), provide an interesting setting to observe how secondary contact between lineages can affect ecotype formation in freshwater environments. In Switzerland natural colonizations of freshwater catchments from two divergent lineages represented in Europe (Fang et al., 2018) coincide with recent anthropogenic introductions of other European lineages among and between catchments (Kottelat and Freyhof, 2007; Lucek et al., 2010; Marques et al., 2019a). In Lake Constance, for example, there is ongoing debate about the origin of the lake and stream stickleback populations, and the importance of secondary contact for ecotype formation (Lucek et al., 2010, 2012, 2013, 2014b; Moser et al., 2012; Roesti et al., 2015; Marques et al., 2016, 2019a). In light of this previous work, and recent observations, there is mounting evidence for three major stickleback ecotypes, two of which are observed in the species complex globally, namely an entirely lacustrine form that breeds within the lake, and a stream resident form. The third form may be rather unique: a potamodromous form that lives in the lake but migrates to streams to reproduce, resembling anadromous ecotypes of the oceans. Some of these ecotypes persist in the face of gene flow, with migratory lake ecotypes (i.e., potamodromous ecotype) breeding in sympatry and parapatry with resident stream ecotypes, suggesting that some populations of Lake Constance stickleback are in the incipient stage of ecological speciation, despite their very recent history in the system (Marques et al., 2016).

In the present paper, we review the genetic, genomic, and phenotypic research to date on threespine stickleback in Central Europe, focusing on Switzerland and the invasion of the Lake Constance region. By providing a review of the existing literature on stickleback populations within Lake Constance and drawing on additional stickleback research from elsewhere, we summarize what is known in this system, highlight knowledge gaps and the utility of this system for studying the genomics and ecology of invasion, range expansion, contemporary ecological diversification, and the evolutionary consequences of secondary contact.

HOW STICKLEBACK DIVERSIFY

The *G. aculeatus* superspecies of threespine stickleback most likely originated in the Western Pacific Ocean (Fang et al., 2020a), and colonized marine habitats around the northern hemisphere during the Pleistocene (Ortí et al., 1994; Mäkinen and Merilä, 2008; Fang et al., 2018, 2020b; Ravinet et al., 2018). It expanded around the Northern Pacific Ocean and through the Bering Sea Strait into the Arctic Ocean, and would have arrived in the Atlantic Ocean between 300 and 50 Kya (Fang et al., 2018, 2020b). After this widespread colonization of the Holarctic Ocean, the ancestors of the Atlantic *G. aculeatus* complex initially split into colonists of freshwaters in Southern Europe and the Atlantic Ocean population between ∼100 and ∼25 Kya (Fang et al., 2020b). These Southern European populations colonized freshwater habitats during the Pleistocene, and persisted in freshwater glacial refugia during the Last Glacial Maximum (Mäkinen and Merilä, 2008; DeFaveri et al., 2012; Lucek and Seehausen, 2015; Sanz et al., 2015; Fang et al., 2018), while the Trans-Atlantic clade colonized freshwater habitats in Northern Europe, and Eastern North America much later during the Holocene (Ortí et al., 1994; Mäkinen et al., 2006; Ravinet et al., 2014; Fang et al., 2018, 2020b). Within the Trans-Atlantic clade, multiple geographically structured subclades exist, including Baltic Sea, Barents Sea, North Sea, and mainland European lineages that diverged 27-11 Kya. In the mainland European clade, lineages diverged 17-5 Kya between large river catchments such as the Rhine, Loire, Vistula etc., likely following deglaciation of Central Europe (Fang et al., 2018).

Despite these advances in understanding phylogenetic relationships in the threespine stickleback superspecies, and despite the evidence for many biological species within it (McKinnon and Rundle, 2002; Dean et al., 2019), the diversity of forms is taxonomically treated by most authors under the singular name *Gasterosteus aculeatus* (Wootton, 1976; Th and Bakker, 1988; Bell, 1995). This "convenient solution" follows a taxonomic history in which over 40 species had been described, often based on phenotypic traits that may well be informative for delimiting two species within a given locale, but not for delimiting local species against all others across the global range (Bertin, 1925; Münzing, 1959, 1963; Penczak, 1966; Miller and Hubbs, 1969; Wootton, 1976; Bell, 1995; Denys et al., 2015). In the European context, for example, *Gasterosteus gymnurus* was described by Cuvier (Cuvier and Valenciennes, 1828; Cuvier, 1829) based on landlocked stickleback from Northern France and Southern England that had lateral plating restricted to the structural plates. Given the historical West-East separation of these plate morphs across much of Europe (Münzing, 1963), others later applied this name to any stickleback from anywhere in Europe and beyond that shared this plating phenotype (Gordon, 1902; Koch and Heuts, 1943; Kottelat and Freyhof, 2007; Denys et al., 2015). Others recognized that this practice had led to a highly polyphyletic nature of *G. gymnurus* and lumped it further with the mostly marine fully plated form into a single taxon, *G. aculeatus* (Wootton, 1976; Paepke, 1983; Bell, 1995; Denys et al., 2015), pending a thorough taxonomic revision that takes biological species into account and that has yet to happen. As a result, the *G. aculeatus* superspecies currently contains both deeply divergent geographical lineages and reproductively isolated biological species within Europe (Jones et al., 2006; Mäkinen et al., 2006; Mäkinen and Merilä, 2008; DeFaveri et al., 2012; Lucek and Seehausen, 2015; Pérez-Figueroa et al., 2015; Berner et al., 2017; Fang et al., 2018; Dean et al., 2019; Marques et al., 2019a).

Phylogeographic and population genetic studies revealed that freshwater ecotypes and species have evolved many times in parallel from marine ancestors, although some freshwater clades have clearly also expanded their ranges across multiple catchments, such that adjacent catchments often share the same lineage, species or ecotype (Mäkinen et al., 2006; Lucek and Seehausen, 2015; Fang et al., 2018; Ishikawa et al., 2019; Marques et al., 2019a). Evidence from the fossil record in Western North America and Eastern Russia shows that members of the family Gasterosteidae have been colonizing freshwater habitats from the Pacific Ocean since the Miocene, so there have been repeated cycles of colonization, adaptation, and extinction over the evolutionary history of the group (Bell, 1977; Bell and Haglund, 1982; Bell et al., 2006, 2009).

Throughout the northern hemisphere, habitat-specific adaptation in allopatry or parapatry is responsible not only for the parallel evolution of freshwater stickleback from marine or anadromous ancestors (Jones et al., 2006, 2012b), but also for most of the parallel evolution of recurrent ecotypes within freshwaters (Hendry et al., 2009; Willacker et al., 2010), albeit this is more pronounced in the Pacific than in European populations (Fang et al., 2020a). Sympatric pairs of benthic and limnetic stickleback are observed in a handful of British Columbian coastal lakes and nowhere else, despite intensive research (McPhail, 1984; Schluter and McPhail, 1992; Baker et al., 2005). These sympatric pairs are thought to have evolved through serial colonizations from the ocean rather than sympatric speciation from a single source population (Hendry et al., 2009; Bolnick, 2011) and this is deemed the "double-invasion hypothesis" (Schluter and McPhail, 1992; McPhail, 1994; Kassen et al., 1995; Taylor and McPhail, 1999). Models predict that sympatric speciation in stickleback is possible, though unlikely, as pressures from disruptive selection and assortative mating tend to be too weak to result in sympatric speciation (Bolnick, 2004, 2011), and indeed no strong case is known. An incipient sympatric species pair has recently been described from a small lake in Switzerland but this pair evolved within the hybrid zone between the Eastern and the Western European lineages (Marques et al., 2017a), and it is currently unclear whether it emerged in sympatry from a hybrid population or represents persistence of the hybridizing and nearly collapsed pre-existing lineages.

Evolution of lake-stream ecotypes and species pairs in parapatry has occurred much more frequently in stickleback than the evolution of species that can persist in sympatry (Reimchen et al., 1985; Lavin and Mcphail, 1993; Hendry et al., 2002, 2009; Hendry and Taylor, 2004; Berner and Grandchamp, 2009; Deagle et al., 2012; Kaeuffer et al., 2012; Moser et al., 2012; Roesti et al., 2012; Lucek et al., 2013, 2014b). This is not surprising given that much weaker selection is sufficient to retain phenotypic distinctiveness when the opportunity for gene flow is geographically constrained (Doebeli and Dieckmann, 2003). Active matching habitat choice between lake and stream may in some ecotonal situations also facilitate the persistence of differentiation (Edelaar et al., 2008; Edelaar and Bolnick, 2012). Stickleback residing in different habitats often show divergence in foraging traits and performance (Arnegard et al., 2014; Best et al., 2017; Schmid et al., 2019), body size (Hendry et al., 2002; Sharpe et al., 2008), migration behavior (Harvey et al., 1997; Kitano et al., 2012), life history (Moser et al., 2012), nuptial coloration (Hagen and Moodie, 1979; Reimchen, 1989; Jenck et al., 2020), and/or in defense traits and defense performance (Reimchen, 1983, 1992b, 1994, 2000; Lucek et al., 2013).

Strong reproductive isolation rarely evolves between parapatric ecotypes of stickleback, and this is likely the chief reason for the extreme rarity of sympatric species persistence (Räsänen et al., 2012). In the few cases where reproductive isolation has been demonstrated, it can result either as a by-product of divergent adaptation to different habitats or ecological niches that lead to assortative mating preferences (Rundle et al., 2000; Boughman, 2001), or from ecological selection that puts hybrids at a fitness disadvantage and may lead to reinforcement (Rundle and Schluter, 1998; Lackey and Boughman, 2017). Previous work on crosses of sympatric benthic and limnetic stickleback demonstrated that hybrids are not at any intrinsic disadvantage, but they may perform worse than either of the parental types in the parental niche (Schluter, 1994, 1995, 2003; Schluter et al., 1996; Hatfield and Schluter, 1999; Vamosi et al., 2000; Jones et al., 2006; Gow et al., 2007; Arnegard et al., 2014; Laurentino et al., 2020). This opens the possibility that hybrids could invade novel (non-parental) niches if the opportunity arises.

THE ENVIRONMENTAL CONTEXT OF THE STICKLEBACK INVASION IN LAKE CONSTANCE

Lake Constance is a peri-alpine lake on the northern edge of the European Alps, and is the third largest lake by surface area in Central Europe (after Lakes Balaton and Geneva), and the second by volume (after Geneva). The lake is part of the Rhine catchment and is located at the intersection between Germany, Switzerland, and Austria (**Figure 1**). Lake Constance consists of a pair of lakes joined by a 4.5 km stretch of river called the Seerhein. Upper Lake Constance is a large (surface area = 472 km^2), deep (max. depth = 254 m, mean depth = 101 m) and monomictic lake, while lower Lake Constance is considerably smaller (surface area = 63 km^2) and more shallow [max. depth = 46 m, mean depth 13 m; (Petri, 2006)].

The geological history of the lake indicates that it was formed by the process of glacial erosion (Müller and Gees, 1968) through the expansion of the Rhine glacier from the inner Alps into the Central European lowlands during the Würm ice age, roughly 115,000–11,700 Kya (Keller and Krayss, 2000). During this period, Lake Constance was covered by ice and its entire surface became ice-free only ∼14,500 ybp (Keller and Krayss, 2000). Thus, all extant freshwater fish species of the lake must have colonized or have been introduced following glacial retreat (Behrmann-Godel et al., 2004). The lake is presently fed by the Alpine Rhine to the south, and drains into the North Sea through the Rhine. Capture of the outflow of Lake Constance by the Rhine formed roughly 7,000–8,000 ybp (Wessels, 1995). Prior to this river capture, Lake Constance drained via the Danube into the Black Sea (Keller and Krayss, 2000). Although this connection no longer exists [with the exception of the underground Danube-Aach system; (Hötzl, 1996)], several freshwater fish species have evidently colonized Lake Constance via the Danube (Nesbø et al., 1999; Bernatchez, 2001; Behrmann-Godel et al., 2004; Gum et al., 2005; Barluenga et al., 2006; Vonlanthen et al., 2007; Hudson et al., 2014; Gouskov and Vorburger, 2016; Lucek et al., 2018).

Naturally oligotrophic, Lake Constance experienced intensive eutrophication beginning in the first half of the 20th century and peaking in the 1980's as a result of human population expansion, agriculture, and industry (Petri, 2006). Total phosphorus concentrations began rapidly increasing from the 1930's primarily from agriculture and sewage runoff. Following concerns of environmental degradation and loss of water quality, the International Commission for the Protection of Lake Constance (IGKB) was formed in 1959 by water management organizations of the bordering countries (Petri, 2006). Efforts to reduce phosphorus concentrations and return the lake to near its original oligotrophic state have eventually been successful in the second decade of the 21st century (Petri, 2006; IGKB, 2018). These rapid shifts in nutrient profiles have had strong impacts on the ecosystem, particularly with regards to primary productivity and oxygen availability in the profundal zone (Numann, 1972; Gaedke and Schweizer, 1993; Sommer et al., 1993; Kümmerlin, 1998; Stich, 2004; Stich and Brinker, 2010), which led to the extinction of at least one endemic fish species (Vonlanthen et al., 2012).

Aside from eutrophication, humans have also facilitated the colonization of many invasive species in the lake. Both the upper and lower Lake Constance have been colonized by considerable numbers of non-indigenous species of fish, crustaceans, and molluscs over the past two centuries, some of which have established large populations (Rey et al., 2005; Alexander et al., 2016). As we discuss in more detail below, threespine stickleback are not native to Lake Constance, but are currently hyper-abundant, representing ∼28% of the total fish biomass, and are the second most abundant fish species in the lake (Zimmermann, 2002; Alexander et al., 2016). Large populations of stickleback

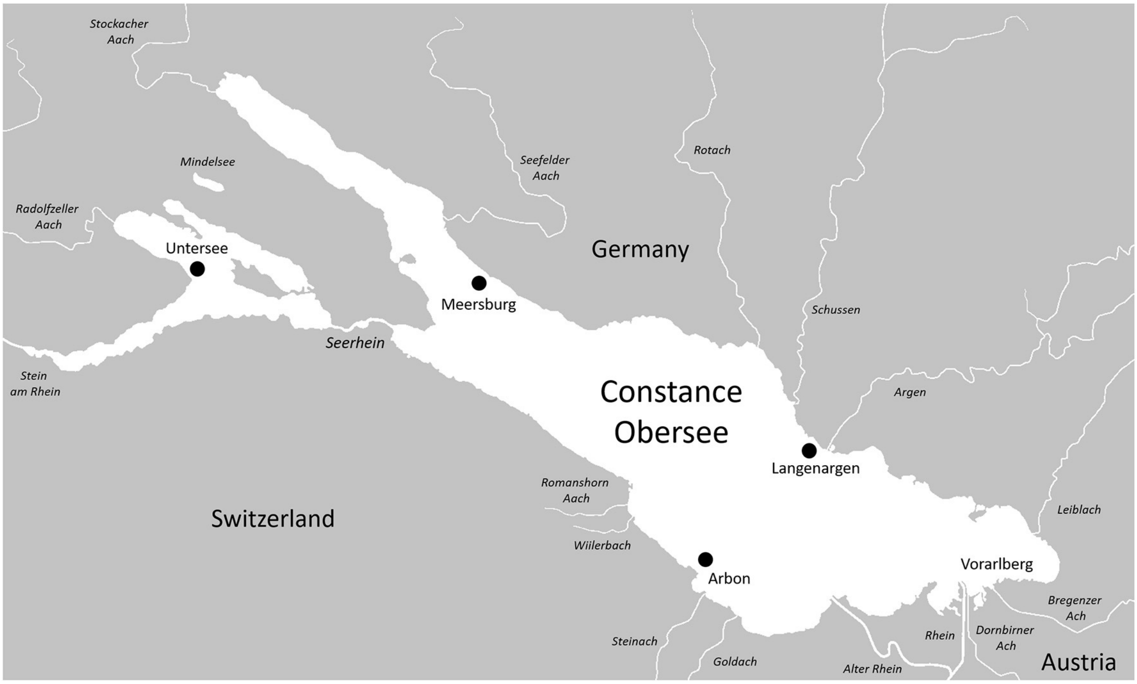


FIGURE 1 | Map of Lake Constance drainage with sampling locations for the 2014 Projet Lac biodiversity sampling campaign. Note the location of Vorarlberg in the southeast of the Lake, where stickleback were first reported by Heller (1871).

are known from other large oligotrophic lakes within their natural range, e.g., in Greenland, Alaska, and the West Coast of Canada (Greenbank and Nelson, 1959; Reimchen, 1992a; Bergersen, 1996; Jeppesen et al., 2017), but such a hyper-abundance is rare in quantitative assessments of lakes that are as large and species rich as Constance. As such, understanding the invasion and establishment of the lake Constance population is also of considerable interest for ecosystem management and conservation.

ORIGINS OF THE THREESPINE STICKLEBACK IN LAKE CONSTANCE

Switzerland lies at the edge of the natural range of threespine stickleback in Europe (Fang et al., 2018). Threespine stickleback historically had a disjunct and very limited distribution in Switzerland, being only represented by two divergent native clades at opposite ends of the country. The first, part of the mainland European and thus Trans-Atlantic clade, was restricted to Rhine tributaries near Basel (Leuthner, 1877; Fatio, 1882; Schulze, 1892; Göldi, 1914) and outside of Switzerland in the Rhine, and parts of Northern France. The second, part of the Adriatic and thus South European clade, was restricted to Lago Maggiore and its tributaries in the Adriatic catchment, and found otherwise in Northern Italy. Recent work suggests that the low plated stickleback of the upper Rhine belong to the same clade as those of Northern France (Mäkinen et al., 2006), which has been referred to as *G. gymnurus*, whereas the low plated stickleback of Lago Maggiore belong to the highly divergent yet taxonomically undescribed South European clade (Mäkinen et al., 2006; Cano et al., 2008; Fang et al., 2018, 2020b).

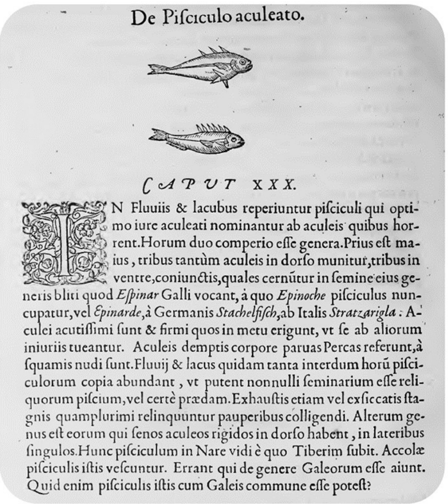
Currently, there are two additional clades that have invaded and colonized Switzerland. First, a lineage colonizing via the middle Rhône that is now dominant in Lake Geneva is a genetically distinctive member of the West European clade (Fang et al., 2018; Marques et al., 2019a). Second, an Eastern European lineage originating from the Baltic Sea Catchment currently dominates Lake Constance (Marques et al., 2019a). However, the history of stickleback in Lake Constance in terms of their time of arrival, source of colonization, and the mode of diversification into lake and stream ecotypes has been debated recently (Roesti et al., 2015; Rösch et al., 2017; Marques et al., 2019a). Notably, some authors proposed a natural postglacial colonization of the Lake Constance basin from the Danube about 9,000 years ago, similar to some other freshwater fish species of which Danube populations inhabit Lake Constance as a consequence of river capture by the Rhine catchment (Nesbø et al., 1999; Bernatchez, 2001; Behrmann-Godel et al., 2004; Gum et al., 2005; Barluenga et al., 2006; Vonlanthen et al., 2007; Hudson et al., 2014; Gouskov and Vorburger, 2016; Lucek et al., 2018). According to this hypothesis, the invading lineage was a stream ecotype that first colonized the tributaries of Lake Constance. These stream populations became isolated from each other because the intermittent lake habitat was presumably ecologically unsuitable (termed "ecological vicariance"). At a later point in time, the lake-adapted population emerged and reconnected previously isolated stream populations through gene flow (Roesti et al., 2015).

Other authors proposed that two or three divergent European lineages of stickleback were introduced into the Lake Constance region in the last 150 years, from catchments south of the Baltic Sea, the upper Rhine, and the Rhône (Lucek et al., 2010; Marques et al., 2019a). In this hypothesis, secondary contact between genetically distinct and previously allopatric lineages resulted in hybridization and variable extents of genetic admixture in different stream populations (Lucek et al., 2010; Roy et al., 2015; Marques et al., 2016, 2019a). The divergent freshwater lineages, including low plated stickleback from Western Europe [i.e., the nominal species referred to as *Gasterosteus gymnurus* (Cuvier and Valenciennes, 1828; Cuvier, 1829)] and a fully plated stickleback from Eastern Europe, formed a hybrid zone across the Swiss midlands including the western end of Lake Constance. As such, the lake populations in the Constance system are predominantly of East European origin, while stream populations around Lake Constance vary in their genetic composition: ranging from predominantly West European with introgression from the East European-derived lake form, to mainly East European-derived with some genomic islands of putative stream adaptation recruited from West European stickleback. In this latter case, introgression of alleles into the East European lineage has probably facilitated ecological speciation into stream vs. lake ecotypes within the East European-derived lineage (Marques et al., 2019a). We review these different hypotheses in the light of the historical ichthyological evidence, phylogeographic, and genomic data.

Historical ichthyological records describe the fish community of Lake Constance dating back to 1557 (Mangolt, 1557). Herein, Mangolt used 76 different common names to refer to the species found in the lake, though many are duplicates or regional names for the same species, of which there are 27 unique species described in total (Ribi, 1942). This text does not include any mention of the threespine stickleback, however he does describe other small fish such as minnows ("*Cyprinus phoxinus*" referring to the genus *Phoxinus*), suggesting that he did not neglect species of the same size as stickleback that have no commercial value. In the same period, an encyclopedia titled *Historiae animalium* (1551–1558) was published by the Swiss naturalist and physician Conrad Gessner (Gessner, 1558) that attempted to describe the animals of the world in detail. Within the *Historiae Piscium & Aquatilium Animantium Natura* (1558) volume, Gessner includes a brief account of the threespine and the ninespine stickleback with text and illustrations that are taken from Guillaume Rondelet's "De Piscibus" (Rondelet, 1554; **Figure 2**) and Albertus Magnus' 13th century text "De Animalibus" (Magnus, 1999; Kitchell Jr. & Resnick translation), referring to them as "Pisiculus aculeatus" and "Pisiculus pungitius," along with other common names. In the corollary added by Gessner, he states that: "They are found elsewhere in Strasbourg [France], Wittenberg [Germany], and in the Alb River [a tributary of the Rhine in Germany]. None are among us." (Gessner, 1558), providing strong evidence that *G. aculeatus* was well-known by natural historians at the time, but absent from Lake Constance. By 1828, Cuvier and Valenciennes write that "Gessner alone says there are [no stickleback] in Switzerland; but we know the opposite" (Cuvier and Valenciennes, 1828), however they do not indicate the regions of Switzerland where stickleback have been found and their information may relate to the native populations from Basel or Lago Maggiore. Further ichthyological descriptions of Lake Constance fish species come from two 19th century fish atlases (Nenning, 1834; von Rapp, 1854) describing 28 and 30 species, respectively [there are presently 42 fish species recognized, of which 11 – including *G. aculeatus* – are introduced (Alexander et al., 2016)], and again make no mention of the stickleback. Given our thorough investigation, and the early interest in describing the fish species of Lake Constance, it seems unlikely that stickleback would have gone unnoticed for so long had the species been present.

The earliest report of stickleback in the region documents their appearance upstream of Lake Constance in Austria just over 150 years ago when they were observed in a tributary to the Alpine Rhine in Vorarlberg (Heller, 1871). In the following years, the presence of stickleback is further noted by both German and Austrian statistical reports (Krafft, 1874; Wittmack, 1875) stating that they are observed in the dead arms of the Alpine Rhine (Wittmack, 1875), and breed in Lake Constance from April to June (Krafft, 1874). Even after these previous sightings, Fehling et al. (1881) writes that "In the river area of the Danube, the stickleback is completely missing, even in Lake Constance it has not been found," suggesting that perhaps the introduced population was at that point in time still isolated in the south-eastern part of the lake and its tributaries. Other documents noted that the stickleback was absent from the upper Danube system until the late 19th century (von Siebold, 1863; Münzing, 1963; Ahnelt, 1986) and most authors agree that the only natural population in Switzerland north of the Alps resided near Basel (**Figure 3**; Schinz, 1837; Wittmack, 1875; Leuthner, 1877; Fehling et al., 1881; Schulze, 1892; Rauther, 1926; Scheffelt, 1926; Muckle, 1972; Ahnelt, 1986; Ahnelt and Amann, 1994; Ahnelt et al., 1998; Paepke, 2002; Altman et al., 2013), that also served as a popular source location of stickleback for aquarium fish traders in Switzerland (Steinmann, 1936). This historical record implies that stickleback were present in the Lake Constance basin before they established in the upper Danube (Vogt and Hofer, 1909; Gaschott, 1941; Berinkey, 1960; Balon, 1967; Ahnelt, 1986; Cakić et al., 2000; Holcik, 2003; Polačik et al., 2008; Lisjak et al., 2015), making a natural colonization from the Danube unlikely.

In the mid-19th century, aquarium keeping emerged in central Europe as a means of popularizing natural sciences (Rossmässler, 1857). Early on, stickleback became popular among European aquarium enthusiasts for their colouration and behavioral displays (Schinz, 1837; Prévost, 1861; Fehling et al., 1881), so it would not be surprising that some unwanted pets would be released into the wild. Indeed, in a previous review of stickleback occurrence in Lake Constance, Muckle (1972) describes a conversation with an aquarist who claims that a fish breeder released some stickleback in a stream that flows into Lake Constance near Allensbach in the late 1920's, originating from a pond in Germany inside the autochthonous range of low plated Rhine stickleback. Muckle (1972) also describes "a release in the years 1933 and 1934 by the "friends of aquaria fish Konstanz" group, of fish imported from a pond near Freiburg im Breissgau [Rhine, Germany]," again within



De Piſciculo aculeato.

CAPUT XXX.

IN Fluuiis & lacubus reperiuntur piſciculi qui optimo iure aculeati nominantur ab aculeis quibus horrent.Horum duo comperio eſſe genera.Prius eſt maius, tribus tantùm aculeis in dorſo munitur,tribus in ventre,coniunctis,quales cernũtur in ſemine eius generis bliti quod *Eſpinar* Galli vocant, à quo *Epinoche* piſciculus nuncupatur,vel *Epinarde*,à Germanis *Stachelfiſch*,ab Italis *Stratzarigla* : Aculei acutiſſimi ſunt & firmi quos in metu erigunt, vt ſe ab aliorum iniuriis tueantur. Aculeis demptis corpore paruas Percas referunt,à ſquamis nudi ſunt.Fluuij & lacus quidam tanta interdum horũ piſciculorum copia abundant , vt putent nonnulli ſeminarium eſſe reliquorum piſcium,vel certè prædam.Exhauſtis etiam vel exſiccatis ſtagnis quamplurimi relinquuntur pauperibus colligendi. Alterum genus eſt eorum qui ſenos aculeos rigidos in dorſo habent , in lateribus ſingulos.Hunc piſciculum in Nare vidi è quo Tiberim ſubit. Accolæ piſciculis iſtis veſcuntur. Errant qui de genere Galeorum eſſe aiunt. Quid enim piſciculis iſtis cum Galeis commune eſſe poteſt?

English translation:

In rivers and lakes a little fish can be found that is named after its sharp spines. I perceive there to be two kinds. The first is larger, protected on the back by only three spines, three in the belly, joined, such as are seen on the seed of a type of Amaranth that the French call *Espinar* [viroflay spinach], which is the reason why the small fish is called *Epinoche* or *Epinarde*, in Germany the *Stachelfisch*, and in Italy the *Stratzarigla*. The sharp and strong spines are raised in alarm, for example to protect themselves from injury. Except for the spines, they resemble a small, scale-less Perch. In rivers and lakes sometimes they are so numerous, that many think they are the origin of the other fish, or at least their prey. When ponds are emptied or dried, many of these fish are left to be collected by the poor. The other species has six spines on its back. I saw this little fish in the river Nar, whence it enters the Tiber. The locals are eating the fish there. Those who say that stickleback fish are a species of Galeorum [catsharks] are mistaken. What can these small fish and the [catsharks] have in common?

FIGURE 2 | Latin descriptions of the threespine and ninespine stickleback from Guillaume Rondelet's 1554 volume "De Piscibus" accompanied by an English translation.

FIGURE 3 | (A) Native low plated specimen belonging to the West European lineage collected in Basel, Switzerland in 1935; **(B)** A fully plated specimen collected in Lake Constance near Langenargen, Germany in 1963; **(C)** A large, fully plated pelagic female captured from Lake Constance near Meersburg, Germany during the 2014 Projet Lac survey.

the native range of low plated Rhine stickleback. Together with the references above regarding introductions to the eastern end of Lake Constance, these historical records provide direct evidence for the introduction of stickleback to the east and west of the lake, and very probably from multiple distinct source populations. Within the same time period that stickleback were first sighted in Lake Constance, a population from the Western lineage of the middle Rhône that was collected from a small stream called Seillon near Vichy was deliberately released into a stream connected to Lake Geneva in Hermance, Switzerland in 1872 by Professor François-Isaac Mayor of Geneva (Fatio, 1882). A few decades later, further introductions and releases were documented in the Lake Neuchâtel catchment and in the upper Rhône upstream of Lake Geneva in the early 20th century (Blanc, 1922; Bertin, 1925).

More recently, analyses of genomic data have been central to the debate over the source location(s), the phenotype of the founding population(s), and timing of stickleback colonization in Lake Constance (Roesti et al., 2015; Marques et al., 2016, 2019a). The ecological vicariance scenario's (Roesti et al., 2015) proposal of a natural colonization of Lake Constance via the upper Danube drainage, was motivated by the genetic and phenotypic similarity between Lake Constance and contemporary upper Danube populations (Moser et al., 2012; Roesti et al., 2015). However, it is noteworthy that there is evidence that these latter populations were themselves also introduced (Ahnelt, 1986; Ahnelt and Amann, 1994). Based on demographic modeling of population genomic data and assuming that the lake and stream populations originated from a single colonization of the Constance system, a colonization time as far back as ~9,000 years ago was estimated, suggesting an early split between lake and stream ecotypes within the Constance catchment (Roesti et al., 2015). Basal placement of stream populations in a phylogeny, increased linkage disequilibrium, and extended selective sweep signatures in lake stickleback genomes were further interpreted in support of this scenario (Roesti et al., 2015). According to this scenario, the Constance stickleback population would represent a natural range expansion from regions close to the Black Sea to freshwater following Pleistocene glacial retreat 12 Kya (McPhail, 1994; McKinnon and Rundle, 2002). Such a colonization route is in principle, plausible, because geological and biological evidence indicates that the Danube drainage was previously connected to Lake Constance (Nesbø et al., 1999; Keller and Krayss, 2000; Bernatchez, 2001; Behrmann-Godel et al., 2004; Gum et al., 2005; Barluenga et al., 2006; Vonlanthen et al., 2007;

Hudson et al., 2014; Gouskov and Vorburger, 2016; Lucek et al., 2018). Today, the only possible corridor connecting the two drainages is the underground Danube-Aach system, which makes contemporary natural colonization by surface water dwelling fishes implausible. However, an early postglacial colonization of threespine stickleback is at odds with the historical ichthyological records described above.

A recent integrative analysis of the phylogeography and demography of Lake Constance basin stickleback in a broader European-wide context (Marques et al., 2019a) supports the hypothesis involving secondary contact between divergent, previously allopatric lineages. Individuals from the Lake Constance catchment possess mitochondrial haplotypes belonging to at least three divergent stickleback lineages contemporarily found in four main Central European river catchments draining into the Baltic Sea, the North Sea, the Mediterranean Sea, and the Black Sea. This is in stark contrast to the ecological vicariance model, where a single threespine stickleback lineage would have colonized the Black Sea from the Mediterranean Sea through the Bosphorus strait after its formation roughly 7,000 ybp (Gökaşan et al., 1997; Fang et al., 2018), continuing into the Danube, and onward into Lake Constance. If this were the case, we would expect Lake Constance stickleback to exhibit a high frequency of Black Sea haplotypes (Mäkinen and Merilä, 2008; DeFaveri et al., 2012; Lucek and Seehausen, 2015; Sanz et al., 2015; Vila et al., 2017), but such haplotypes are absent in both the Lake Constance catchment as well as in the upper Danube. Instead, the lake population itself is dominated by a haplotype from the Baltic Sea catchment in Poland (Lucek et al., 2010). Further mitochondrial analyses revealed the presence of four additional mitochondrial haplotypes within the Lake Constance catchment, in stream habitats at low to moderate frequencies (Lucek et al., 2010; Moser et al., 2012; Marques et al., 2019a). These haplotypes are otherwise known from populations native to the North Sea, the Rhine catchment, and the Rhône catchment (Mäkinen and Merilä, 2008; Lucek et al., 2010; Marques et al., 2019a). This secondary contact model suggests that introduced stickleback from two or three ancient European lineages met in the Constance basin and have introgressed, facilitating differentiation between lake and stream ecotypes via retention of historical lineage differentiation and/or likely adaptive recruitment of western lineage alleles into stream populations (Marques et al., 2019a). Genome-wide RAD-sequencing data showing admixture between Rhine, Rhône, and Baltic lineages in Lake Constance, a higher Rhine and Rhône ancestry in stream stickleback, and an enrichment of genomic islands of differentiation for Rhine lineage alleles in stream ecotypes support this view (Marques et al., 2019a).

Phenotypic analyses also support the secondary contact scenario. Stickleback native to the Rhine and Rhône originating from natural colonization from the Sea (Lucek et al., 2010), including Rhine populations from Basel (**Figure 3**), were historically fixed for the low plated phenotype (Fatio, 1882; Münzing, 1963), prior to hybridization and introgression with the introduced Eastern European lineage (Lucek, 2016). In contrast, Baltic Sea freshwater populations were fixed for the fully plated phenotype until recent introductions of low plated fish from Western Europe (Bańbura, 1994) and the likely introduced freshwater populations in the upper and middle Danube which contain a mix of low and fully plated morphs (Ahnelt, 1986). Lake Constance is dominated by fully plated stickleback, while the populations in inlet streams to the North and West of the lake are polymorphic with high frequencies of low plated fish. In contrast, streams to the south of Lake Constance have low frequencies of low plated fish. These phenotypic patterns are in line with the inferences from mitochondrial and genomic data of an origin of lake stickleback from the Baltic region and an admixed origin of stream stickleback north and west of the lake, supporting the secondary contact hypothesis.

In summary, the historical ichthyological evidence, phylogeographic analysis in a European context, and demographic modeling of genomic data, all suggest that the most plausible scenario for the origin of stickleback in Lake Constance is that beginning in the late 1800's stickleback were introduced by aquarium hobbyists or fishermen from multiple sources (Heller, 1871; Fatio, 1882; Steinmann, 1936; Muckle, 1972) that represent at least three different European lineages and a minimum of three different introductions (Marques et al., 2019a). Following these introductions, the lake population then underwent an expansion, becoming abundant by the 1960's (Laurent, 1972; Numann, 1972; Deufel, 1985; Zimmermann, 2002; Alexander et al., 2016), and has experienced fluctuations in density over the past 50 years. This debate, and growing support for the "secondary contact scenario," reveals the value of integrative analyses of invasion dynamics that include inferences about historical fish occurrences from ichthyological records (where available), historical colonization pathways from geomorphological evidence, and both demographic history and phylogeographic patterns from genomic data.

In the following sections, we review general patterns of ecological and evolutionary diversification in the *G. aculeatus* species complex, and develop contemporary parallels associated with the invasion of Lake Constance.

EXAMINING THE LAKE CONSTANCE STICKLEBACK POPULATION FROM A GLOBAL PERSPECTIVE

Their historical and geographic origins notwithstanding, Lake Constance stickleback are unique among central European freshwater populations in that a high number of individuals are foraging in the pelagic zone of the lake, and appear to be phenotypically adapted for a lifestyle in large pelagic environments. A lake-wide fish diversity survey of Swiss lakes, conducted in 2014, demonstrated that lacustrine stickleback are not only hyper-abundant within Lake Constance (**Table 1**), but are also distributed along a variety of habitats throughout the water body, with some individuals found foraging in the profundal zone as deep as 40 m (Alexander et al., 2016). Similar distribution patterns in freshwater lakes are only known from Lake Michigan, where stickleback invaded the ecosystem on a comparable time scale, occupy qualitatively similar depth ranges (Stedman and Bowen, 1985), and consume a diet that is mostly zooplankton and mysids (Turschak and Bootsma, 2015). During the breeding season, lacustrine stickleback have been observed to spawn both within lakes along the shoreline, and in adjoining streams (Snyder, 1991; Harvey et al., 1997; Lucas et al., 2001). We observe this pattern in Lake Constance as well, though it remains to be investigated whether these resident and potamodromous lacustrine fish constitute a panmictic population or are reproductively isolated. Potamodromous individuals migrate in large numbers several kilometers up into streams in April, where the males build nests and where they breed before they leave the streams again in May/June, much like anadromous marine stickleback. These potamodromous individuals demonstrate genomic differentiation from stream residents in both sympatry and parapatry (Marques et al., 2016), and differ from the stream populations in an array of phenotypic traits. Lake fish have elongated gill rakers and a distinct head shape that allows feeding on small planktonic prey, as opposed to stream resident individuals, which feed predominantly on larger benthic prey (Berner et al., 2010; Lucek et al., 2012, 2013; Moser et al., 2012, 2015; Karvonen et al., 2015; Roesti et al., 2015; Marques et al., 2016, 2019a). Lake and stream ecotypes also differ in defensive morphology, with lake stickleback possessing elongated spines and a set of tall bony lateral plates that cover most of the body, whereas stream resident fish have shorter spines and a reduced plate coverage which they achieve by having either fewer, or shorter, lateral plates (Berner et al., 2010; Moser et al., 2012; Lucek et al., 2013, 2014b; Marques et al., 2016). Lastly, the ecotypes differ in life history, where lake resident and potamodromous fish live longer and start to reproduce on average 1 year later than stream residents (Lucek et al., 2012; Moser et al., 2012, 2015). Lake-stream divergence has been reported elsewhere in Switzerland (and in other locations globally; **Table 2**) but is much less pronounced in other large Swiss lakes (Lucek et al., 2013, 2014b). This suggests that naturally colonized systems and anthropogenic introductions of threespine stickleback have the underlying process of ecotypic differentiation in common, but that the rate at which phenotypic and genetic divergence occur, and its dimensionality, are system specific.

In a recent assessment, sticklebacks in Lake Constance represent ∼28% of the fish biomass, and accounted for 96% of fish captured in the pelagic zone of the upper lake during the Projet Lac survey (Alexander et al., 2016). Despite being identified as hyper-abundant from the 1960's to the 70's (Numann, 1972), and again in littoral habitats almost two decades ago (Zimmermann, 2002) threespine sticklebacks were first recorded as bycatch in the pelagic zone of Constance by commercial fisheries in 2013 (Rösch et al., 2017). Previous reports (Numann, 1972; Deufel, 1985) indicate that stickleback have been a nuisance to fishermen in the past, with populations reaching high abundances, but then declining rapidly, presumably from parasitic infections (e.g., whitespot disease *Ichthyophthirius multifiliis* and carp louse *Argulus foliaceus*), though evidence of this is largely speculative.

The massive recent increase in stickleback abundance coincides with a sharp decline in pelagic whitefish (*Coregonus wartmanni*, Bloch, 1784) yields, both in the number of individuals caught, and their weight-at-age (Rösch et al., 2017). Previous work has speculated that the invasive stickleback population could have a negative impact on whitefish growth and abundance, and shows that stickleback will prey on whitefish larvae in laboratory foraging experiments (Roch et al., 2018; Ros et al., 2019) or following stocking (Roch et al., 2018). However, the first stickleback population expansion during the eutrophication period in Constance coincides with population size increase in whitefish (Numann, 1972), so the relationship between whitefish and stickleback abundances is either mediated by some other factors in the environment, or it is not causal. It has been proposed that either competition for pelagic zooplankton resources such as *Daphnia* - that have declined in abundance with the re-oligotrophication of Lake Constance (Straile and Geller, 1998; Stich and Brinker, 2010; Rösch et al., 2017) - or direct predation on whitefish eggs and larvae (Roch et al., 2018; Ros et al., 2019) are responsible for this reduction in yield. Predation by sticklebacks on eggs and juveniles of their own species occurs frequently (Whoriskey and FitzGerald, 1985; Hyatt and Ringler, 1989; Smith and Reay, 1991; Foster and Bell, 1994; Manica, 2002; Mehlis et al., 2010) along with predation on larvae of other fish species (Hynes, 1950; Manzer, 1976; Delbeek and Williams, 1988; Kean-Howie et al., 1988; Gotceitas and Brown, 1993; Nilsson, 2006; Kotterba et al., 2014; Byström et al., 2015), while previous studies on stickleback populations in the Baltic Sea have suggested that intraguild predation on eggs and juvenile fish is responsible for the observed declines in perch (*Perca fluviatilis*, Linnaeus, 1758) and pike (*Esox lucius*, Linnaeus, 1758) recruitment (Nilsson, 2006; Bergström et al., 2015; Byström et al., 2015; Nilsson et al., 2019; Eklöf et al., 2020). It is possible that the same is occurring with Lake Constance whitefish populations, although evidence for this is currently lacking. Our analysis of stickleback gut contents (see below) did not detect any whitefish eggs or larvae in wild stickleback, nor did other studies (Lucek et al., 2012; Moser et al., 2012; Roch et al., 2018), though intraspecific egg predation was observed. However, no study to date has sampled lacustrine stickleback during or shortly after whitefish spawning season, so the hypothesis about whether direct predation on whitefish eggs by invasive stickleback is responsible for population declines requires further testing.

For piscivorous predators, increased stickleback abundance may provide a new food source that could be particularly important for overwintering birds. In a similar fashion, the presence of introduced zebra mussels in Lake Constance (*Dreissena polymorpha*, Pallas, 1771) has resulted in a 4-fold increase in overwintering molluscivorous waterbird densities since their introduction in the 1960's (Werner et al., 2005). Following environmental protection legislation under the EU Birds Directive, a breeding population of great cormorants (*Phalacrocorax carbo*, Linnaeus, 1758) has established in lower Lake Constance since 1997, and has grown steadily despite population culling (Gaye-Siessegger, 2014). Both cormorant, and great crested grebe (*Podiceps cristatus*) populations have increased since 2010, and it has been suggested that the abundance of stickleback in the lake is responsible for this change (Werner et al., 2018). Stomach content analysis of great cormorants from lower Lake Constance between 2011 and 2013

TABLE 1 | Stickleback caught during the Projet Lac sampling campaign, and other Swiss lakes where they are known to occur but were not captured.

Lake	Information	Stickleback catch per unit effort (Projet Lac)	Habitats occupied by stickleback
Constance - upper	Projet Lac	2,527	Littoral/benthic/limnetic (< 47 m)
Constance - lower	Projet Lac	249	Littoral/benthic/limnetic (< 42 m)
Biel	Projet Lac	63	Littoral/benthic (< 5 m)/limnetic (< 25 m)
Geneva	Projet Lac	24	Littoral/benthic (< 10 m)
Lucerne	Projet Lac	2	Littoral (1.5 m)
Maggiore	Projet Lac	1	Littoral (1.6 m)
Murten	(Zaugg and Huguenin, 2018) + EAWAG	0	Littoral
Neuchâtel	(Zaugg and Huguenin, 2018) + EAWAG	0	Littoral
Zug	(Zaugg and Huguenin, 2018)	0	Littoral
Zurich	(Zaugg and Huguenin, 2018)	0	Littoral

during the autumn and winter seasons showed that 19.1% of birds had eaten stickleback, and 24.3% of fish eaten were threespine stickleback. This indicates that cormorants frequently target *G. aculeatus*, although they only contribute a small proportion of total diet content by weight (Gaye-Siessegger, 2014). A later study (Rey and Becker, 2017) documented an increase in the proportion of cormorant stomachs containing stickleback in comparison to the previous survey, with 39% of individuals hunted in the spring of 2016 containing *G. aculeatus*. Thus, it is possible that these abundant prey items in the pelagic zone (Eckmann and Engesser, 2018) are particularly beneficial when other species of dietary importance, such as *Perca fluviatilis*, move to deeper water to overwinter (Wang and Eckmann, 1994; Eckmann and Imbrock, 1996). Many other avian species in the region such as grebes, herons, mergansers, kingfishers, gulls, and terns are known to consume threespine stickleback (Foster and Bell, 1994; Werner et al., 2018), so we may observe an increase in their abundance, or changes in migration patterns as well in response to increased pelagic stickleback densities in the future.

In addition to their high abundance, broad habitat use, and interactions with other species, one of the most compelling characteristics of the Lake Constance stickleback is their exceptional body size. In freshwater, *G. aculeatus* typically attains between 30 and 80 mm in standard length (SL) (Wootton and Wootton, 1976; Foster and Bell, 1994) and has an average lifespan of 2–4 years (Pennycuick, 1971; Moodie, 1984; Baker, 1994). Stickleback caught in Lake Constance during the Projet Lac survey demonstrated large body sizes and complete defensive complexes (**Figure 3**), with the largest individual measuring 101 mm in SL (Alexander et al., 2016; **Figure 4**). Since many individuals captured during this survey were above the typical size range of the species, we compiled data from our own work, along with published sources on body size distributions of freshwater stickleback populations from around the globe (**Figure 4**). This data shows that their large body size is not necessarily unique for the species, but that Lake Constance individuals are larger than those from most other European freshwater populations. There are two other regions where freshwater stickleback have been measured at comparable body sizes, the Haida Gwaii archipelago (British Columbia, Canada), and Lake Towada, Japan. In Haida Gwaii, stickleback in a small number of very distinctive populations or species (the giant threespined stickleback) have been observed to grow up to 106 mm in SL (Gambling and Reimchen, 2012) and can live to be 8 years old (Reimchen, 1992a), while in an introduced Japanese population in Lake Towada females with a SL > 100 mm have been collected (Mori and Takamura, 2004). Furthermore, marine individuals of threespine stickleback have been reported at sizes up to 110 mm (Muus and Nielsen, 1999), and evidence from the fossil record suggests that such "gigantism" in stickleback has evolved previously, with fossilized individuals measured at 110 mm (Bell, 1984). Large body size hence is repeatedly observed but uncommon in the *G. aculeatus* species complex, especially in freshwater populations.

What could be responsible for this pattern of gigantism, and how do Lake Constance stickleback compare to other systems? For Haida Gwaii, Gambling and Reimchen (2012) suggest that large body size has evolved in order to escape gape-limited piscivores that are abundant in the ecosystem, but this is not the case in Lake Towada because there are no predatory fish (Mori and Takamura, 2004). Instead, Mori and Takamura (2004) suggest that either an abundant supply of planktonic prey or greater fish longevity are responsible for the large sizes they observed, although they did not directly measure stickleback ages in their study. In Lake Constance stickleback the majority of lake breeding fish are 2 years of age when they first reproduce (Moser et al., 2012), but fish of 3 years of age are also common and occasionally they are older (Lucek et al., 2012; Moser et al., 2012). There is some experimental evidence that predation pressure on Swiss stickleback populations can select for faster growth rates (Zeller et al., 2012), and that the lake population in Constance grows faster than one of the stream populations when reared on limnetic prey, although they grow at a similar rate when reared on benthic prey (Lucek et al., 2012). This latter experiment suggests that the lake ecotypes are better adapted to grow quickly on limnetic prey than are stream ecotypes. However, whether the larger body sizes of the lake populations are due to adaptations allowing sustained rapid growth under limnetic food resources, or the result of selection for larger body size as a mechanism to escape predation is still unknown.

TABLE 2 | Mechanisms of ecotype formation in threespine stickleback.

Ecotypes	Mechanism of selection	Location	Publications
Lava and nitella	Predation by arctic char, substrate background matching	Iceland	Kristjansson et al. (2002), Doucette et al. (2003), Ólafsdóttir and Snorrason (2007, 2009), Millet et al. (2013)
Lake and stream	Divergent selection in trophic niche and habitat use	Throughout European and North American populations	Lavin and Mcphail (1993), Hendry et al. (2002), Hendry and Taylor (2004), Berner and Grandchamp (2009), Deagle et al. (2012), Kaeuffer et al. (2012), Moser et al. (2012), Lucek et al. (2013), Ravinet et al. (2013), Feulner et al. (2015), Marques et al. (2016), Hanson et al. (2017), Stuart et al. (2017), Paccard et al. (2019), Rennison et al. (2019)
Benthic and limnetic	Divergent selection in trophic niche and habitat use	Pacific North West, Canada and Alaska	Bentzen and McPhail (1984), McPhail (1984, 1994), Schluter and McPhail (1992), Foster and Bell (1994), Baker et al. (2005), Gow et al. (2008), Willacker et al. (2010), Østbye et al. (2016)
White and common	Sexual selection, assortative mating associated with male color polymorphism, lack of parental care in white ecotype	Atlantic North East, Canada	Blouw and Hagen (1990), Haglund et al. (1990), Jamieson et al. (1992a,b); Macdonald et al. (1995), Blouw (1996), Samuk et al. (2014), Haley et al. (2019)
Benthic and limnetic	Female preference for male shape and size promotes sexual isolation between ecotypes	Pacific North West, Canada	Head et al. (2013)
Benthic and limnetic	Female preference for male nuptial coloration in different light environments	Pacific North West, Canada	Boughman (2001)
Blackwater and clearwater	Predation and sexual selection, aquatic light environment influences color vision evolution	Pacific North West, Canada	Hagen and Moodie (1979), Reimchen (1989), Flamarique et al. (2013), Rennison (2016), Marques et al. (2017b)
Red and orange nuptial colouration morphs	Female preference for male throat colouration promotes sexual isolation between morphs	Switzerland	Feller et al. (2016), Marques et al. (2017a)
Brackish and oceanic	Reduced gene flow along thermal and salinity gradients	Baltic Sea	deFaveri et al. (2013), Guo et al. (2015)
Benthic and limnetic	Differential predation pressure produces divergent body pigmentation between ecotypes	Pacific North West, Canada	Gygax et al. (2018)
Lake and stream	Sexual selection, females use male MHC olfactory cues to assortatively mate	Germany	Eizaguirre et al. (2011), Andreou et al. (2017)
Plate morphs	Calcium availability, salinity, and predation regime	North Uist, Scotland	Giles (1983), Cresko et al. (2004), Spence et al. (2012, 2013), Magalhaes et al. (2016)
Adaptive radiation in body size and defensive complex	Predation regime, ecosystem size, and light spectrum	Pacific North West, Canada	Reimchen et al. (2013)

Understanding variation in dietary niche and metabolism might yield insights into the uniqueness of the Constance population with respect to European and Global populations. In Swiss populations, there is some evidence for differences in the dietary niche between the West European and East European lineages, but insufficient data to quantitatively compare dietary niche variation between the native and introduced range. These lineages have colonized freshwater independently, with populations diverging in the late Pleistocene (Fang et al., 2020b) or early Holocene (Marques et al., 2019a), many thousands of years before their introduction to Swiss lakes and before any known instances of secondary contact. As a result, the lineages have a different evolutionary history of adaptation to freshwater environments. In light of this, previous work has suggested that the Constance population has a more pelagic phenotype and feeds more efficiently on plankton than the population of Lake Geneva (Best et al., 2017), which originates from the West European Lineage (Fang et al., 2018) that invaded Switzerland from the middle Rhône. Indeed, in our analysis of stomach contents from 253 individuals, following similar methods as Lucek et al. (2012), and Anaya-Rojas et al. (2016), and presented here for the first time, we confirm that individuals in both

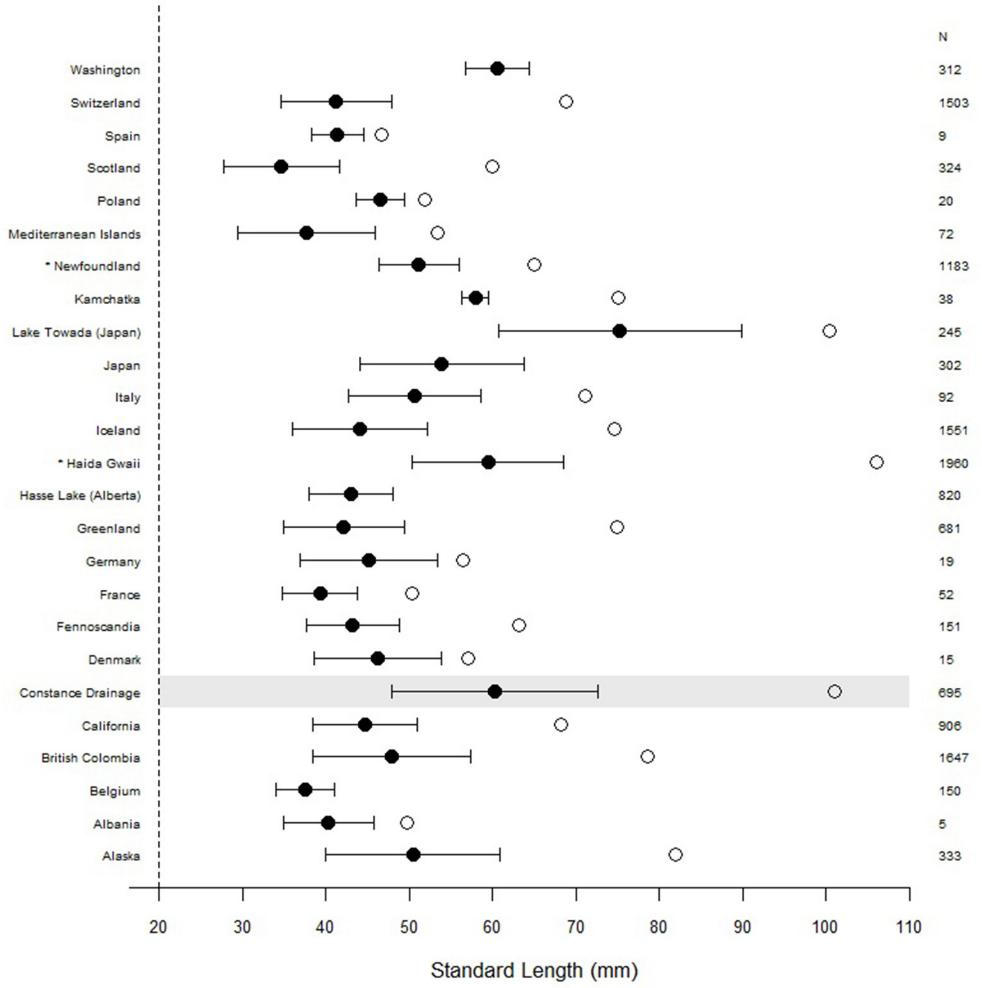


FIGURE 4 | Mean standard length of stickleback captured in the Constance drainage (highlighted in gray) with standard deviation compared to body sizes of other freshwater populations around the globe. Open circles represent maximum body sizes, when available from the data. We obtained body size data from published studies (**Supplementary Table 1**) and calculated means for each region by pooling all individuals and both sexes. For studies that did not provide raw data, we re-calculated mean and standard deviation values by combining means between studies using standardized methods (Altman et al., 2013). *Haida Gawii populations are represented by the top 10 largest individuals of each sex from 98 populations (Reimchen et al., 2013), and thus the distribution is right-skewed, Newfoundland populations are measured as total length and not SL.

upper and lower Lake Constance are predominantly feeding on plankton (e.g., cladocerans and copepods; **Figure 5**), and a low proportion of chironomid larvae. We also found some evidence for within-lake dietary niche variation between the upper and lower lake (**Figure 5**): Individuals caught in the lower lake consumed a higher proportion of bosmina (two-tailed z-test, $z = 2.45$, $p = 0.012$; upper lake $N = 232$; lower lake $N = 21$) compared to those from the upper lake. Furthermore, within the upper lake, pelagic individuals consumed a higher proportion of bythotrephes than benthic individuals did (two-tailed z-test, $z = 2.8$, $p = 0.005$; benthic $N = 133$, pelagic $N = 99$). More of such comparative dietary work is needed in other large lakes in order to test how niche variation within populations in large lakes (e.g., Lake Geneva) compares among lineages in the natural and invaded range.

There is also compelling evidence for differences in the extent of metabolic adaptation by the two stickleback lineages to the lower average food quality of freshwater compared to marine prey. During the colonization of freshwater by marine species, organisms need to adapt to an environment where essential fatty acids are low in abundance (Arts et al., 2009). When encountering nutritional constraints, organisms can evolve metabolic or ecological adaptations to overcome this environmental scarcity. In freshwater fish, *in vivo* biosynthesis of long-chain fatty acids by desaturation of short-chain derivatives is performed by enzymes produced by the *Fads2* gene (Castro

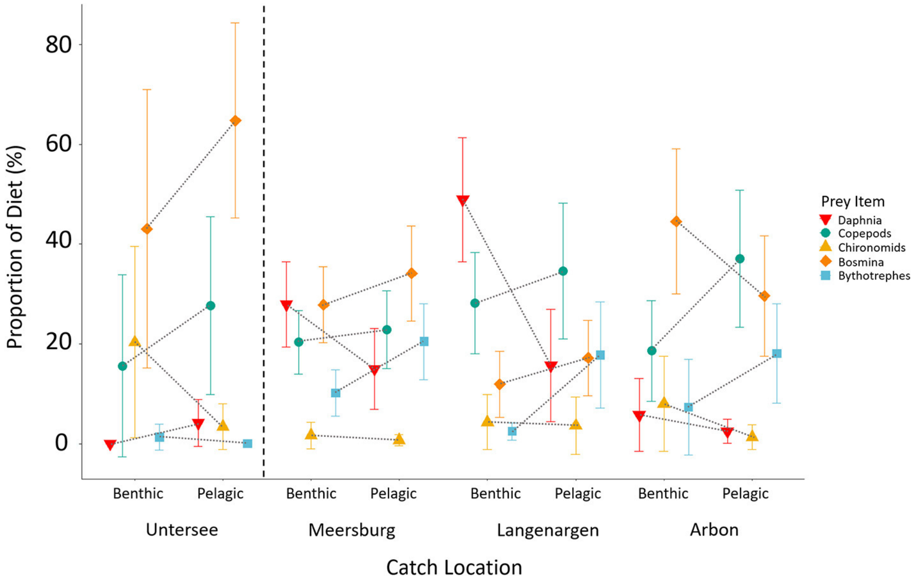


FIGURE 5 | Gut content proportions of the top five most common prey items in Projet Lac stickleback split by region and catch location. Individuals caught in the benthic habitat were those caught <25 m from the lakebed, while individuals caught in the pelagic habitat were captured >25 m. Stickleback caught in Untersee have a higher proportion of Bosmina in their diet, and within Obersee fish caught in deeper pelagic regions had a higher proposition of Bythotrephes in their diet (see main text).

et al., 2012). *Fads2* has recently been identified as a key metabolic gene for several freshwater fish species in that they exhibit multiple independent gene duplications across different lineages, and this suggests that copy number variation is under positive selection for its role in long chain fatty acid biosynthesis (Ishikawa et al., 2019). From the same study, an investigation of copy number variation in European freshwater stickleback populations showed that the Western European lineage possesses higher copy numbers of *Fads2* than the population in Lake Constance (Ishikawa et al., 2019), implying that they are capable of more efficient metabolic desaturation and elongation of polyunsaturated fatty acids. It is possible that this has enabled the Western lineage to persist in environments with low food quality such as stream or benthic lake littoral habitats. If organisms are incapable of fatty acid biosynthesis, another evolutionary strategy is to adapt their morphology and behavior to more efficiently prey on food sources that are rich in essential nutrients. A planktonic diet can provide these essential nutrients [i.e., fatty acids; (Smyntek et al., 2008)], although some of the high quality prey items are also evasive (e.g., copepods). Based on the gut content data and the prolific use of the open water habitat, it is possible that Lake Constance stickleback are well-adapted to exploit the abundant zooplankton populations in Lake Constance, and this may help them compensate for the fewer copies of *Fads2* that they possess in comparison to the Western lineage.

PUTTING THE INVASION OF LAKE CONSTANCE INTO PERSPECTIVE FOR DRAINAGES IN SWITZERLAND

Invasive species vary widely in both their ability to invade, and their impact following invasion (Williamson and Fitter, 1996; Zenni and Nuñez, 2013). In the case of stickleback in Switzerland, success of establishment varies within the introduced taxon: the population of stickleback in Lake Geneva seems to be much less dominant than the population in Lake Constance, despite similar timescales of invasion. Stickleback are present in many of the large freshwater bodies within Switzerland, including Lakes Biel, Neuchâtel, Lucerne, and Geneva, where they range from exceedingly rare (Lucerne) to locally common (Geneva) but are hyper-abundant only in Lake Constance. Here we will discuss this phenomenon in the context of the two largest lakes, Constance and Geneva. Both are large peri-alpine lakes that harbor introduced populations of stickleback of similar age (Heller, 1871; Fatio, 1882) so why are there striking differences in stickleback abundance between the two? During peak eutrophication in the 1980's stickleback were similarly abundant in both lakes (Laurent, 1966, 1972; Numann, 1972), but this is no longer the case. It is only after re-oligotrophication that Geneva populations have declined, while Constance populations have become hyper-abundant again (Alexander et al., 2016). As the ecosystems are similar in some respects, such as lake depth, size, and community composition, and the time since colonization is

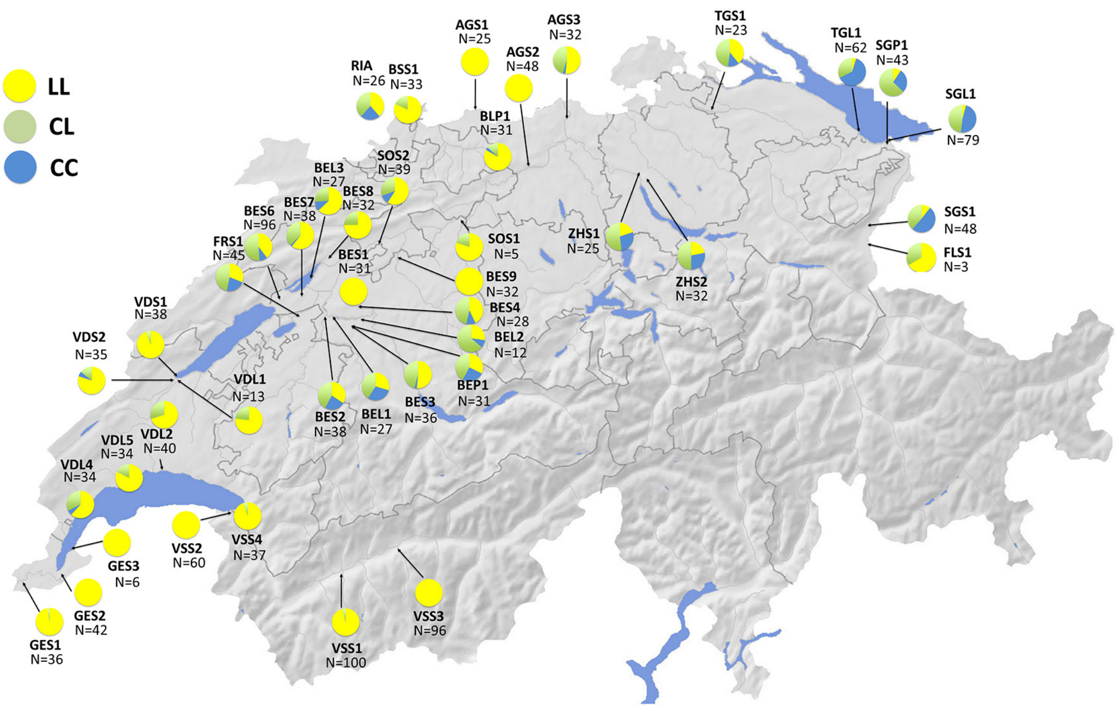


FIGURE 6 | Ectodysplasin (*Eda*) STN382 allele frequency distributions across Swiss stickleback ($n = 1{,}598$) populations ($n = 42$) generated by KL as in Lucek et al. (2010). Yellow (LL) = homozygous for the low plated allele; Green (CL) = heterozygous individuals; Blue (CC) = homozygous for the complete plated allele. Information on site codes and capture locations can be found in **Supplementary Table 2**.

roughly the same, differences between the colonizing lineages in phenotype, genetic makeup, or ecology may be responsible for this pattern.

As discussed above, we know that stickleback populations in Switzerland are made up of several divergent European lineages that vary in their evolutionary history in freshwater, and that these lineages differ both phenotypically and genotypically (Mäkinen and Merilä, 2008; Lucek et al., 2010; Moser et al., 2012; Fang et al., 2018, 2020b; Marques et al., 2019a). Stickleback in Geneva are genetically dominated by a freshwater lineage from the middle Rhône (Mäkinen and Merilä, 2008; Marques et al., 2016, 2019a; Fang et al., 2018) while those in Constance most likely originate from the Baltic Sea drainage of Eastern Europe (Lucek et al., 2010). The Geneva population is phenotypically similar to West European stream stickleback, while the Constance population has a pelagic phenotype and is fully plated. Gene flow into Lake Geneva from the East European lineage has recently introduced the fully plated Ectodysplasin (*Eda*) allele into the population of Lake Geneva where it seems to be under positive selection in the lake but not in the streams (Lucek et al., 2014a). This introgression of the fully plated *Eda* allele has likely occurred through the large hybrid zone that spans the Swiss plateau between Lake Constance and Lake Geneva, and here we present new data on stickleback populations genotyped for the STN382 allele (**Figure 6**) as in Lucek et al. (2010) using the protocols of Colosimo et al. (2005). The spread of this allele suggests that genetic contributions from the East European lineage may increasingly permit adaptation to exploitation of pelagic habitats in other invaded lakes as well (Lucek et al., 2014a). Conversely, introgression from the West European lineage (largely from Rhine populations) into Lake Constance has been found among stream populations (Marques et al., 2019a), but not in the lake population. Thus, it is likely that we are observing an inverse scenario of invasion and secondary contact between the two lakes, lineages, and freshwater habitat types.

When we compare these two lakes, genetic constraints (e.g., limiting genetic variation due to drift at the invasion front or the lack of required adaptive genetic variation for the colonization of a specific habitat) may have limited the invasiveness of each introduced population to colonize multiple habitat types initially, but this constraint was alleviated by hybridization at each invasion front (Lucek et al., 2014a; Marques et al., 2019a). In other words, we have evidence that hybridization between East and West European lineages in Lake Constance has enabled the colonization of stream habitats (Marques et al., 2019a), but we know much less about how introgression of Eastern European alleles might facilitate a habitat expansion of the Lake Geneva population from littoral habitats into the pelagic zone. Further testing of this phenomenon within other Swiss lakes in the hybrid zone (e.g., Lakes Biel and Neuchâtel) could be fruitful, and may reveal similar patterns.

CONCLUSIONS

Invasive species allow us to observe the process of evolution on an ecological timescale, as non-native organisms adapt to new environmental conditions. Whether they are ecologically detrimental or not, we can use invasions to explore how organisms from one genetic background perform in an environmental circumstance that differs from that at its origin. Multiple introductions and admixture through hybridization can provide new genetic material for selection to act upon, and teach us about the consequences of secondary contact for adaptation, divergence and associated ecosystem impact. Here we have a system with multiple recent introductions, rapid population divergence, and the potential for large ecosystem effects in an area that has already experienced intense human habitat alteration. The Swiss stickleback system provides a model to study multiple axes of ecological diversification in threespine stickleback, and insights from this system can be applied both to the global threespine stickleback radiation in particular, and to our understanding of invasive species in general. We observe rapid parallel diversification along the lake-stream ecotype axis, and threespine stickleback have become hyper-abundant within the pelagic zone of Lake Constance, now representing one of the most common species in the lake. Whether the lacustrine population has begun to diversify into genetically distinct groups along the benthic-limnetic or lake resident-migratory axes, additional to the lake-stream axis is currently unknown and remains to be investigated. Furthermore, as hybridization has occurred between the same introduced stickleback lineages in other regions of Switzerland, we may see adaptive population divergence and possibly the evolution of invasiveness in other Swiss lakes as well.

AUTHOR CONTRIBUTIONS

OS started the long-term research on invasion biology of stickleback in Switzerland and on the ecology, genomics of hybridization, and ecotype formation in Lake Constance. OS, BM, CH, and TA conceived of the review. KL, DM, MM, TA, BM, and OS collected data and assisted CH in producing figures and tables. CH analyzed the data and led the writing of the manuscript with assistance from BM, KL, DM, TA, and OS. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.611672/full#supplementary-material

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Mapping the Establishment and Invasiveness Potential of Rainbow Trout (*Oncorhynchus mykiss*) in Turkey: With Special Emphasis on the Conservation of Native Salmonids

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Rainbow trout (*Oncorhynchus mykiss*) has become by far the most frequently farmed freshwater fish species in Turkey, whereas very little is known about its establishment and invasiveness potential. We explored this potential through a combination of Maxent habitat suitability model and the Aquatic Species Invasiveness Screening Kit (AS-ISK) on the river basin scale by generating an overall risk score (ORS). The outcome of this approach was also incorporated with the spatial analysis of native salmonid species by generating a relative vulnerability score (RVS) to prioritize susceptibility of native species (or populations) and to propose risk hotspots by identifying their potential geographic overlap and interaction with *O. mykiss*. Results suggest that the northern basins (Eastern Black Sea, Western Black Sea and Marmara basins) are the most suitable basins for *O. mykiss*. According to the Basic Risk Assessment (BRA) threshold scores, *O. mykiss* is classified as "high risk" for 3 (12.0%) of the 25 river basins screened (Western Black Sea, Eastern Black Sea and Maritza-Ergene), and as "medium risk" for the remaining basins. The climate change assessment (CCA) scores negatively contributed the overall invasiveness potential of *O. mykiss* in 22 (88.0%) of the river basins and resulted in zero contribution for the remaining three, namely Aras-Kura, Çoruh river and Eastern Black Sea. The ORS score of river basins was lowest for Orontes and highest for Western Black Sea, whereas it was lowest for Konya-closed basin and highest for Eastern Black Sea, when CCA was associated. The micro-basins occupied by *Salmo rizeensis* had the highest mean habitat suitability with *O. mykiss.* Among the all species, *S. abanticus* had the highest RVS, followed by *S. munzuricus* and *S. euphrataeus.* The overall outcome of the present study also suggests that the establishment and invasiveness potential of

O. mykiss may decrease under future (climate warmer) in Turkey, except for the northeast region. This study can provide environmental managers and policy makers an insight into using multiple tools for decision-making. The proposed RVS can also be considered as a complementary tool to improve IUCN red list assessment protocols of species.

Keywords: aquaculture, aquatic species invasiveness screening kit, Maxent habitat suitability model, climate change, invasiveness potential

INTRODUCTION

The spectrum of the impacts of invasive species on overall biodiversity is so wide that ranges from behavioral changes of native species to entire collapsing of ecosystem services and extinction of native taxa (Charles and Dukes, 2008; Simberloff et al., 2013; Gallardo et al., 2016). The economic burden is also significant, reaching billions of dollars all over the world, particularly in the United States, Australia and Western Europe (Pimentel et al., 2005; Hoffmann and Broadhurst, 2016; Bertolino et al., 2020). Therefore, environmental agencies have struggled to manage the introduction and impact of invasive species by strengthening their legislations and policies for nature conservation and socio-economic issues globally (Copp et al., 2005a). The most important step of this endeavor is to predict species' invasiveness– the capacity of a species to reproduce, spread from its place of introduction, and establish in new locations (Rejmánek, 2011)-, and which sources of introductions are likely to become more prominent under current and future climatic and socio-economic conditions (Rahel and Olden, 2008; Banerjee et al., 2019; Raffini et al., 2020). Escape from farms, breeding/cultivation facilities, gardens or laboratories into natural systems constitute important and unregulated sources of introductions by which animals and plants become invasive (Hulme et al., 2008; Essl et al., 2015). Concordantly, the majority of aquatic inland species introductions are considered as escapees from aquaculture, which have resulted in serious aquatic invasions worldwide (Ju et al., 2020). This pathway is even more crucial for countries like Turkey where aquaculture predominantly depends on the farming of non-native species and represents approximately 25% (by value) of the total production of the fishery sector (TUIK, 2014). This pathway is also highly important due to Turkey's current efforts to align with international quality standards (e.g., European Commission, 2014).

In Turkey, inland fisheries constitute a considerable proportion of the total fish production both by capture (5%) and aquaculture (44%) and are concentrated in inland lakes (including reservoirs) and the coastal lagoons (FAO, 2019). Aquaculture dates back to the late 1960s (Lök et al., 2018) with farming of rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*), with non-native *O. mykiss* being the most frequently farmed aquaculture species over the past few decades. According to the up-to-date aquaculture statistics provided by the Turkish General Directorate of Fisheries and Aquaculture, the species is now being farmed in 74 of the 81 provinces with ∼1,550 farms which are mostly located in inland waters (dataset available at https://www.tarimorman.gov.tr/BSGM/Belgeler/Icerikler/Su%20%C3%9Cr%C3%BCnleri%20Yeti%C5%9Ftiricili%C4%9Fi/Su-Urunleri-Tesisleri-2019.pdf). On one hand, *O. mykiss* has been listed as one of the 100 worst invasive alien species identified globally by the IUCN (Lowe et al., 2000) and ranks high in the list of the top 18 fish species that cause severe ecological impacts, compiled from establishment and impact assessment data contained in FISHBASE (Casal, 2006). Therefore, the impact mechanisms of the species, which can be summarized as competition/monopolizing resources, hybridization with native salmonids and predation, are very well-documented (Scott and Irvine, 2000; Weigel et al., 2003; Stanković et al., 2015). On the other hand, a recent review of risk screenings conducted for the eastern Mediterranean region (Vilizzi et al., 2019), indicated that the potential of *O. mykiss* to be a highly invasive species is lower (i.e., yielded moderate risk) than suggested by previous risk assessments where future climate change may negatively affect its spread and impact. In a recent study, the threats posed by *O. mykiss* in Greece has also been reported to be lower than suggested by recent risk assessments (Koutsikos et al., 2019), though its self-sustaining population has become established on the Island of Crete (Stoumboudi et al., 2017). In contrast, Hasegawa (2020) demonstrated that brown trout in some Japanese islands show higher invasiveness than that was suggested in previous studies, and this was associated with the similarities in the life histories of native salmonids in Japan. These results, therefore, indicate that the current approaches in risk assessments tend to under- or overestimate the potential risk of salmonids, probably due to the lack of integrated quantitative habitat and niche suitability (or species distribution) and climate change models. Furthermore, selecting coarse scales for the risk assessments (e.g., biogeographical, climatic, or country scale) especially for geographically heterogeneous risk assessment (RA) areas (e.g., Turkey) may result in under- or overestimated risk scores.

Although *O. mykiss* has had a relatively long history in aquaculture in Turkey (Tarkan et al., 2015), escapes from farms, which are known to occur very frequently, have been poorly documented. Also, little is known on whether escapes have resulted in viable (or resilient) populations in natural habitats. These incidents are of great importance especially in Turkey, which covers the entire natural distribution of at least 13 native trout species, 6 of which are classified as threatened, 6 are not evaluated and only one is classified as least concern by the International Union for Conservation of Nature (IUCN). Despite this rich diversity of native salmonids in Turkish freshwaters, there remains a paucity of research exploring the habitat suitability and establishment risk of *O. mykiss,* which may help to address its potential spatial overlap and interaction with native salmonids. Previous studies on the effects of *O. mykiss* aquaculture in Turkey have been focused on water quality of natural waters around the facilities (Demir et al., 2001; Cromey et al., 2002; Brooks et al., 2003), and there is only one study reported some adverse impacts of escapee rainbow trout in some streams (Biga peninsula, Çanakkale, NW Turkey) on native ichthyofauna through abundance, diet preferences and intraspecific feeding interactions (Ertürk-Gürkan and Yalçin Özdilek, 2019).

The main goal of this study was therefore 2-fold: to evaluate the establishment and invasiveness potential of *O. mykiss* in Turkey on the river basin scale, and to identify its geographic overlap probability with the native salmonid species of conservation concern. The first step was addressed by predicting the establishment potential of non-native *O. mykiss* in Turkey following the habitat suitability approach using the Maxent species distribution modeling, and by assessing its invasiveness potential across the main river basins of Turkey using the Aquatic Species Invasiveness Screening Kit (AS-ISK). By collecting and assessing the available occurrence records of farm escapements, this study also has the potential to contribute to fill up the common gaps by minimizing possible inconsistencies between current and future invasiveness of species and current and projected risk assessments.

The second objective was met by conducting spatial analysis for documenting the distribution of native salmonids and for examining the suitability of their natural geographic range against *O. mykiss*. It is a well-known fact that cost-effective conservation of species requires prioritization. Therefore, to prioritize and categorize the sensitivity of native salmonids against the overlap and interaction (including hybridization) with rainbow trout, we proposed a metric by blending the benefited risk assessment approach with the spatial analysis of the native trout species. By this way, hotspots for potential hybridization and antagonistic interactions between the non-native *O. mykiss* and the native trout species are also proposed. The overall outcome of the present study is also expected to provide environmental managers and stakeholders insight into the potential use of AS-ISK with habitat suitability models as an integrated set of decision-support tools for informing policy makers and for suggesting management options for the potential, existing and future undesired non-native fishes of aquaculture importance.

MATERIALS AND METHODS

Habitat Suitability Model

Since *O. mykiss* is a non-native species in Turkey, and no established populations have been officially confirmed yet, the distribution data of the species which is needed for calibration of the habitat suitability was downloaded from the Global Biodiversity Information Facility (GBIF, 2020). The spatial data represented the natural distribution of *O. mykiss*, which extends from Alaska to Mexico in the northwest of North America (Behnke, 1979; Crawford and Muir, 2008) (**Figure 1**). The occurrence data is comprised of 94,275 georeferenced records from 4,476 unique locations which were compiled from 40 published datasets covering this native range. To model the habitat suitability, common topographical and climatic parameters within BasinATLAS database were used and seven environmental variables were significant in terms of explaining the habitat suitability of *O. mykiss*. These variables are average annual minimum temperature, average annual maximum temperature, average annual temperature, slope, aridity index, elevation and precipitation. All these variables were examined on the basis of hydrological subunits (i.e., micro-basin scale provided by the BasinATLAS Attributes) and the highest resolution of sub basin division (level 12) (Lehner and Grill, 2013) were used. Commonly used species distribution model Maxent (Phillips et al., 2006, Version 3.3.3) was employed to generate the habitat suitability of *O. mykiss* across Turkey with 10-fold cross-validation method. Maximum iterations were set to 10,000, random test percentage was 20, and maximum number of background points were set as 10,000. For each run, 2,974 presence records were used for training, 743 records for testing and 12,590 points were used to determine distribution including both background points and presence points. First, the model was calibrated for North America (native range) for the occurrence data downloaded from GBIF and then, the calibrated model was projected for habitat suitability of *O.*

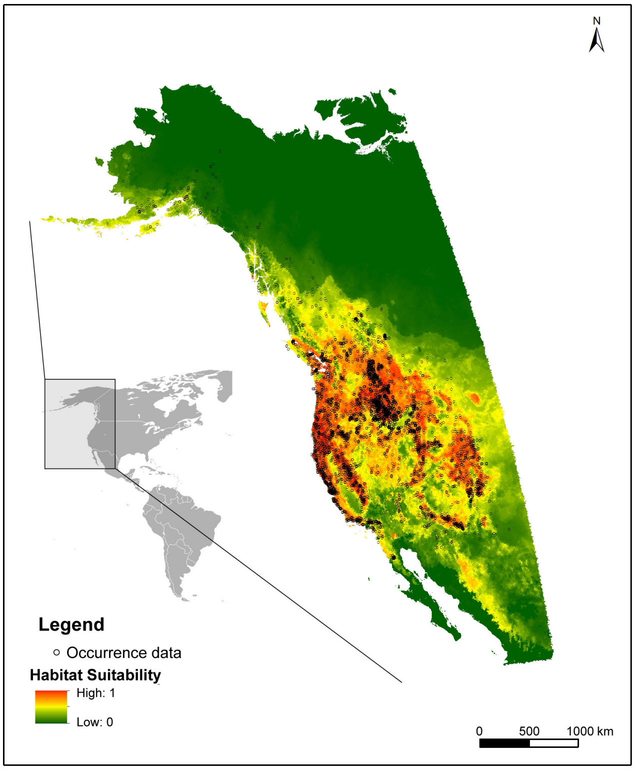

FIGURE 1 | Occurrence data and the output of the habitat suitability map of *Oncorhynchus mykiss* in calibration phase. GBIF Occurrence Download. https://doi.org/10.15468/dl.r7ht8c.

mykiss in Turkey. Multivariate Environmental Similarity Surfaces (MESS, Elith et al., 2010) analysis, which were also embedded in Maxent software were applied to assess the similarity of the predictor variables between the training dataset (United States) and projection dataset (Turkey). The accuracy of the results was evaluated by True Skill Statistics (TSS) and the area under the receiver operating characteristic (ROC) curve (AUC), which are commonly used as threshold-independent measures for model performance (Manel et al., 2001; Allouche et al., 2006). These model evaluation statistics range between 0 and 1 for AUC and between −1 and 1 for TSS, where AUC and TSS values close to 1 indicates excellent model performances.

Screening of Invasiveness

Aquatic Species Invasiveness Screening Kit (AS-ISK) v2.2 (available at www.cefas.co.uk/nns/tools) was used to identify the invasiveness potential of *O. mykiss*. AS-ISK is a risk screening decision-support tool that is fully compliant with the "minimum standards" (Roy et al., 2018) for the assessment of non-native species for the European Commission Regulation on the prevention and management of the introduction and spread of invasive alien species (European Union, 2014). This decision-support tool has already been used successfully to screen non-native fishes in several RAs including biogeographic (Glamuzina et al., 2017; Tarkan et al., 2017a) and climatic risk RAs (Dodd et al., 2019). AS-ISK consists of 55 questions, which are grouped into 49 Basic Risk Assessment (BRA) and six Climate Change Assessment (CCA) questions to assess the biogeographical and historical traits of the taxon and its biological and ecological interactions. The BRA questions have two main sections: the Biogeography/Historical and the Biology/Ecology that are complemented by an additional six questions asking the assessor to assess how predicted future climate conditions are likely to affect their responses to Qs related to the risks of introduction, establishment, dispersal and impact. For each question in AS-ISK, the assessor must provide a response, justification and level of confidence. The screened species eventually receives both a BRA and a BRA+CCA (composite) score, which respectively range from −20.0 to 68.0 and from −32.0 to 80.0 (Dodd et al., 2019). AS-ISK scores < 1.0 suggest that the species is unlikely to become invasive in the RA area and is therefore classified as "low risk" whereas higher scores classify the species as posing either a "medium risk" or a "high risk" of becoming invasive. The ranked levels of confidence (1 = low; 2 = medium; 3 = high; 4 = very high) associated with each response in AS-ISK mirror the confidence rankings recommended by the International Programme on Climate Change (IPCC, 2005).

Turkey is divided into 25 main hydrological units which herein we called the river basins. For *O. mykiss*, AS-ISK assessments were undertaken at the river basin level to increase the resolution of the scores as much as reasonably possible. The assessments were carried out by a subset of co-authors, whose knowledge of the species is specialized to a certain region, namely the western basins were assessed by AST, the central and northern basins by BY, and the eastern and north-eastern basins by CK. All assessments then were peer-reviewed by all authors. A literature survey was carried out pertinent to the species and the RAs to obtain valid evidences for each of the question in the risk assessment panel. Although there is extensive peer-reviewed literature on the species itself, we mainly utilized review papers with relatively broader extent and some common databases (e.g., FishBase and Global Invasive Species Database) in which respectable and goal-directed information are available. Information on the general climate and future predictions inherent in the CCA questions were based on the Köppen–Geiger climate classification system (Peel et al., 2007).

The calibrated AS-ISK threshold score (i.e., 27.75) established for categorization of potential risk status of non-native freshwater fish species (i.e., distinguishing between high risk from low-to-medium risk) for whole Turkey (Tarkan et al., 2017b) was used in the present study for *O. mykiss* in the assessed river basins. However, as no BRA+CCA threshold in Tarkan et al. (2017a) was provided, Receiver Operating Characteristic (ROC) curve analysis (Bewick et al., 2004) was used to assess the predictive ability of AS-ISK to discriminate between non-native freshwater fish species posing a high risk and those posing a medium or low risk of being invasive for the RA area. For ROC curve analysis to be implemented, species need to be categorized a priori in terms of their documented invasiveness (i.e., non-invasive or invasive). Since this information is readily available in Tarkan et al. (2017a), we used it for a new threshold value calculation of BRA+CCA. Briefly, a ROC curve is a graph of sensitivity vs. 1 – specificity (or alternatively, sensitivity vs. specificity) for each threshold value, where in the present context sensitivity and specificity will be the proportion of a priori invasive and non-invasive species, respectively, that are correctly identified by AS-ISK as such. The Area Under the Curve (AUC) known as a measure of the accuracy of the calibration analysis typically ranges from 0.5 to 1, and the closer to 1 the greater the veracity in differentiating between invasive and non-invasive species. When the test is 100% accurate, AUC is equal to 1. Because both sensitivity and specificity are 1, and there are neither "false positives" (a priori non-invasive species classified as high risk, hence invasive) nor "false negatives" (a priori invasive species classified as low risk, hence non-invasive). Conversely, when the test is 0% accurate it means the AUC is equal to 0.5, as it cannot discriminate between "true positives" (a priori invasive species classified as high risk, hence invasive) and "true negatives" (a priori non-invasive species classified as low risk, hence non-invasive).

Following ROC analysis, the best AS-ISK threshold value that maximizes the true positives rate and minimizes the false positives rate was determined using Youden's J statistic; whereas, the "default" threshold of 1 was set to distinguish between low risk and medium risk species (Copp et al., 2005b). ROC analysis was carried out with package pROC (Robin et al., 2011) for R x64 v4.0.0 (R Core Team, 2020) using 2000 bootstrap replicates for the confidence intervals of specificities, which were computed along the entire range of sensitivity points (i.e., 0–1, at 0.1 intervals). The ranked levels of confidence (1 = low; 2 = medium; 3 = high; 4 = very high) associated with each response in AS-ISK mirror the confidence rankings recommended by the International Programme on Climate Change (IPCC, 2005; see also Copp et al., 2016a). Based on the confidence level (CL) allocated to each response for a given basin, a confidence factor

(CF) is obtained as:

$$\sum (CL_{Qi}) \ / \ (4 \ x \ 55) \ (i \ = \ 1, \ \ldots, \ 55)$$

where $\mathrm{CL}_{\mathrm{Q}i}$ is the CL for question i (Qi); 4, is the maximum achievable value for confidence (i.e., very high: see above) and 55 is the total number of questions comprising the AS-ISK questionnaire. Based on the BRA and the CCA only, the $\mathrm{CL}_{\mathrm{BRA}}$ and $\mathrm{CL}_{\mathrm{CCA}}$ are also computed. The CF ranges from a minimum of 0.25 (i.e., all 55 questions with CL equal to 1) to a maximum of 1 (i.e., all 55 questions with CL equal to 4). Two additional CFs are also computed, namely the $\mathrm{CF}_{\mathrm{BRA}}$ and the $\mathrm{CF}_{\mathrm{CCA}}$, as per the CL.

Overall Assessment

We employed an integrated approach in which both habitat suitability and the invasiveness potential are jointly considered in evaluating the overall risk of *O. mykiss*. A common way for estimating overall risk exposure is to multiply probability and impact. Here, we consider habitat suitability (Maxent scores) as the establishment probability of *O. mykiss*; whereas, we considered invasiveness screening outputs as the impact. To obtain the possible maximum resolution to incorporate establishment probability and the invasiveness screening was to estimate the habitat suitability and to screen the invasiveness potential on the same scale. To this end, we selected each river basin as RA area, and performed AS-ISK separately for all river basins. After that, to calculate the descriptive statistics of Maxent habitat suitability values for each of the river basin, we aggregated the micro-basin level habitat suitability values. Therefore, the overall risk score (ORS) of basin "i" is calculated by the following equation;

$$\mathrm{ORS}_i = \mathrm{Maxent}_i \ \times \ \mathrm{AS\text{-}ISK}_i$$

Where "Maxent_i" is the average habitat suitability of *O. mykiss* for basin "i", and "$\mathrm{AS\text{-}ISK}_i$" is the invasiveness screening score of *O. mykiss* for basin "i". We generated two ORSs for each basin, one is based on BRA ($\mathrm{ORS}_{\mathrm{BRA}}$) and the other is on BRA+CCA ($\mathrm{ORS}_{\mathrm{BRA+CCA}}$) scores.

To propose risk hotspots and to prioritize native endemic trout, we estimated their extent of occurrence (EOO) by calculating the area (km^2) of minimum convex polygon around all present native occurrences of a species. We also took the number of independent populations (IP) of a species into account. IP was primarily determined by considering the number of hydrologically independent river catchments that actually occupied by the native trout species. Secondly, each of the discrete (or isolated) populations within the same river catchment, if available, are regarded as IP. Following this, we complemented the assessment of their occurrence ranges with the habitat suitability of *O. mykiss*. For this, the habitat suitability of *O. mykiss* was estimated for the all of the micro-basins which bear at least one site-scale record of a native salmonid species. The EOO calculations of the native species were based on these site-scale records and achieved using the spatial analysis module of RAMAS Red List Pro software (Akçakaya and Root, 2007). To avoid overestimating the EOOs for the species with multiple discrete populations that dispersed over different river basins, EOOs were calculated separately for each river basin and then were summed up to obtain the species' EOO. The occurrence datasets of the native trout species were derived from the available recent literature (Turan et al., 2010, 2011, 2012, 2014a,b; Turan et al., 2017, 2020; Yoğurtçuoğlu et al., 2020). The confirmed occurrences of *O. mykiss* from natural water bodies was also compiled from both published literature and data obtained by our own field explorations, which were dotted on the habitat suitability map together with the occurrence of native salmonids in order to visually match the model outcome and the real escape cases of *O. mykiss*.

To prioritize the native salmonid species and their habitats against the potential of establishment and invasiveness of *O. mykiss*, we proposed a Relative Vulnerability Score (RVS) based upon blending the ORS, Maxent outputs and the spatial range of the native species. RVSs were calculated for two ORSs i.e., both with BRA based ORS ($\mathrm{RVS}_{\mathrm{BRA}}$) and BRA+CCA based ORS ($\mathrm{RVS}_{\mathrm{BRA+CCA}}$). If a species occurs in more than one river basin, RVSs were calculated separately for each of those basin. Therefore, RVS of species i in basin a (RVS_{ia}) was calculated by the following equation;

$$\mathrm{RVS}_{ia} = (\mathrm{ORS}_a \ x \ \frac{\mathrm{MB}_1 \ + \ \mathrm{MB}_2 \cdots \mathrm{MB}_{ni}}{ni}) \ / \ \log\,(\mathrm{EOO}_i \ \mathrm{x} \ \mathrm{IP}_i);$$

Where MB_{ni} is the mean Maxent value of the micro-basins that contain at least one site-scale record of species i in basin a. EOO_i is the extent of occurrence of species i, and IP_i is the number of independent populations of species i. The extreme difference between EOO × IP values of the widespread and localized species was down-weighted by applying a log-transformation. After detecting the outliers (scores outside 1.5 times the interquartile range), we simply considered the mean of the RVSs that were calculated by excluding outliers as the threshold i.e., distinguishing between high and low vulnerability. Finally, we highlighted and proposed the micro-basins that are occupied by a native species with high RVS as the hotspots for potential antagonistic interactions with *O. mykiss*.

Statistical Analyses

Differences between mean confidence levels of Basic Risk Assessment (BRA) and Climate Change Assessment (CCA) (in AS-ISK) were tested by permutational analysis of variance (PERANOVA) based on a one-factor design with two levels: BRA and CCA (note that testing of the same differences for the Confidence Factor leads to the same results as the two measures differ only by a constant).

Significant difference among the mean Maxent suitability of the micro-basins of the native species was also tested by PERANOVA based on a one-factor design with 13 levels (all native species) (The dataset for Maxent descriptive data of microbasins and related statistical design is deposited in **Supplementary Data sheet 3**).

PERANOVA was carried out in PERMANOVA+ for PRIMER v6, where we normalized the data and used an Euclidean distance

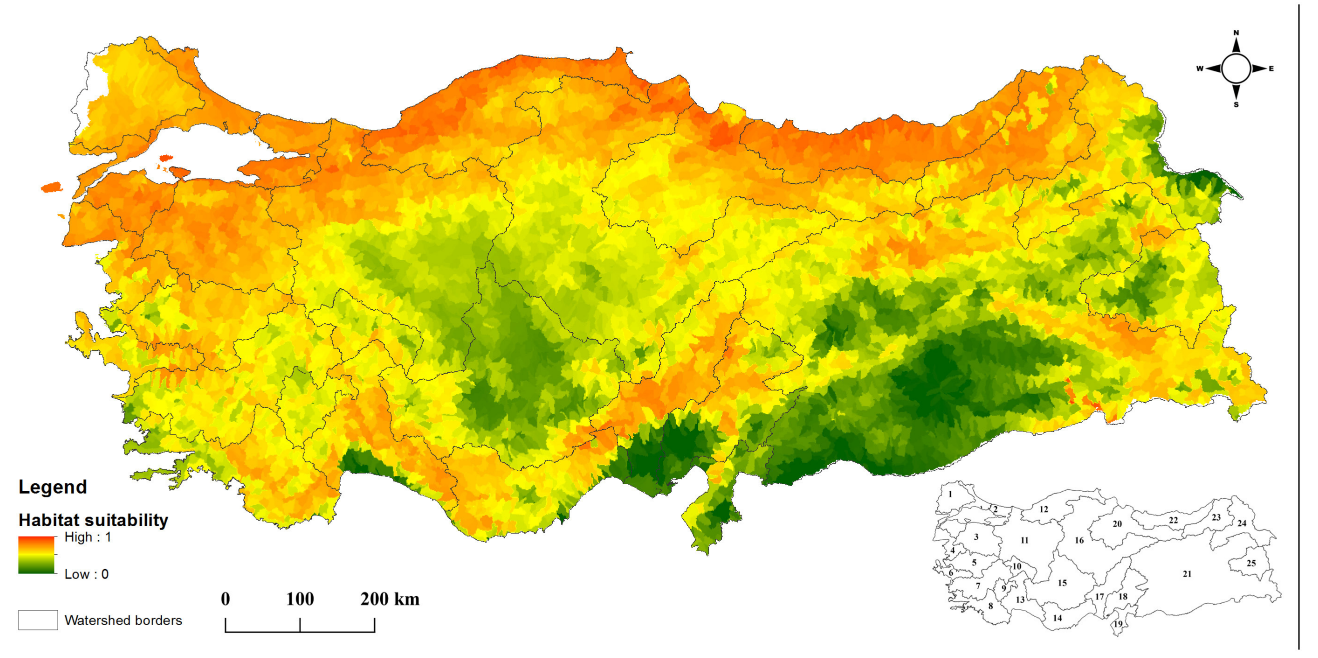

FIGURE 2 | Habitat suitability map of *Oncorhynchus mykiss* in Turkey. The inset at the bottom-right corner shows the codes for 25 river basins namely 1, Maritza-Ergene; 2, Marmara; 3, Susurluk; 4, North Aegean; 5, Gediz; 6, Small Menderes; 7, Great Menderes; 8, Western Mediterranean; 9, Burdur; 10, Akarçay; 11, Sakarya; 12, Western Black Sea; 13, Antalya; 14, Eastern Mediterranean; 15, Konya Closed Basin; 16, Kizilirmak; 17, Seyhan; 18, Ceyhan; 19, Orontes; 20, Yeşilirmak; 21, Euphrates and Tigris; 22, Eastern Black Sea; 23, Çoruh; 24, Kura and Aras; 25, Lake Van Basin.

measure (for BRA-CCA); or square rooted the data and applied a Bray-Curtis dissimilarity measure (for micro-basins). Both analyses ran with 9999 unrestricted permutations of the raw data (Anderson et al., 2008) with statistical effects evaluated at $\alpha = 0.05$.

RESULTS

Habitat Suitability

The output of the Maxent model for *O. mykiss* habitat suitability during the calibration phase is given in **Figure 1**. There is a good concordance between the occurrence data and model outputs and AUC (area under ROC curve) value for training 0.82, while it was 0.83 for testing (the ROC curve simulation results were depicted in **Supplementary Figure 1**). Average TSS results for 10 replicate runs was also calculated to be 0.54. The most significant variables determining the distribution of *O. mykiss* were average annual minimum temperature (59.5%), slope (12.2%) and average annual maximum temperature (9.7%). Considering the results of the MESS analysis, similarity between the environmental variables of the training dataset and projected dataset was >0 for all microbasins, indicating native range data was suitable for modeling the potential distribution of *O. mykiss* in Turkey (see also the map of the MESS analysis in **Supplementary Figure 2**). Hence, the calibrated model was extended to project the habitat suitability of *O. mykiss* across the river basins in Turkey and the output was given in **Figure 2**. Given the average Maxent values, the most suitable river basins for *O. mykiss* were predicted as the northern basins namely Eastern Black Sea, Western Black Sea and Marmara basins (**Figure 2**). According to the model, Orontes river basin, Konya-closed

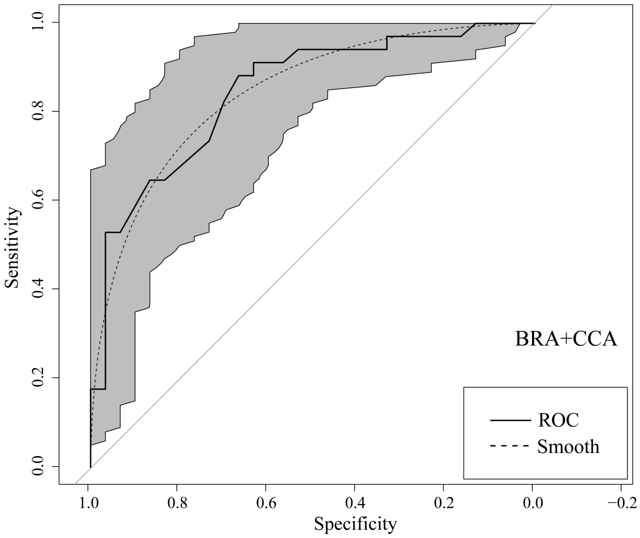

FIGURE 3 | Receiver Operating Characteristic (ROC) curve (solid line) for BRA+CCA (Climate Change Assessment) of the non-native freshwater fish species screened with the Aquatic Species Invasiveness Screening Kit (AS-ISK) for Turkish freshwaters. Smoothing line and confidence intervals of specificities (gray area) are also provided. The raw data analyzed is taken from Tarkan et al. (2017a).

TABLE 1 | *Oncorhynchus mykiss* screened with Aquatic Species Invasiveness Screening Kit (AS-ISK) for each of the river basins, i.e., risk assessment (RA) areas, that comprise Turkey, numbered (in [ ]) as per **Figure 2**.

Basin	**Assessment component**				**Confidence**						
	BRA		**BRA+CCA**		**CL**				**CF**		
	Score	**Outcome**	**Score**	**Outcome**	**Delta**	**Total**	**BRA**	**CCA**	**Total**	**BRA**	**CCA**
Akarçay [10]	14.0	Medium	8.0	Medium	−6.0	2.8	2.8	2.7	0.69	0.69	0.67
Antalya [13]	21.0	Medium	15.0	Medium	−6.0	2.8	2.8	2.0	0.69	0.71	0.50
Aras and Kura [24]	21.0	Medium	21.0	Medium	0.0	2.4	2.4	2.2	0.59	0.60	0.54
Burdur [9]	15.0	Medium	9.0	Medium	−6.0	2.8	2.8	2.7	0.70	0.70	0.67
Ceyhan [18]	18.0	Medium	14.0	Medium	−4.0	2.7	2.8	2.2	0.68	0.69	0.54
Çoruh [23]	24.0	Medium	24.0	Medium	0.0	2.6	2.6	3.0	0.65	0.63	0.75
Eastern Black Sea [22]	28.0	High	28.0	Medium	0.0	2.5	2.5	3.0	0.62	0.61	0.75
Eastern Mediterranean [14]	19.0	Medium	13.0	Medium	−6.0	2.7	2.8	2.0	0.68	0.70	0.50
Euphrates and Tigris [21]	27.5	Medium	23.5	Medium	−4.0	2.5	2.6	2.0	0.63	0.64	0.50
Gediz [5]	18.0	Medium	16.0	Medium	−2.0	2.9	2.9	2.5	0.71	0.72	0.63
Great Menderes [7]	17.5	Medium	13.5	Medium	−4.0	2.9	2.9	2.5	0.71	0.72	0.63
Kizilirmak [16]	21.0	Medium	13.0	Medium	−8.0	2.8	2.8	2.5	0.69	0.69	0.63
Konya [15]	16.0	Medium	6.0	Medium	−10.0	2.7	2.8	2.2	0.68	0.69	0.54
Marmara [2]	27.0	Medium	21.0	Medium	−6.0	2.9	3.0	2.2	0.72	0.74	0.54
Meriç-Ergene [1]	28.0	High	24.0	Medium	−4.0	2.8	2.9	2.3	0.71	0.72	0.58
North Aegean [4]	19.0	Medium	19.0	Medium	0.0	2.9	2.9	2.5	0.72	0.73	0.63
Orontes [19]	18.0	Medium	14.0	Medium	−4.0	2.7	2.8	2.2	0.68	0.70	0.54
Sakarya [11]	24.5	Medium	16.5	Medium	−8.0	2.8	2.8	2.2	0.69	0.70	0.54
Seyhan [17]	20.0	Medium	14.0	Medium	−6.0	2.7	2.8	2.0	0.68	0.70	0.50
Small Menderes [6]	13.5	Medium	11.5	Medium	−2.0	2.8	2.9	2.5	0.71	0.72	0.63
Susurluk [3]	22.5	Medium	18.5	Medium	−4.0	2.9	2.9	2.5	0.72	0.73	0.63
Van [25]	23.5	Medium	19.5	Medium	−4.0	2.5	2.5	2.0	0.62	0.63	0.50
Western Black Sea [12]	31.0	High	29.0	Medium	−2.0	2.8	2.9	2.5	0.70	0.71	0.63
Western Mediterranean [8]	24.5	Medium	20.5	Medium	−4.0	2.9	2.9	2.7	0.73	0.73	0.67
Yeşilirmak [20]	22.5	Medium	18.5	Medium	−4.0	2.7	2.8	2.3	0.68	0.69	0.58

Basic Risk Assessment (BRA) and BRA plus Climate Change Assessment (BRA+CCA) scores and corresponding risk outcome rankings, difference (Delta) between BRA+CCA and BRA, Confidence Level (CL) and Confidence Factor (CF) for all questions (Total) and separately for the BRA and CCA questions are given. Risk outcomes for the BRA are based on a threshold of 27.75 ("Medium" for 1 to 27.75 and "High" for >27.75) and for the BRA+CCA on a threshold of 34.5.

basin and the lower parts of Tigris & Euphrates basin (the southeast of Turkey) were the least suitable regions for *O. mykiss.*

AS-ISK

The receiver operating characteristic curves for the Basic Risk Assessment+ Climate Change Assessment resulted in an AUC of 0.85 (0.75–0.94 95% CI) (**Figure 3**). These AUCs indicated that AS-ISK was able to discriminate between non-invasive and invasive *O. mykiss* for the assessed basins. Youden's J provided a threshold of 34.5 for the BRA+CCA, which was used for calibration of the AS-ISK risk outcomes. Accordingly, the BRA+CCA threshold allowed to distinguish between medium risk species with scores within [1.0, 34.5[, and high-risk species with scores within]34.5, 80.0]. Species classified as low risk were those with BRA+CCA scores within [−32.0, 1.0[(note the reverse bracket notation indicating in all cases an open interval).

According to the BRA threshold, *O. mykiss* was classified as "high risk" for 3 (12.0%) of the 25 river basin screened (Western Black Sea, Eastern Black Sea, and Maritza-Ergene), and as "medium risk" for the remaining ones. The species achieved the highest BRA score in Western Black Sea and lowest BRA score in Small Menderes Basin (31.0 and 13.5, respectively) (**Table 1**).

The CCA negatively contributed the BRA+CCA scores of *O. mykiss* in 22 (88.0%) of the river basins and resulted in zero contribution for the remaining three, namely Aras-Kura, Çoruh river, and Eastern Black Sea. The largest negative change in score was obtained for Konya-Closed basin ($\Delta = -10$). *Oncorhynchus mykiss* was classified as "medium risk" in all of the river basins screened. The highest BRA+CCA score was achieved for the Western Black Sea basin (**Table 1**).

The CL (over all 55 Qs) was 2.744 ± 0.030 SE, the CL_{BRA} 2.784 ± 0.030 SE, and the CL_{CCA} 2.376 ± 0.059 SE (hence, in all cases indicating medium to high confidence), and the CL_{BRA} was significantly higher than the CL_{CCA} ($F^{\#}_{1.48} = 37.84$, $P = 0.001$). Similarly, mean values for CF = 0.683 ± 0.007 SE and $CF_{BRA} = 0.692 \pm 0.008$ SE were higher than the mean value for the $CF_{CCA} = 0.593 \pm 0.015$ SE, and the mean CF_{BRA} was

TABLE 2 | Overall Risk Score (ORS) of the river basins calculated based on their mean *Oncorhynchus mykiss* habitat suitability values (Maxent) and the Basic Risk Assessment (ORS_{BRA}) and Climate Change Assessment ($ORS_{BRA+CCA}$) scores. MB, microbasin.

Basin	**Overall Risk Score**		***O. mykiss* habitat suitability (Maxent)**	
	$\mathbf{ORS_{BRA}}$	$\mathbf{ORS_{BRA+CCA}}$	**Number of MB**	**Range (mean ± s.d.)**
Akarçay [10]	6.09	3.48	7,027	0.30–0.56 (0.43 ± 0.05)
Antalya [13]	10.09	7.21	17,428	0.13–0.63 (0.48 ± 0.11)
Aras and Kura [24]	9.08	9.08	25,110	0.09–0.61 (0.43 ± 0.10)
Burdur [9]	6.49	3.89	5,470	0.27–0.55 (0.43 ± 0.04)
Ceyhan [18]	6.90	5.36	18,840	0.11–0.63 (0.38 ± 0.14)
Çoruh [23]	13.36	13.36	18,317	0.34–0.63 (0.56 ± 0.05)
Eastern Black Sea [22]	17.16	17.16	20,664	0.49–0.69 (0.61 ± 0.03)
Eastern Mediterranean [14]	8.80	6.02	18,639	0.11–0.64 (0.46 ± 0.10)
Euphrates and Tigris [21]	10.00	8.54	154,394	0.10–0.71 (0.36 ± 0.12)
Gediz [5]	8.97	7.97	15,086	0.25–0.61 (0.50 ± 0.06)
Great Menderes [7]	7.96	6.14	22,600	0.24–0.60 (0.45 ± 0.05)
Kizilirmak [16]	9.55	5.91	73,611	0.25–0.69 (0.45 ± 0.08)
Konya [15]	5.68	2.13	43,652	0.22–0.64 (0.35 ± 0.08)
Marmara [2]	16.13	12.55	20,695	0.48–0.71 (0.60 ± 0.02)
Meriç-Ergene [1]	14.75	12.65	13,254	0.46–0.63 (0.53 ± 0.02)
North Aegean [4]	10.38	9.29	8,719	0.33–0.63 (0.55 ± 0.05)
Orontes [19]	5.51	4.28	6,707	0.11–0.56 (0.31 ± 0.10)
Sakarya [11]	11.10	7.48	56,516	0.28–0.67 (0.45 ± 0.09)
Seyhan [17]	9.34	6.54	18,739	0.13–0.64 (0.47 ± 0.14)
Small Menderes [6]	6.76	5.76	6,034	0.37–0.60 (0.50 ± 0.05)
Susurluk [3]	12.67	10.42	21,686	0.41–0.66 (0.56 ± 0.04)
Van [25]	9.28	7.70	15,722	0.19–0.59 (0.39 ± 0.08)
Western Black Sea [12]	18.27	17.10	26,248	0.44–0.74 (0.59 ± 0.05)
Western Mediterranean [8]	11.40	9.54	17,927	0.18–0.61 (0.47 ± 0.07)
Yeşilirmak [20]	11.62	9.56	35,664	0.37–0.68 (0.52 ± 0.06)

numbers in ([ ]) stand for geographic ranges as per ***Figure 2****.*

significantly higher than the mean CF_{CCA} (same significance values as for the CL_{BRA} vs. CL_{CCA} comparison due to the two indices being related). In all cases, the narrow standard errors indicated overall similarity in CLs and CFs across the river basins screened. Output reports of AS-ISK analyses of *O. mykiss* for all river basins are provided in **Supplementary Data sheet 1**.

Overall Risk Assessment and Its Implications for Native Trout Conservation

The Overall Risk Score calculated with only basic risk assessment of river basins (ORS_{BRA}) ranged from 5.51 to 18.27 (mean 10.29 ± 0.70 SE) for Orontes river basin and the Western Black Sea, respectively. The ORS calculated with basic risk and climate change assessments ($ORS_{BRA+CCA}$) ranged from 2.12 to 17.16 (mean 8.36 ± 0.78 SE) for Konya-Closed basin and Eastern Black Sea basin, respectively. The ORS_{BRA} calculated for Aras-Kura, Çoruh river, and Eastern Black Sea were the same with their $ORS_{BRA+CCA}$ due to the zero contribution of the CCA (**Table 2**).

We identified 13 native trout species whose known distribution range fall entirely (or largely for *S. coruhensis*) within Turkey. The most widespread species, distribute in more than two basins are *Salmo coruhensis* (15 populations), *S. opimus* (10 populations), and *S. rizeensis* (9 populations). The species which are restricted to a single independent lake or stream catchment are *S. abanticus, S. chilo, S. fahrettini, S. kottelati, S. munzuricus, S. tigridis,* and *S. platycephalus.* The mean *O. mykiss* habitat suitability of micro-basins that a native species occupies ranged from 0.23 for *S. opimus* (Ceyhan population) to 0.67 for *S. rizeensis* (Western Black Sea population) (see **Table 3**); and were significantly different among the species (or populations) examined (Pseudo-$F = 7.327$; $P = 0.001$; see **Supplementary Data sheet 4** for the entire outputs of the main and pair-wise tests). The mean of the Relative Vulnerability Scores that were calculated by excluding outlier values were 1.81 for RVS_{BRA} and 1.50 for $RVS_{BRA+CCA}$ (**Figure 4**). The four highest RVS_{BRA} and $RVS_{BRA+CCA}$ were detected as the outliers and belonged to *Salmo abanticus, S. munzuricus, S. euphrataeus,* and *S. fahrettini* (by descending order). Other populations that ranked as high in terms of both RVS_{BRA} and $RVS_{BRA+CCA}$ were *S. coruhensis* (Eastern Black Sea populations), *S. kottelati, S. labecula* (Antalya population), *S. platycephalus, S. rizeensis* (Eastern and Western Black Sea and populations), and *S. tigridis* (**Figure 5**; **Table 3**). *Salmo labecula* from Seyhan river basin and *S. chilo* ranked as high for RVS_{BRA}, but not for $RVS_{BRA+CCA}$, suggesting that their vulnerability to *O. mykiss* will significantly decrease by climate change. RVS_{BRA} was also higher

TABLE 3 | Native salmonid species and populations in Turkey with their Extent of Occurrence (EOO) and total independent population numbers (IP#).

Species	**Spatial analysis**			***O. mykiss* suitability of micro-basins**	**Vulnerability**	
	Basin code	**EOO (km^2)**	**IP#**	**Range (mean ± s.d.)**	**RVS$_{BRA}$**	**RVS$_{BRA+CCA}$**
Salmo abanticus	12	8.0	1	0.53–0.61 (0.56 ± 0.01)	**11.17**	**10.45**
Salmo chilo	18	89.7	1	0.44–0.54 (0.51 ± 0.01)	**1.81**	1.41
Salmo coruhensis	2	21225.7	15	0.57–0.58 (0.58 ± 0.01)	1.22	0.82
Salmo coruhensis	11	21225.7	15	0.56–0.61 (0.60 ± 0.01)	1.70	1.32
Salmo coruhensis	20	21225.7	15	0.44–0.57 (0.51 ± 0.01)	1.07	0.88
Salmo coruhensis	22	21225.7	15	0.55–0.62 (0.59 ± 0.01)	**1.82**	**1.82**
Salmo coruhensis	23	21225.7	15	0.41–0.63 (0.58 ± 0.03)	1.41	1.41
Salmo euphrataeus	21	8.0	1	0.41–0.58 (0.56 ± 0.01)	**6.22**	**5.31**
Salmo fahrettini	21	8.0	1	0.41–0.51 (0.47 ± 0.01)	**5.24**	**4.48**
Salmo kottelati	8	46.8	1	0.44–0.58 (0.55 ± 0.01)	**3.73**	**3.13**
Salmo labecula	13	215.5	2	0.50–0.60 (0.58 ± 0.02)	**2.22**	**1.59**
Salmo labecula	17	215.5	2	0.45–0.59 (0.54 ± 0.02)	**2.08**	1.45
Salmo munzuricus	21	3.5	1	0.43–0.58 (0.53 ± 0.02)	**9.77**	**8.35**
Salmo okumusi	21	2,214	2	0.34–0.45 (0.43 ± 0.01)	1.18	1.00
Salmo opimus	13	488	10	0.23–0.60 (0.49 ± 0.04)	1.33	0.95
Salmo opimus	14	861.5	10	0.47–0.59 (0.53 ± 0.01)	1.19	0.82
Salmo opimus	18	2513.4	10	0.25–0.61 (0.53 ± 0.03)	0.70	0.54
Salmo platycephalus	17	75.8	1	0.42–0.59 (0.55 ± 0.01)	**2.85**	**2.00**
Salmo rizeensis	12	5132.9	9	0.58–0.67 (0.65 ± 0.01)	**2.54**	**2.37**
Salmo rizeensis	20	5132.9	9	0.43–0.60 (0.52 ± 0.01)	1.30	1.07
Salmo rizeensis	22	5132.9	9	0.58–0.62 (0.61 ± 0.00)	**2.24**	**2.24**
Salmo rizeensis	23	5132.9	9	0.52–0.61 (0.56 ± 0.01)	1.61	**1.61**
Salmo tigridis	21	135.7	2	0.50–0.59 (0.58 ± 0.01)	**2.37**	**2.02**

*Basin codes stand for the basins per **Figure 2**. RVS$_{BRA+CCA}$ and RVS$_{BRA}$ are the relative vulnerability scores calculated from ORS$_{BRA+CCA}$ and ORS$_{BRA}$ respectively. Numbers in bold stand for high vulnerability (RVSs over thresholds).*

than RVS$_{BRA+CCA}$ for all species except the Çoruh and Eastern Black Sea populations of *S. coruhensis* and *S. rizeensis* (**Table 3**). The known distribution ranges of the species (populations) with high RVS$_{BRA}$ and RVS$_{BRA+CCA}$ are proposed as the hotspots for potential overlap and interaction with *O. mykiss* (**Figure 6**). The site descriptions of hotspots were provided in **Table 4**. All the occurrence records of *O. mykiss* escapes compiled from literature and the occurrence dataset is provided in the **Supplementary Table 1**. The river tributaries where possess at least one site-scale record of *O. mykiss* were highlighted. These river patches located on the moderate to high dark areas indicate a reasonable accuracy of the potential map (**Figure 6**). According to the occurrence map, *S. tigridis*, *S. kottelati* and populations of *S. coruhensis* and *S. rizeensis* in the Eastern Black Sea Basin and *S. labecula* in Seyhan river basin seem to already exposed to escapees of *O. mykiss*.

DISCUSSION

Establishment and Invasiveness Potential of *Oncorhynchus mykiss*

As a risk screening (or identification) and decision support tool, AS-ISK inherently makes use of expert opinion to respond the assessment questions. There has been a debate on whether the climate change assessment of AS-ISK is subjective as it allows the use of Köppen-Geiger climate types, thus resulting in the qualitative evaluation for climate matching (Marcot et al., 2019). However, AS-ISK also primarily recommends to benefit from a climate-matching approach (e.g., Climex, GARP, Climatch) or physiological tolerances of species in question (Copp et al., 2016a; Hill et al., 2020). To this end, we developed an alternative way to the risk assessment by integrating AS-ISK with the outputs of a species distribution model (Maxent), which potentially reflects both environmental (including current climate) matching and physiological tolerance. In the present study, Maxent results constituted the quantitative base for the establishment potential of *O. mykiss* in Turkey. However, our ensemble approach can be applied in various ways such as the addition of different climatic scenarios (future climate) into the establishment potential component, that can, in turn improve the Climate Change Assessment part of the AS-ISK. For risk screening, using Maxent to predict the potential distribution of an invasive species is a powerful method (Bosso et al., 2017; Rodríguez-Merino et al., 2018) yet it has been inadequate in utilizing such inputs as the potential impacts, introduction pathways, successful introduction events or biogeographical and historical traits of the taxon. AS-ISK mitigates these gaps by its related query sections. For example, in the present study

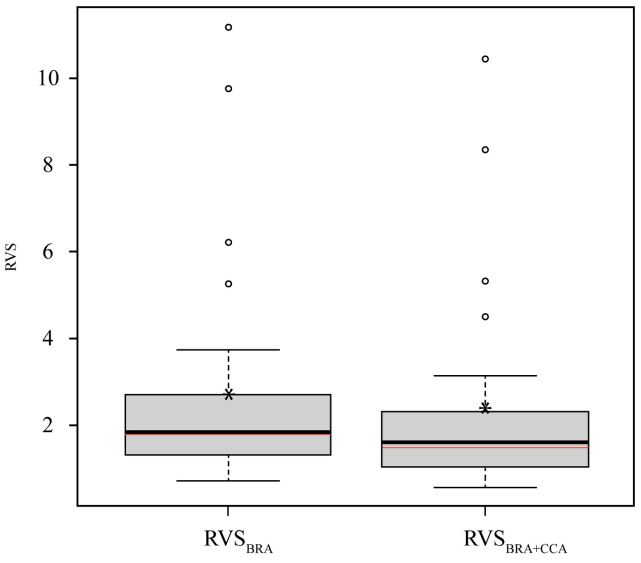

FIGURE 4 | Box-whisker plot representation of the Relative Vulnerability Scores (RVS) of native salmonid species and populations in Turkey. RVSs were calculated for two kinds of overall risk score (ORS), i.e., Basic Risk Assessment (BRA)-based ORS (RVS$_{BRA}$) and Climate Change Assessment (CCA)-based ORS (RVS$_{BRA+CCA}$). Boxes extend from the 25th to the 75th percentile of each RVS's distribution of values; whiskers above and below the box indicate the 90th and 10th percentiles, respectively. Circles indicate the outliers (scores outside 1.5 times the interquartile range). horizontal black lines within the boxes denote median, asterisks denote the mean of the values. Horizontal red lines within the boxes denote the mean RVSs calculated by excluding the outliers.

Marmara, Susurluk and North Aegean basins ranked high for their Maxent values, whereas AS-ISK scores substantially decreased their ranks, suggesting that *O. mykiss* is likely to survive in these basins but less likely to become invasive. Therefore, Overall Risk Score has the potential to balance between these two main components by combining the probability and impact.

One of the most frequently used spatial scales on which species' invasiveness potential is screened is the country-level (Perdikaris et al., 2016; Tarkan et al., 2017a; Uyan et al., 2020; Zieba et al., 2020), however, using national boundaries for risk screening of taxa whose distribution is strongly related to climatic conditions (like Salmonids) may result in rough estimates, if the country covers great climatic and geographical heterogeneity. As for risk screening of *O. mykiss* in Turkey, where several different climate types and a diverse topography are found (Deniz et al., 2011; Iyigun et al., 2013), use of an alternative spatial scale than the country-level is needed. Selecting the river basins as a spatial scale in this regard is more appropriate, as they are compact and coherent hydrological units that also reflect biogeographical and ecological boundaries to a certain extent. This can also enable assessors to predict certain cases (e.g., species distributions and traits) more precisely (e.g., Dodd et al., 2019) than considering the whole country level. Still, increasing resolution of assessments by decreasing spatial scale of risk assessment area would enhance the predictions for the establishment and invasiveness likelihood of salmonid species since they frequently show local adaptation to each environment. For instance, in a country-level basin screening *O. mykiss* yielded 20 in both its BRA and BRA+CAA which resulted it in being categorized as medium risk potential of being invasive in Turkey (Tarkan et al., 2017a). However, our river basin screenings resulted in high potential risk for three basins, which suggest that coarser scale RA selections may mask true invasiveness potential, at least in cases of geographically heterogenous RAs such as Turkey.

Oncorhynchus mykiss is a cold-water species native to the Pacific drainages of North America and northeastern Siberia (Behnke, 1979). It is therefore not surprising that the most explanatory variables determining the distribution of *O. mykiss* was the average annual minimum and maximum temperature (ca 70% in the present study) (Clark et al., 2001; Coleman and Fausch, 2007). This might largely explain why the northern river basins are found to be more suitable for *O. mykiss* compared to those warmer regions located in central and southern Turkey. This was also clearly reflected by the CCA (Climate Change Assessment) scores, which resulted in a negative contribution for *O. mykiss* in most assessed basins under predicted climate warming conditions (**Table 1**). However, there are still some river basins with no change in CCA scores suggesting habitat suitability of *O. mykiss* will not be affected in the short term by means of climatic variables. Although BRA+CCA is significantly lower than BRA (Basic Risk Assessment) due to the intrinsically higher level of uncertainty in future climate change projections (Killi et al., 2020, Uyan et al., 2020), this is supported by the high confidence ranking of CCA reflecting both extensive literature information available on the screened species and the similar level of expertise amongst assessors, hence increasing the reliability of the screening outcomes.

Some other factors such as flow regime (Fausch et al., 2001), stream size (Rahel and Nibbelink, 1999), and gradient (Adams, 1999) have been shown to affect distribution and establishment of *O. mykiss*. In the present study, slope was the second most important defining variable that might explain higher habitat suitability for northern RAs. This could be attributed to the occurrence of steep and fast-flowing streams in Black Sea region (northern part of Anatolia) that could have contributed the species' persistence (e.g., Fausch et al., 2001). However, the low invasiveness potential of *O. mykiss* was attributed to the poor persistence of rainbow trout populations to be locally established, mainly due to biotic resistance from competition, predation and parasites or diseases (Fausch, 2007). Angling and wild collecting as other crucial factors hamper invasion success of *O. mykiss* in the U.K (Fausch, 2007), and these cannot be ruled out for Turkey.

Priority and Conservation of Native Salmonids

The diversity of trout species in Western Palearctic has long been a subject of dispute among taxonomists. *Salmo trutta* had been considered a widespread polymorphic species that distributed throughout Europe reaching southwards to the Atlas Range (Morocco, Algeria) and eastwards to the upper Amu-Darya drainage in Afghanistan (Kottelat, 1997). In recent decades, large

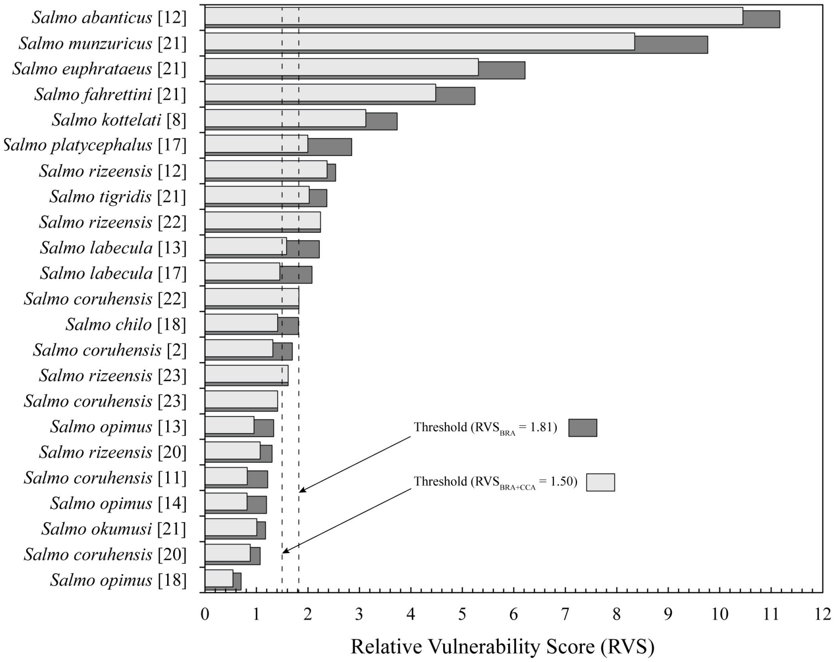

FIGURE 5 | Relative Vulnerability Scores (RVS) of native *Salmo* species and populations in Turkey. Brackets ([ ]) after species (or populations) present river basin codes are as per **Figure 2**.

numbers of studies have uncovered the diversity of trout in Europe by the recognition of several species that had been earlier identified as morphs or ecotypes of *S. trutta* (Delling, 2003, 2011; Delling and Doadrio, 2005). The case for the Turkish trout species has followed similar path. Only five species of trout had been recognized up to 2010 (*S. abanticus, S. caspius, S. labrax, S. macrostigma*, and *S. platycephalus*). In the last decade, 11 additional species have been described from Turkey, as well as the existence of *S. labrax* and *S. macrostigma* in Turkey was disproven by extensive molecular and morphological studies (Turan et al., 2010, 2011, 2012, 2014a,b, 2017, 2020). Although the number of trout species in Turkey is still tentative, and some have been questioned for their validity (Kalayci et al., 2018; Ninua et al., 2018), here we follow in-state taxonomy. In the light of the Aichi Biodiversity Targets, agreed in 2010 (https://www.cbd.int/sp/targets/), biodiversity has been recognized not only at the ecosystem and species level, but also at the genetic diversity level. Thereof, regardless of whether the current trout taxonomy is established or not, we consider the geographically and/or genetically distinct native trout populations as conservation and management units.

Before the present study, the number of species or populations of trout that have been directly exposed to *O. mykiss* in nature was poorly documented and anecdotal in Turkey. The present research, has demonstrated, for the first time, to what extent the spatial overlap currently exists between *O. mykiss* and native salmonids, and how this might change in the future. On one hand, stocking of non-native salmonids for aquaculture and recreational fishing, and escapement from these systems into nature, is an important issue in salmonid conservation. Several studies have documented negative impacts of escapee non-native salmonids, mainly *O. mykiss* and the brown trout (*Salmo trutta*), on native salmonids, with the latter not being a farmed species in Turkey (for a detailed account, see Fausch, 2007; Stanković et al., 2015; Hasegawa, 2020). Among the pronounced impacts, displacement of native salmonids by non-natives through reduced fitness and survival (Muhlfeld et al., 2009; Houde et al., 2015), competitive exclusion (Fausch, 1988; Seiler and Keeley, 2009), hybridization (Weigel et al., 2003; Boyer et al., 2008) or predation (Budy and Gaeta, 2018) have been well-studied in many parts of the world, especially in the North America, Europe, New Zealand and Japan, yet none of these impacts have been documented in Turkey. On the other hand, interpopulation introductions of native salmonids for wild stock enhancements is another conservation issue, as mixing species is always problematic. In Turkey, such an effort has been made to support wild populations of salmonids, especially *S. rizeensis*, *S. coruhensis* and *S. abanticus* (Akkan et al., 2016), and much more care is needed to avoid mixing the populations of different species and the mismatching of species to their native ranges during introductions.

According to our proposed relative vulnerability measure, *S. abanticus* received the highest score, suggesting that the species is represented by a low number of independent populations (IP = 1) in a restricted area with relatively a higher potential than other native salmonids to be exposed to *O. mykiss*. Among the native trout species with the five highest Relative Vulnerability Scores, only *S. abanticus* has an IUCN Red List category (VU, Freyhof, 2019a), whereas the others have not been evaluated. This calls for an urgent need to specify their conservation status. The

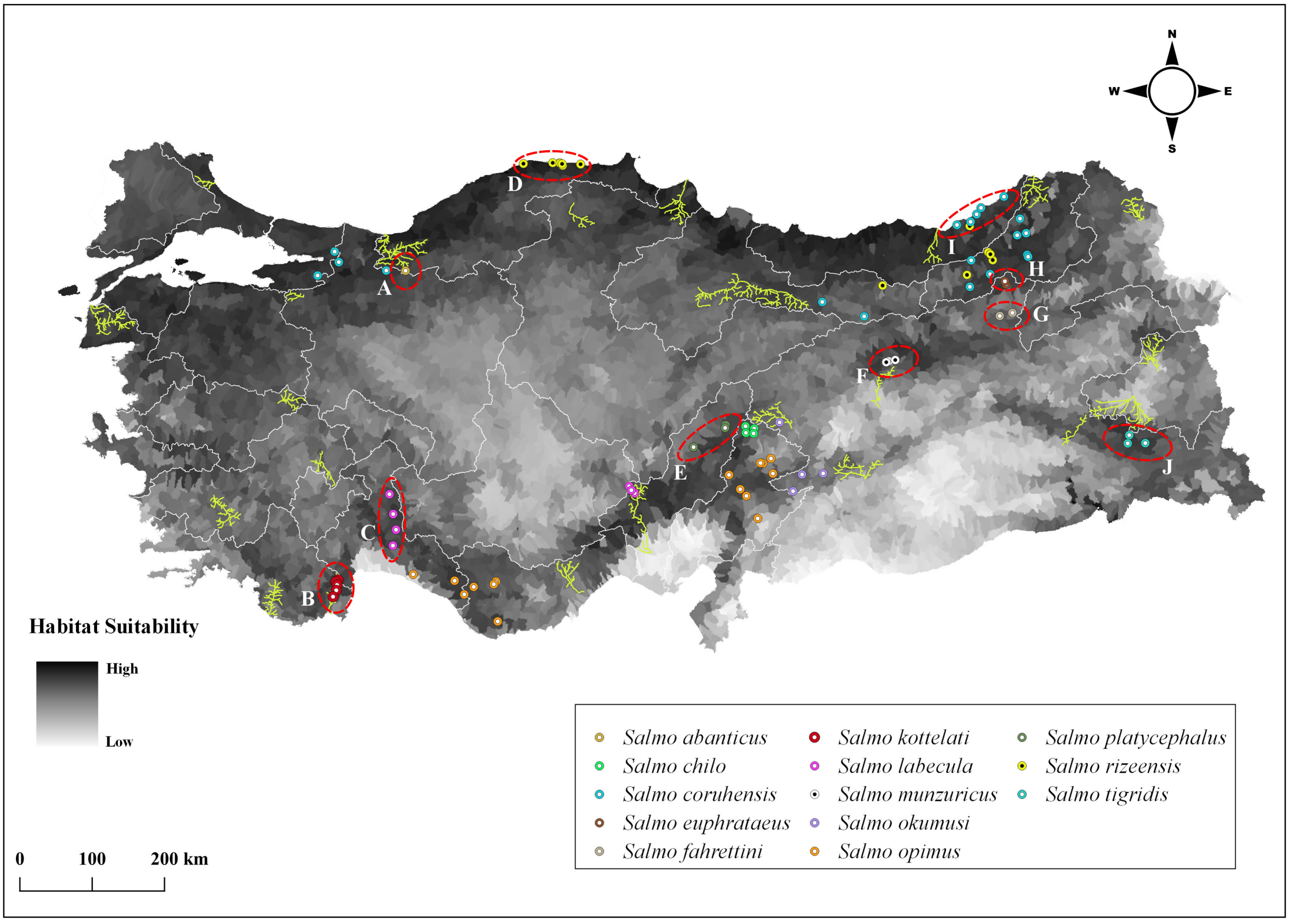

FIGURE 6 | The distribution of native Salmo species and the range of *Oncorhynchus mykiss* farm escapes in Turkey. The colored circles represent site scale records per native species; the river network patches (in yellow) represent the tributaries where *O. mykiss* farm escapements have been recorded. The map is black-white equivalent of **Figure 2** (i.e., habitat suitability map of *O. mykiss*). The red-dashed circles denote proposed hotspot areas, and are also coded by letters. The coordinates and sources from which *O. mykiss* escape records taken were provided in **Supplementary Table 1**.

TABLE 4 | Description and geographic ranges of hotspots proposed for species and populations of native *Salmo* spp. in Turkey. Map codes correspond to the red circles depicted in **Figure 6**.

Map Code	Species	Basin	Hotspot description	Latitude range	Longitude range
A	*S. abanticus*	Western Black Sea	Lake Abant drainage nearly 30 km southwest of Bolu	40.53–40.81	31.19–31.65
B	*S. kottelati*	Western Mediterranean	Alakir river drainage between the eastern Beydaglari mountain range and the western Gulf of Antalya. The area from Alakir reservoir to the headwaters is of great importance	36.31–36.86	30.10–30.27
C	*S. labecula*	Antalya	Köprüçay River drainage between the western Taurus Mountain range around Sütçüler (Isparta) and south of the Köprülü Canyon National Park (Antalya)	37.12–37.94	30.94–31.40
D	*S. rizeensis*	Western Black Sea	Small coastal streams from the west (Cide-Devrekani) to the east of (Çatalzeytin) Kastamonu	41.76–42.01	33.32–34.25
E	*S. platycephalus*	Seyhan	Zamanti River between Pinarbaşi and Kizlarsekisi in the upper Seyhan River drainage.	37.56–38.78	35.55–36.56
F	*S. munzuricus*	Euphrates and Tigris	Munzur river (upper Euphrates) from the Gözeler springs (headwaters of the river) to the north of Tunceli province.	39.19–39.52	38.77–39.68
G	*S. fahrettini*	Euphrates and Tigris	Headwaters of Karasu river in the upper Euphrates. Streams Tekke and Ömertepesuyu in west of Palandöken Montain (Erzurum)	39.71–39.98	40.84–41.25
H	*S. euphrataeus*	Euphrates and Tigris	Streams Kuzgun, Senyurt and Ilica around Kuzgun Dam Lake in Karasu river drainage (upper Euphrates), about 35 km northeast of Erzurum.	39.91–40.35	40.75–41.27
I	*S. rizeensis* and *S. coruhensis*	Eastern Black Sea	Coastal streams between Hopa (Artvin) and Iyidere (Rize) in the eastern Black Sea region.	40.82–41.27	40.33–41.45
J	*S. tigridis*	Euphrates and Tigris	Two headwaters of Botan River (uppermost Tigris drainage). Stream Müküs at Bahçesaray and Çatak River at Çatak (Van)	37.98–38.24	42.65–43.35

RVS alone reflects the relative vulnerability of the examined trout species to the interaction potential with *O. mykiss*. Therefore, this metric can be considered as a complementary tool to improve conservation assessments (e.g., IUCN Red list assessment), though it can help to propose relative priority for a range of species (or populations) examined, when applied solely. In this context, our tentative attempt, in which we determine the threshold can be modified by any case-specific conditions, such as, for example, considering RVS of certain species (or populations) that is known to have a virtual interaction (reported hybridization or competition) with *O. mykiss* as the threshold or reference point.

As reviewed and suggested by Coates et al. (2018), infra-specific entities have always been problematic for evolutionary and conservation biologists in deciding conservation units. However, it is widely recommended that distinct populations within a species would be better accepted and treated as distinct genetic lineages for the practical assessment of conservation status or impact assessments (Frankham et al., 2012; Taylor et al., 2017; Coates et al., 2018). In agreement with this conservative approach, although *S. rizeensis* (LC) and *S. coruhensis* (NT) are relatively wide-spread, some of their populations received high RVS as their distribution ranges overlap with habitats suitable to *O. mykiss*. Conversely, all populations of *S. opimus* from southern drainages have been represented by relatively low RVS, although the species is classified as Endangered by the IUCN (Freyhof, 2019b). This result could be attributed to the potential failure of *O. mykiss* in southern basins (**Table 2**). This potential is expected to increase for this spatial range by the predicted climate change. In parallel with this assumption, $\mathrm{RVS}_{\mathrm{BRA+CCA}}$ of *S. labecula* population from Antalya and *S. chilo* from Ceyhan remained under the proposed threshold, even though their $\mathrm{RVS}_{\mathrm{BRA}}$ was above the corresponding threshold.

CONCLUDING REMARKS

Whilst the use of non-native species has an indispensable importance for international aquaculture, it also poses considerably high risks to native biodiversity, as escaped aquatic species may become invasive in certain environments. Despite the fact that preventing organisms from escaping is the best interest of aquaculturists—who engage in the farming of fish, mollusks, crustaceans and aquatic plants—, it is widely known that species in fact do escape. This is not only true for fish species, as is the case in the present study, but also for some mammals (American minks, Anderson and Valenzuela, 2011; Nutria, Simberloff, 2011), marine shellfish (Galil, 2011), garden plants (Reichard and White, 2001) and aquatic plants, as the most popular example being the killer algae, *Caulerpa toxifolia* (Meinesz and Hesse, 1991), all of which could have been successfully introduced to many parts of the World.

As for future prospects, it is crucial that we incorporate escape probability from the farms to better quantify the overall risk exposed by *O. mykiss*. Fish escapes from farms can be both detrimental to the natural species and can also reduce income of fish farms. For this aim, we highly anticipate receiving the benefit of aquaculture-specific and post-screening risk analysis tools such as European Non-native Species in Aquaculture Risk Analysis Scheme (ENSARS) (Copp et al., 2016b; Tarkan et al., 2020), by also implementing the information on farm intensity (number of fish farms per unit area) and farm capacity. Fish escape should also be taken seriously especially for the hotspots we proposed, and related measures should be amended to prevent or minimize it. According to the confirmed escape records, several species and populations of native trouts have already been exposed to escapees of *O. mykiss*, and we propose to give priority to monitor those species or populations in near future.

There is still ongoing debate as to whether species distribution models are accurate enough to predict potential establishment and spread of introduced species (Bertolino et al., 2020). Therefore, collecting field data would be needed to support the validation of our modeling. Considerable gaps in the knowledge have been identified in both the science and policy for the effective management of biological invasions, which indicate that non-native species are not properly managed because they receive less attention from a wide range of related groups (i.e., scientists, policy-makers, and managers). This is substantially important in tackling invasive non-native species through prevention, early detection, rapid response, eradication and control, which are all considered to be financially demanding (Britton et al., 2011). In cases where river basins contain both native trout species and *O. mykiss*, implementation of such measures significantly depend on the local authorities and public perception mainly because of the economic value of *O. mykiss*. However, as the results of the present study suggest a potential impact on native and endemic trout species, a balance should be found between the ecological impacts and the economic benefits of *O. mykiss*.

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.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the study has based on the methodology that includes the risk screening and spatial modeling approaches. No animals were used.

AUTHOR CONTRIBUTIONS

BY and AST conceived and designed the study, employed risk screening of the river basins, interpreted results, and drafted the manuscript. TB performed Maxent modeling, interpreted results, and revised the manuscript. FGE conceived and designed the study, interpreted results, and revised the manuscript. CK conceived and designed the study, employed risk screening of the river basins, and developed native salmonid occurrence dataset.

All authors contributed to the paper, read, and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.599881/full#supplementary-material

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