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Comparing morphological, parasitological, and genetic traits of an invasive minnow between intermittent and perennial stream reaches

Alejandra Cruz[1] | Carla Llinares[1] | Inés Martín-Barrio[1] | Gema Castillo-García[1] | Pilar Arana[2] | Emili García-Berthou[3] | David H. Fletcher[4] | David Almeida[1,3]

[1]Department of Basic Medical Sciences, School of Medicine, Universidad San Pablo-CEU, CEU Universities, Urbanización Montepríncipe, Boadilla del Monte, Spain

[2]Department of Genetics, Physiology & Microbiology, Complutense University of Madrid, Madrid, Spain

[3]GRECO, Institute of Aquatic Ecology, University of Girona, Girona, Spain

[4]UK Centre for Ecology & Hydrology, Environment Centre Wales, Gwynedd, UK

Correspondence
David Almeida, Department of Basic Medical Sciences, School of Medicine, Universidad San Pablo-CEU, CEU Universities, Urbanización Montepríncipe, Boadilla del Monte, Spain.
Email: david.almeidareal@ceu.es

Abstract

1. Invasive fishes are a major environmental issue at the global scale, particularly for their impacts on freshwater ecosystems via the mechanisms of hybridisation, competition, predation, and disease transmission. This is of special conservation concern on the Iberian Peninsula due to the high level of endemism. With the aim to improve our knowledge on the invasion process of non-native fishes, the present study consists of analysing key biological traits potentially related to their colonisation capacity: morphology, parasite communities, and genetic diversity.

2. A non-native population of Languedoc minnow *Phoxinus septimaniae* (leuciscid species native to south-east France) was assessed in Tordera Stream (north-eastern Iberian Peninsula). Fish were sampled in mid-May (reproductive period) 2018 and 2019. Biological traits were compared between colonising and resident individuals from stream reaches of intermittent and perennial flow, respectively.

3. After adjusting for fish size/body mass with analysis of covariance, body condition and health status (external and internal examination) tended to be better in the intermittent reach. Parasite load (number) and diversity (Shannon index) were significantly higher in the perennial reach. Fish development was also more stable in colonising minnow, as detected by low levels of fluctuating asymmetry for the eye diameter and the length of pectoral fins (adjusted for bilateral character size). Genetic diversity (microsatellite markers) was lower in resident minnow. Minnow genetic homozygosity (index of internal relatedness) was positively associated with parasite load and developmental instability (high fluctuating asymmetry levels).

4. Our results show that colonising minnows display particular profiles of morphological, parasitological, and genetic traits that apparently allow these individuals to increase their dispersal capacity to survive and reproduce in harsher environmental conditions. The potential mechanisms may be related to a wider physiological tolerance, better immune response, parasite resistance/tolerance, and genetic diversity in colonising specimens.

Alejandra Cruz and Carla Llinares are co-first authors.

5. Such information reveals the specific traits of successful fish invaders under a seasonal colonisation dynamic. These data are relevant for environmental managers, to predict and identify areas vulnerable to invasion, in order to establish monitoring programmes for early detection, which will help to reduce the spread of non-native fish populations.

1 | INTRODUCTION

Biological invasions have resulted in serious disturbances and deleterious effects on natural ecosystems, being a major driver of biodiversity loss at the global scale (Butchart et al., 2010; Mooney & Hobbs, 2000). This is of particular concern in freshwater ecosystems, where multiple non-native plant and animal species have been introduced world-wide (Francis, 2012; Mooney & Hobbs, 2000), with special emphasis on fishes (Leprieur et al., 2008; Rahel, 2000). On the Iberian Peninsula, the disruptive effect of fish invasions is particularly acute (Almeida, Ribeiro, et al., 2013), as endemism is high in these freshwater ecosystems. Indeed, >50% of the native fish species are unique to this eco-region, many of them being threatened (Almeida, Ribeiro, et al., 2013; Reyjol et al., 2007). A wide variety of adverse impacts on Iberian native communities have been caused by invasive fish via the mechanisms of hybridisation, competition, predation, and acting as disease vectors (Leunda, 2010; Ribeiro & Leunda, 2012).

Growing concern about the impacts of non-native fishes has led to research on these species within their introduced ranges under the particular environmental conditions of those areas, where they may confront new ecological stressors, such as floods, droughts, fluctuating food supply, or native predators (Almeida et al., 2014; Latorre et al., 2020). Nevertheless, there is still an urgent need to improve our knowledge on the invasion process of non-native fishes, particularly the key biological traits that increase colonisation capacity throughout novel habitats (Brandner et al., 2013; Rubenson & Olden, 2017). This information would enable us to characterise the specific features of successful invaders in the Iberian Peninsula (García-Berthou, 2007; Ribeiro et al., 2008). Thus, environmental managers could use such data to identify new vulnerable areas for fish invasion, in order to avoid the expansion across Iberian fresh waters (Almeida, Ribeiro, et al., 2013; Latorre et al., 2018, 2020).

Most research on biological traits of invasive fishes focuses on particular aspects (e.g. growth, reproduction or diet) independently, or a couple of traits at the most, with multiple sets of parameters being rarely assessed on the same specimens. For instance, in the Iberian Peninsula, Almeida et al. (2009) assessed body condition and dietary flexibility of pumpkinseed Lepomis gibbosus, Benejam et al. (2009) provided data on life-history and parasitology in mosquitofish Gambusia holbrooki along a latitudinal gradient, and Latorre et al. (2018) analysed growth and reproduction strategies on different populations of

bleak Alburnus alburnus as adaptations to contrasting environmental conditions. For a more holistic approach, diverse biological traits should be simultaneously measured at the individual level to more accurately identify particular colonising profiles within invasive fish populations. Among the various of biological traits to be assessed, three are highlighted for their potential interactions in non-native fishes. Morphology, such as body condition or asymmetry level, along with parasitology, are useful indicators of host status in invasive fish species in Iberia (Almeida et al., 2008). Indeed, fish parasite communities can be also used as a proxy for structure of biotic interactions from the wider aquatic ecosystem (Lafferty, 2008; Marcogliese, 2005). In fact, the invasion process of fishes is likely to be modulated by parasites via enemy release (parasite loss after introduction, with invaders becoming more competitive than native species) and parasite acquisition (infections by pathogens from the native range, potentially resulting in parasite spill-back to native species) mechanisms (Brandner et al., 2013; Sheath et al., 2015). Unfortunately, there is a dearth of parasitological information on invasive fishes in Iberia (but see some data in Almeida et al., 2008; Benejam et al., 2009; Muñoz-Mas & García-Berthou, 2020). The third biological trait of particular relevance is genetics, as the interaction between environmental factors and genetic diversity may determine establishment success and future colonisation status of non-native fishes (Ağdamar et al., 2015). Combined, these data would aid to provide better insights into the specific mechanisms that facilitate colonisation, such as synergistic effects between different biological parameters or environmental-specific responses (Almeida et al., 2017). However, this holistic approach (i.e. the simultaneous assessment of multiple traits) has been poorly attained under natural conditions, because of the difficulties and complexity of the study design, execution, and interpretation. Furthermore, data from invasive populations subjected to temporal colonisation dynamics is of pivotal importance for conservation purposes in Mediterranean water courses, where strong flow variations occur seasonally (Gasith & Resh, 1999). For example, identifying particular traits that aid recolonisation of dry stream sections, after the hot summer drought, could be relevant to understand, predict, and manage bio-invasions within these eco-regions. However, to the best of our knowledge, non-native fish populations displaying this seasonal colonisation effort have been virtually undescribed in the Iberian Peninsula. Additionally, if such an environmental context of colonisation is repeated from year to year, the observed variations in colonising fish traits will be more appropriately considered as general ecological patterns.

Consequently, the aims of the present study consist of analysing a variety of biological parameters within an invasive fish population under a seasonal colonisation dynamic since its introduction. Specifically, morphological, parasitological, and genetic traits were compared between *colonising* and *resident* specimens from *intermittent* and *perennial* reaches (respectively) in an Iberian stream. In fish species displaying partial migration (such as many leuciscids), individuals showing poor body condition are usually displaced to sub-optimal areas, and they form the *colonising-migrating* fraction of the population (Chapman et al., 2012). In this respect, Mediterranean temporary-streams pose *harsh* (sub-optimal) habitats to be re-colonised by fish assemblages (Gasith & Resh, 1999; Larned et al., 2010). This profile of lower condition (and less intra-specific competitive ability) displayed by dispersing individuals has also been described for invasive fish (Grabowska et al., 2019). Theoretically, fluvial habitats closer to the ecological optimum for a fish (e.g. permanent water flow) should provide better environmental conditions of stability during development, with individuals displaying a lower asymmetry level (Dongen, 2006). In terms of taxonomy, a wide variety of parasites are commonly found in freshwater fish, with several species displaying complex life-cycles that include up to four contrasting hosts, such as snails, crustaceans, fish, birds, or mammals (Lafferty, 2008). Thus, more fluctuating aquatic habitats may hamper the presence of one particular host in the cycle, which could affect parasitological parameters in fish, e.g. by reducing abundance or diversity (Marcogliese, 2005). Regarding genetic traits, environmental instability may pose a pressure on colonising individuals, favouring specimens with elevated genetic diversity for better dispersal within that ecological context (e.g. see a case study on cichlid fishes in Nevado et al., 2013). Following this general background, the hypotheses tested were that: (1) body condition (eviscerated mass) and health assessment (e.g. internal organs) might indicate a poor physical status in colonising fish, i.e. competitively inferior individuals, along the intermittent reach; (2) resident individuals should be morphologically less asymmetric, i.e. higher environmental stability during development along the perennial reach; (3) parasitological traits should reflect that biotic interactions are less complex in the intermittent reach; and (4) genetic diversity will be lower in fish from the perennial reach, i.e. a limnologically more stable habitat.

2 | METHODS

2.1 | Study area

Tordera Stream is located in north-eastern Iberian Peninsula (Figure 1). Climatic, geo-morphological, limnological, and ichthyological features, along with anthropogenic impacts, have been well-described in Bae et al. (2016), Benejam et al. (2008, 2010), and Merciai et al. (2017, 2018). Briefly, Tordera Stream is a water course that rises in the mountainous area of Montseny Natural Park (>1,600 m above sea level) and flows into the Mediterranean Sea after 65 km, draining an area of around 900 km^2 (Figure 1). The

flow regime is typically Mediterranean, with winter floods and summer drought (Gasith & Resh, 1999). In terms of geo-morphological and hydrological features, the mainstem of Tordera Stream should be naturally permanent all year. However, a particular stream reach completely dries out in summer because of the severe water abstraction after the presence of a small weir (2-m height). Multiple impacts of this weir on Tordera Stream have been well-studied in Bae et al. (2016), Benejam et al. (2008), and Merciai et al. (2018). This barrier is used for water diversion to a lateral channel and it is the cause of a seasonally intermittent stream reach (13 km; Figure 1), which remains dry between July and October (Bae et al., 2016), i.e. dramatically intensifying the effect of summer drought. The rest of Tordera mainstem has a persistent flow throughout the year (Figure 1). Substratum in the study area mainly consists of Palaeozoic slates, Hercynian granites, and some areas of Quaternary clay soils. The climate in north-eastern Iberia is Mediterranean, with rainfall concentrated in autumn and winter (80–100 mm) and summer drought (<25 mm). The average annual temperature ranges between 12 and 17°C. The lowest temperatures occur in winter (–5°C) and the highest in summer (35°C; Ministry of Environment Spain, 2021). The upper reach runs from the headwaters to the Montseny foothills across a forested area dominated by oak *Quercus* spp. and alder *Alnus glutinosa*. The middle and lower reaches have dense riparian vegetation of elmleaf blackberry *Rubus ulmifolius* and invasive giant reed *Arundo donax*. Along these stream reaches, land use is mainly agricultural and residential (Benejam et al., 2008).

The most representative native species within the fish assemblage includes the migratory European eel *Anguilla anguilla* and two Cypriniformes: the Mediterranean barbel *Barbus meridionalis* and the Catalan chub *Squalius laietanus*, which are endemic to north-eastern Spain and southern France (Doadrio et al., 2011). Brown trout *Salmo trutta*, which is abundant in the headwaters, is assumed to be non-native or, at least, under an *uncertain* status (Benejam et al., 2008). The invasive eastern mosquitofish *Gambusia holbrooki* is commonly found in the lowermost part of the stream, whereas common carp *Cyprinus carpio* and other non-native species (e.g. largemouth bass *Micropterus salmoides*) are captured occasionally (Benejam et al., 2008, 2010).

2.2 | Study species and Tordera population

The Languedoc minnow *Phoxinus septimaniae* (hereafter, *minnow*) is a small-bodied Cypriniformes and Leuciscidae fish native to south-east France. Currently in Iberia, this non-native species in only found in north-eastern Spain (Corral-Lou et al., 2019; Doadrio et al., 2011). This fish species was introduced into Tordera Stream at the beginning of the 2000 decade (Corral-Lou et al., 2019; García-Berthou et al., 2017), probably as a *forage fish* for stocked brown trout (see above). Since its introduction, the minnow has displayed a strong invasive character along Tordera Stream (>30 km; Figure 1) and is currently the most abundant fish species in this water course, out-competing native barbel and chub for food and
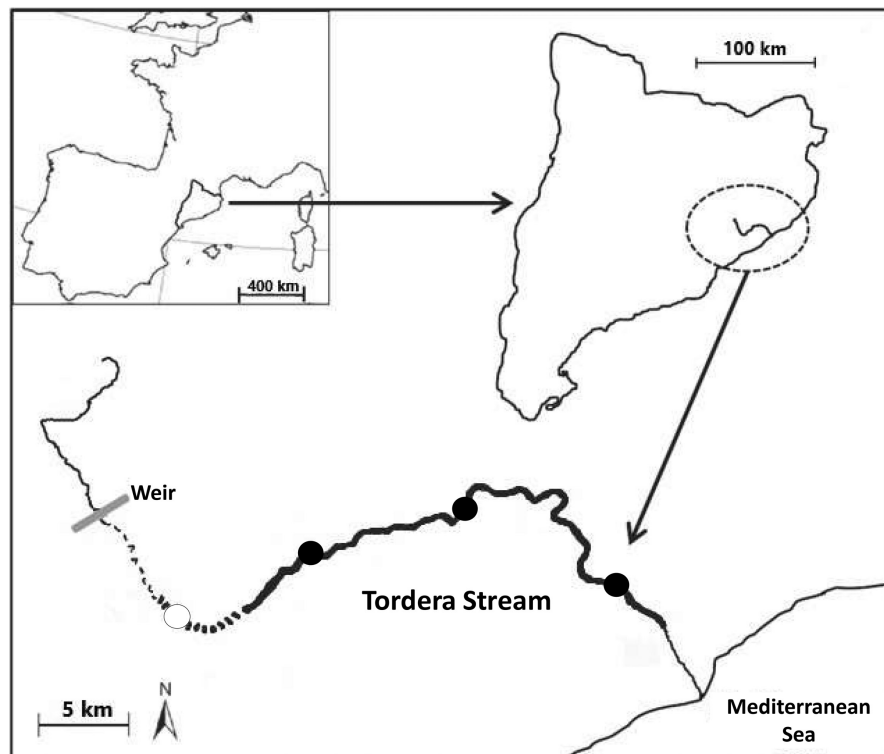
FIGURE 1 Map of the study area showing the geographic location for Tordera stream (Catalonia region, north-eastern Spain). Thick line: minnow presence; thin line: minnow absence; dashed line: intermittent reach; solid line: perennial reaches; white circle: sampling site in the intermittent reach; black circles: sampling sites in the perennial reach. The location of the weir causing the intermittent stream flow is also shown

habitat (García-Berthou et al., 2017; D. Almeida, personal observations in 2021). The minnow is present and spawns along the middle reach of Tordera Stream (D. Almeida, personal observations of minnow fry in the same sampling years 2018 and 2019), partly including the intermittent stream reach (*c.* 6 km) as the upstream limit (Figure 1). That *ecological limit* is due to the decrease in channel width and the strong increase in slope, water velocity, and particle size (mainly pebble and boulder, according to the river-bed classification by Platts et al., 1983), i.e. typical features of piedmont at the foot of Montseny Mountains. Such habitat conditions are not suitable for the upstream natural dispersal of this small-bodied leuciscid. Indeed, their spawning areas typically include sand and gravel bottoms (Doadrio et al., 2011). Because of the disturbed flow regime by the weir (i.e. channel partly dry), minnow do not start to re-colonise the intermittent stream reach until the beginning of spring (end of March). More specifically, fish activity is low from October to March (cold temperatures) and re-colonisation of this reach must first be accomplished by the fauna of benthic invertebrates (Almeida, Merino-Aguirre, & Angeler, 2013), to provide a food supply for the minnow (Oscoz et al., 2006). In relation to this, previous surveys were carried out to compare the abundance of benthic invertebrates between the intermittent and perennial stream reaches during the same sampling period for fish (mid-May, see below), focusing on the main prey for the genus *Phoxinus* in the Iberian Peninsula (Oscoz et al., 2006). Statistical differences ($p < 0.05$) were found after using a Surber sampler at the same sampling sites as per minnow, according to the meso-habitat variability. In particular, abundance of benthic invertebrates was significantly lower in the intermittent reach (mean = 0.83 g dry mass/m², *SEM* = 0.12) than in the perennial reach (mean = 2.52 g

dry mass/m², *SEM* = 0.15; see specific methods for *Invertebrate Sampling* and *Data Analyses* in Almeida, Merino-Aguirre, & Angeler, 2013). From March onwards, temperatures increase and thus also biotic activity (both benthos and fish). In particular, minnows reach their upstream distributional limit at the beginning of May, i.e. the onset of the reproductive period in this population (D. Almeida, personal observations). Indeed, the highest minnow abundance is found in the intermittent reach by mid-May (prior surveys by multi-pass electrofishing and block-nets: minnow density *c.* 2,100 ind./ha), although this abundance is much lower than in the perennial reach during the same period (>4,000 ind./ha). Moreover, individuals from the intermittent reach are mainly (*c.* 80%) large reproductive adults for this minnow population (size range: 55–75 mm standard length, SL), whereas the proportion for this size range is much lower (<20%) in the perennial reach. Previous surveys during the pre-spawning period (i.e. end of April) did not find statistically significant differences ($p > 0.05$) for reproductive investment (adjusted gonad mass, covariate: eviscerated body mass) between colonising (mean = 390 mg, *SEM* = 42) and resident (mean = 388 mg, *SEM* = 34) minnows (see specific *Laboratory Procedures* and *Data Analyses* in Latorre et al., 2018). Young of the year (YOY) minnows are observed at the end of June along the intermittent reach, when flow starts to rapidly decrease (D. Almeida, personal observations). These juvenile individuals probably get to the perennial reach in July, when the intermittent reach completely dries out (some juveniles remain in isolated pools and ultimately perish). All these environmental features ensure that adult individuals found in the intermittent reach entirely come from the perennial reach, i.e. playing a role as the source for natural dispersal. Although flow is artificially disturbed by the

weir in the intermittent reach, this system as whole may be an appropriate model to better understand how non-native species can invade novel habitats comprising harsh environmental stressors (e.g. stream reaches under strong summer drought), which are less frequent in their native areas (see Almeida et al., 2014 for a similar example). Finally, two advantages of using the minnow as a model species in this stream are: (1) there is no restriction to capture a large number of specimens, as this species has an *invader* status (invasive fish cannot be released back to the stream, according to the regional legislation); and (2) because of the high abundance, the extraction of a large fish sample has virtually no effect on this population and, consequently, on subsequent data acquisition of the present (2-year) study.

2.3 | Field sampling

The selected sampling period was in mid-May, corresponding to the highest minnow abundances (see above). This was a 2-year study, 2018 and 2019, which allowed us to acquire a more representative dataset than a single-year study, as recommended by Fausch et al. (2002). In the intermittent reach (only 6 km with minnow presence), one sampling site (100-m river stretch) was located at 500 m from the maximum limit of minnow presence (Figure 1). This site was selected because it was the most distant to the perennial reach with enough abundance of minnows to collect a representative number of specimens (see sampling size below in Section 2.4). For a better spatial representativeness in the perennial reach, three sampling sites were selected at the upstream beginning, middle, and the downstream end (Figure 1). These sampling sites were separated by >10 km to minimise spatial dependence (Figure 1). Also, sampling sites were located in the vicinity of similar natural surroundings, with dense riparian vegetation and far from influences of main tributaries, towns, and farm-lands. Each site was randomly sampled on different dates during the same week per year. Fish collection was carried out always in the morning and weather conditions (e.g. temperature, humidity, visibility) were similar among sampling dates. A consistent sampling protocol was followed by wading according to the European legislation (CEN/ISO Standards, EC Directive 2014/101/ UE; EC, 2014). Sampling effort was similar per site, in terms of time (1.5 h) and research team (the same three people), to ensure comparability between stream reaches. As in previous fish surveys in Tordera Stream (Merciai et al., 2018), the catch method consisted of following a zigzagging upstream direction at both banks at each site by electrofishing (pulse DC at 200–250 V, 2–3 A). To encompass the existing environmental variability, fish were collected from all meso-habitats present in the study stream (e.g. runs, pools, shallows). All these sampling conditions allowed the acquisition of a representative and comparable sample of the study fish species at each site and stream reach.

After each survey was concluded per site, captured fish were identified and the target species (i.e. Languedoc minnow) was separately placed in tanks with cooled water (by using cooling accumulators) and supplied oxygen (two battery-operated aerators with portable pumps). Fish were then transported to a laboratory facility at the University of Girona, which is located <1 h from the study area. Thus, all fish collected at one particular site (see sampling size below in Section 2.4) were processed on the same date of capture, in the afternoon. This overall procedure helped to: (1) minimise the effect of fish stress; and (2) reduce disturbances in their parasite infra-communities (e.g. specimen loss).

All field procedures complied with animal use and care regulations of Europe and Spain (specific licences were granted for scientific capture by the Catalan authorities). Fish were collected by trained personnel (i.e. the holder of the licences, D. Almeida). Thus, no adverse effects were caused on the wildlife in the study habitats. All native fish fully recovered from the collection method, and were released at the same site of Tordera Stream after recovery. In accordance with regional legislation, non-native species were euthanised, similarly to minnow at the laboratory (see details below).

2.4 | Morphological examination

On arrival at the laboratory, 50-L aquaria were filled with water collected from the same sampling site of Tordera Stream. Aquaria were equipped with constant oxygen supply, water filter, temperature/ pH controller, and environmental enrichment (e.g. similar substrate and submerged vegetation as per each site), following Näslund and Johnsson (2016). Fish were allowed to acclimatise for a 30-min period by swapping water between the laboratory aquarium and the transportation tank, before being completely released into the aquarium. Minnow were individually euthanised by immersion in an overdose solution of anaesthetic (dropwise addition of clove oil) for 5 min (adapted from Chapman et al., 2015), followed by severance of the spinal cord. Immediately after euthanasia, fish were measured for SL (±1 mm). As per Latorre et al. (2020), this particular fish length was selected because it avoids potential *noise* given by variation of caudal fin length not related to body size (e.g. wounds and cuts in the fish skin and rays). To minimise the effect of fish size on data and ensure an age enough to effectively detect developmental instability (see details below about *Asymmetry*), the largest individuals of each fish sample per site were selected for examination. In total, 300 individuals (50×2 years from the intermittent reach +33– 34×3 sites×2 years from the perennial reach) were examined. Size range was 55–75 (mean = 62.1, *SE* = 0.46) and 55–74 (mean = 61.5, *SE* = 0.33) mm SL from the intermittent and perennial reaches, respectively. After SL measurements, fish were dissected and sex determined.

Body condition (BC) is often estimated by using the body mass relative to size, and then compared to a reference value. Therefore, the BC concept emerges as the plumpness, fatness, or well-being of the animal (García-Berthou, 2001; Jakob et al., 1996). Indeed, a BC index can also be used as a morphological approach to measure variations in physical health of fish at a short-term scale (e.g. weeks or months; Le Cren, 1951; Nyboer & Chapman, 2017). In this study,

eviscerated body mass (eBM, ±1 mg) was measured using an electronic balance to provide an integrated quantification of BC. Thus, this procedure avoids bias from gonad mass and gut content. The data on fish size were then used for the estimation of BC (see details in Section 2.7).

Following Adams et al. (1993), a health assessment index (HAI) was computed. This is another approach to measure variations in fish physical health, but unlike other *external* condition indices (e.g. BC), a variety of internal organs are usually examined to compute the HAI (see details below). Thus, the simultaneous use of both BC and HAI (i.e. on the same fish individuals) provides a more complete picture of the physiological state of individuals. Moreover, HAI helps to complement data on parasite infra-communities (see details below in Section 2.5) as a proxy of *tolerance* (Blanchet et al., 2010; Råberg et al., 2007) and it can also reveal the effect of poor physiological conditions over short periods (e.g. YOY fish, see Chapman et al., 2015). Specifically, skin (fins and scales), eyes, and gills were externally examined for abnormalities. Then, major organs were internally examined to assess the colour, size, and condition: alimentary tract, heart, liver, spleen, and gonads. Special care was taken to avoid damaging parasite fauna during this health assessment (see below in Section 2.5). Scores for each examined category range from 0 to 30 and were combined to compute a particular HAI value per fish. An HAI score of zero indicates a fish that did not have any appreciable abnormality, whereas a high score reflects an individual perceived to be unhealthy. Theoretically, HAI could range between 0 and 240 (i.e. 30×8 anatomical regions/organs) in this study, although no fish exceeded a total score of 100.

Given that most morphological and parasitological traits can vary significantly after relatively recent disturbances (see Chapman et al., 2015 for an example in parasites of juvenile fish), it may be appropriate to use other parameters to detect variations in physiological state arising as a result of drivers operating over longer time scales. In this respect, fluctuating asymmetry (FA) is considered a morphological trait that acts as a good indicator of developmental instability throughout an animal's life span (Parsons, 1992). Such an approach has previously been applied successfully in selected fish taxa (Almeida et al., 2008). The study minnow species can reach a maximum age of 5 years in Tordera Stream (D. Almeida, personal observations), although the examined fish were specifically found to be between 3+ and 4+, through scale observations (true annuli). As mentioned above, fish individuals cannot be too young to effectively detect developmental instability. To test for differences in FA between stream reaches, three morphometric traits were measured: eye diameter, and pectoral and pelvic fin lengths. These bilateral characters were chosen because they commonly respond to developmental instability in fish (Almeida et al., 2008; Almeida & Grossman, 2014). A random sub-sample comprising 30% of the examined individuals was re-measured to estimate measurement error (see details below in Section 2.7). To minimise such an error, all measurements were taken by the same person (D. Almeida). To obtain more accurate morphometric measurements (±1 μm), bilateral characters (i.e. eyes and fins) were examined using a dissecting microscope (40×) with integrated camera (Leica Microsystems EZ4). Images were then processed and paired traits were measured by using the software Aperio ImageScope v.12 (Aperio Technologies).

2.5 | Parasitological examination

Ecto- and endo-parasites were identified and quantified per fish during the HAI examination (i.e. the same anatomical regions and organs, see above in Section 2.4). In this study, the number of fish examined for parasites was much higher than the number selected after applying species accumulation curves in previous papers (e.g. see figure 1 in Chapman et al., 2015; <20 fish individuals per site). Thus, the sample size (>30 individuals per site) in the present work achieved a good representativeness of parasite infra-communities in the study stream reaches. Fish were examined under a dissecting microscope (40×) for the presence of parasites according to standard protocols (Stoskopf, 2010; Weber & Govett, 2009). Parasites were also identified using a light microscope (1000×) equipped with phase contrast and differential interference contrast. Parasites were excysted, where necessary, by using fine forceps, and fixed in 70% ethanol for later staining (e.g. iron acetocarmine) and identification. Parasites were identified using the keys/databases in Brewster (2016), Bruno et al. (2006), and Illán-Aguirre (2012). For particular parasite taxa, family or genus were the lowest practical taxonomic levels, since these categories provide enough information to assess the ecological role of parasites as potential bio-indicators (Chapman et al., 2015; Poulin, 1997). After examination and counting, specimens were deposited in the Parasitological Collection of the Department of Basic Medical Sciences, San Pablo-CEU University (Boadilla del Monte, Spain).

2.6 | Genetic analysis

Microsatellite markers are used in riverine fishes as a proxy of genetic diversity (Blanchet et al., 2009, 2010). Adapted from Aljanabi and Martinez (1997), a sample (*c.* 25 mg) of muscle tissue was taken from each minnow individual (right flank) to extract total DNA by using the DNeasy® Blood & Tissue Kit (Qiagen). DNA was quantified in a Nanodrop® Lite Spectrophotometer (ThermoScientific). As per Blanchet et al. (2009), 15 polymorphic microsatellite *loci* were selected for this study (Table 1) due to their high variability. These markers were isolated and developed on Cypriniformes fish phylogenetically close to the study species *Phoxinus septimaniae* (Grenier et al., 2013). After microsatellite selection, this particular set of markers was tested on the minnow tissue samples to ensure that genetic data were suitable for the study species (see below in Section 2.7). For each minnow specimen, locus markers were co-amplified by using the QIAGEN Multiplex PCR Kit (Qiagen). Polymerase chain reactions (PCRs) were carried out in a 16-μl final volume containing 20–25 ng of genomic DNA, 8 μl of 2 × QIAGEN Multiplex PCR Master Mix, and

TABLE 1 Description of the microsatellites ($n = 15$) used in this study to estimate genetic diversity in Languedoc minnows *Phoxinus septimaniae* from Tordera stream

Locus (GenBank ID)	Primers 5′-3′	Allele size range	NA	Core motif	References
CypG9 (AY439127)	F-FAM: GCAGTCACGTATTAAGGCGAGCAG R: GAGCGGACTCTCAGGCACCTACC	106–114	3	$(CAGA)_5$	Baerwald and May (2004)
CtoA-247 (GU254031)	F-FAM: TGCAAACATATAAACTGAAACAAGG R: GCAGGTATATTCCCAGCC	160–179	7	$(ATC)_7$	Dubut et al. (2010)
LleC-090 (FJ601722)	F-FAM: TCAGACACAACTAACCGACC R: GGCGCTGTCCAGAACTGA	230–313	13	$(TC)_{15}GG(TC)_3$	Dubut, Martin, Costedoat, et al. (2009)
Ppro132 (AY254354)	F-ATTO565: GCATTTCCTTTTGCTTGTAAGTCTCAA R: GGTTTAACCCGATCAATGGCTGTGC	117–124	4	$(CT)_{18}$	Bessert and Ortí (2003)
LleB-072 (FJ601720)	F-ATTO565: TCATTAGGGAGGCTGCTTATTC R: CCTTTTCAACAATTTGTCACGG	159–181	7	$(TG)_{13}$	Dubut, Martin, Gilles, et al. (2009)
LceC1 (AY962241)	F-Bodipy530: AGGTGTTGGTTCCTCCCG R: TGTTATCTCGGTTTCACGAGC	89–119	12	$(CA)_{15}$	Larno et al. (2005)
Rru4 (AB112740)	F-Bodipy530: TAAGCAGTGACCAGAATCCA R: CAAAGCCTCAAAAGCACAA	174–180	3	$(CA)_{15}$	Barinova et al. (2004)
Lco3 (AY318779)	F-ATTO550: GCAGGAGCGAAACCATAAAT R: AAACAGGCAGGACACAAAGG	241–251	3	$(TG)_9$	Turner et al. (2004)
BL1-44 (FJ468355)	F-FAM: AAGACCAGCATGTGCTT R: ACATAGACTAACCAGTTTCACTT	112–130	7	$(CA)_{13}$	Dubut, Martin, Gilles, et al. (2009)
Lsou5 (EF209002)	F-FAM: CTGAAGAAGACCCTGGTTCG R: CCCACATCTGCTGACTCTGAC	188–229	12	$(CA)_{17}$	Muenzel et al. (2007)
Lsou19 (EF209008)	F-ATTO565: TCCCGTGGAGAAACTACAGG R: TTCTTCGGTGAGTGTCGATG	164–181	4	$(GT)_{32}$	Muenzel et al. (2007)
BL1-98 (FJ468349)	F-ATTO565: ATTGTTTTCATTTTGTCAG R: CCGAGTGTCAGAGTTATT	273–330	14	$(CA)_9N_4(CA)_3N_{50}(CA)_4$	Dubut, Martin, Gilles, et al. (2009)
Rhca20 (DQ106915)	F-Bodipy530: CTACATCTGCAAGAAAGGC R: CAGTGAGGTATAAAGCAAGG	100–113	6	$(GA)_{17}$	Girard and Angers (2006)
BL1-84 (FJ468346)	F-Bodipy530: CATTACTACGGCAACCACAT R: GCGAAAAGGAAAGAGACTGA	187–194	4	$(AC)_4N_{24}(CA)_9$	Dubut, Martin, Gilles, et al. (2009)
CtoG-075 (GU254035)	F-Bodipy530: TCATTTGGATAACAATCCATCATCAC R: ACTATGTTAGCATCCACACC	220–230	5	$(GC)_4$	Dubut et al. (2010)

Note: For each pair of primers (F-forward, R-reverse), the following information is provided: locus name and GenBank ID, specific sequences, and fluorophore name (F-attached), allelic size range, allelic richness (N_A), core motif, and particular bibliographic reference.

locus-specific optimised combination of primers (detailed protocols are available from the authors upon reasonable request). PCR amplifications were performed in a Sure Cycler 8800 machine (Agilent Technologies) under the following program: 15 min at 95°C, followed by 30 cycles × (30 s at 94°C, 90 s at 56°C, and 60 s at 72°C), and finally a 45-min final elongation step at 72°C. Amplified fragments were separated on an ABI PRISM 3730 automated capillary sequencer (Applied Biosystems) in the Department of Genomics and Proteomics at the Complutense University (Madrid, Spain). Allelic sizes were scored using the molecular weight marker GeneScan-500 LIZ® (Applied Biosystems). Genoypes were analysed by using the software Peak Scanner v2.0 (Applied Biosystems) to generate individual profiles with the 15 microsatellites.

2.7 | Data analyses

Linear models were used to test for differences between males and females on the examined traits, as per Almeida et al. (2014). Given that no difference was found (overall *p*-values >0.05), this categorical factor (i.e. sex) was not included in subsequent models. Data were pooled because the effects of *sampling site* and *year* were not significant within each stream reach, after previous linear mixed models by using *site* and *year* as the random factors.

For FA analysis in fish, statistical techniques followed Almeida & Grossman (2014; see specific details and a comprehensive list of references therein). Neither directional asymmetry, anti-asymmetry, nor a significant contribution of the measurement error were detected. Consequently, differences in size between both sides were

considered as true FA for the examined bilateral characters. The unsigned (absolute) values, i.e. right minus left ($|R-L|$), were used to assess the FA levels between stream reaches.

To explore spatial patterns, quantitative descriptors were used to properly analyse the variation in parasitological traits between stream reaches. Two parameters of parasite infra-communities were calculated for each fish individual (adapted from Bush et al., 1997): *total load* (TL, total number of parasite individuals detected from the examined anatomical regions/organs); and *diversity* (Shannon index H', computed with $\log_2$). TL can be used as a proxy of host *resistance* (Blanchet et al., 2009, 2010), since a highly parasitised fish usually exhibits low resistance due to a poor immune response (Råberg et al., 2007). Parasite H' can be used as a proxy of *complexity* in freshwater ecosystems (Lafferty, 2008; Marcogliese, 2005). Additionally, *prevalence* (the proportion of examined hosts infected with parasites) was also calculated.

With respect to genetic traits, no null alleles or linkage disequilibrium for each pair of *loci* were detected (see specific details on the statistical procedure in Blanchet et al., 2009). Two genetic indices of homozygosity were calculated: *internal relatedness* (IR, see specific algorithm in Amos et al., 2001); and *homozygosity by locus* (HL, see specific algorithm in Aparicio et al., 2006). As per Blanchet et al. (2009), both indices were highly correlated ($r = 0.94$, $p < 0.001$), with only IR (range: from −0.18 to 0.39) being finally reported in the present study.

A Chi-square test was used to compare prevalence between stream reaches. Despite the largest individuals being selected for data analyses (see above in Section 2.4), the effect of body size was controlled for subsequent comparisons by using analysis of covariance (ANCOVA), to account for its potential effects. In particular for BC, ANCOVA allowed the comparison of eBM between stream reaches, after controlling for fish size (covariate: SL). This analytical procedure is preferable to the use of residuals from linear regressions between the study parameters and the size (García-Berthou, 2001) or computing indices/ratios (e.g. Fulton's condition factor; García-Berthou, 2001; Packard & Boardman, 1999). As with BC, ANCOVA (covariate: eBM) was used to test for significant differences between stream reaches for the response variables HAI and the two parasite descriptors (TL and H'). FA was also assessed by means of ANCOVA, with *character size* (i.e. the average between both sides, $[R+L]/2$) as the covariate (Almeida et al., 2008; Almeida & Grossman, 2014). One-way ANOVA was used to detect significant differences in IR between stream reaches. Relationships between the three groups of biological traits were fitted by generalised additive models (GAMs). This technique was performed because, unlike more conventional regression methods, it does not require the assumption of a particular shape for the relationship (e.g. linearity) between variables. Indeed, Blanchet et al. (2009) found a curvilinear association (polynomial/quadratic function) between parasite load and genetic diversity. The complexity of GAMs was selected by a stepwise procedure according to Akaike information criterion. Thus, only models showing the highest goodness of fit and parsimony were extracted.

Data were transformed by using $\log_{10}(x+1)$. In particular for absolute FA distributions, these data were normalised using the two-parameter Box–Cox transformation (Almeida & Grossman, 2014 and references therein). Assumptions of normality of residuals and homogeneity of variances were verified through residual plots and Levene's tests, respectively. Statistical analyses were performed with R v. 3.6.3 (R Core Team, 2020). The significance level was set at $\alpha = 0.05$. Values reported in results are means $\pm$ *SEM*.

3 | RESULTS

The eBM range was 1,971–4,633 and 1,920–4,385 mg from the intermittent and the perennial reaches, respectively. The difference in eBM was marginally non-significant (ANCOVA: $F_{1,297} = 3.05$, $p = 0.081$) between stream reaches, after controlling for the effect of fish size, with minnows displaying a slightly better BC (i.e. adjusted eBM, mg) in the intermittent reach ($2,604 \pm 82$) than the perennial reach ($2,427 \pm 51$). Statistical differences were found for HAI (ANCOVA: $F_{1,297} = 4.43$, $p = 0.036$), with this index (specific range: 0–100) being significantly lower (i.e. better fish health) in the intermittent reach (28.9 ± 1.5) than in the perennial reach (33.2 ± 1.2). Significant morphological differences were also found for FA. In particular for the eye diameter and the length of pectoral fins, with minnows showing a lower FA level (i.e. higher developmental stability) in the intermittent stream reach. FA for pelvic fins was slightly lower in the intermittent reach; however, this was not statistically significant ($p = c$. 0.10; Figure 2).

In the intermittent reach, minnows showed a lower percentage of parasite prevalence (62%) relative to the perennial reach (71%), although this difference was not statistically significant ($\chi^2_1 = 2.48$, $p = 0.115$). A total of 13 and 20 parasite taxa were identified in minnows from the intermittent and perennial reaches, respectively. Moreover, many of the parasite taxa not observed in the intermittent reach display complex life-cycles, i.e. more than one host, such as *Ligula*, Digenea and *Camallanus* (Table 2). The most abundant parasites found in minnows from both stream reaches were Monogenea and Digenea taxa, particularly the genus *Gyrodactylus* and the species *Posthodiplostomum cuticola*. Most of the parasite taxa were more abundant in minnows from the perennial reach. Consequently, the difference between stream reaches was very significant for TL (ANCOVA: $F_{1,201} = 8.79$, $p = 0.003$), which was lower (number of parasites) in minnows from the intermittent reach (20.0 ± 1.7) than from the perennial reach (28.4 ± 1.6). Similarly, parasite diversity (H') was also significantly (ANCOVA: $F_{1,201} = 6.48$, $p = 0.012$) lower for colonising individuals (2.24 ± 0.04) than for resident minnow (2.38 ± 0.03).

The difference of IR was very significant between stream reaches (ANOVA: $F_{1,298} = 8.83$, $p = 0.003$). This homozygosity index was significantly lower in minnows from the intermittent reach (0.074 ± 0.020) than the perennial reach (0.143 ± 0.013). Concerning relationships in biological traits, the GAM technique provided only two significant and positive associations. One model consisted of

a linear regression between (log-transformed) parasite TL and IR (TL = 1.85×IR+2.79, $r = 0.40$, $F_{1,202} = 27.58$, $p < 0.001$). A relationship was also detected by GAM between (Box–Cox transformed) FA for pectoral fins and IR (FA = 0.01×IR+0.98), although this association was weaker ($r = 0.19$, $F_{1,298} = 5.39$, $p = 0.021$).

4 | DISCUSSION

Non-native species commonly display a great *flexibility* in a variety of biological traits (e.g. growth, reproduction, diet) when invading new habitats, with this being particularly apparent in freshwater fishes (Copp et al., 2005; Vilizzi et al., 2019). Specifically for the Iberian Peninsula, which mostly has a Mediterranean climate, invasive Cypriniformes fishes from the rest of Europe, which mostly has a Temperate climate, usually display high variability under contrasting environmental conditions. This contributes to enhance the invasion process throughout this eco-region of high richness, in terms of fish biodiversity (Latorre et al., 2018, 2020; Ribeiro & Veríssimo, 2014). Accordingly, the examined biological traits were clearly variable within the study population, which is surely linked to particular fluvial conditions (i.e. flow regime, habitat constraints; Gasith & Resh, 1999). Indeed, this study showed how such a variability was also observed in other traits (morphology, parasitology, and genetics) very different from the typical analyses of life-history, fecundity or feeding tactics (see above in Section 1 for study examples). This variety of biological strategies is probably displayed as a pre-adaptation to invade novel habitats, facilitating fish establishment, reproduction, and spread (e.g. see a comprehensive review on invasion steps in Sakai et al., 2001).

Regarding morphology, minnows from the intermittent stream reach displayed better body condition (close to statistical significance) and health index, and lower developmental instability (low FA level). These traits may simply reflect a colonisation process due to a breeding migration. Thus, minnow migrate upstream to reproduce, with the most vigorous specimens being able to reach these sites. Indeed, the sampling period (mid-May) included the breeding season of this species in Tordera Stream and large reproductive individuals (e.g. gravid females, nuptial tubercles in males) were observed and captured in the intermittent reach. However, this case does not appear to be a simple breeding colonisation process, where *better* specimens reach *better* upper zones. In the present study, the intermittent reach could be considered as a sub-optimal habitat for a Euro-Siberian fish species, such as the Languedoc minnow. Thus, the intermittent reach is ecologically similar to a temporary river, where the seasonal cessation and onset of flow imply a sequential re-colonisation of different biological communities (e.g. biofilm, submerged vegetation, benthic invertebrates) prior to fish arrival and settlement (Larned et al., 2010). Moreover, minnow can spawn along the middle reach of Tordera Stream, as it was mentioned in the description of this study population. Consequently, a *partial migration* scenario may be occurring during breeding season. In this ecological context, Chapman et al. (2012) observed a similar pattern after reviewing scientific literature on a variety of taxonomic cases, with large reproductive fish being mainly found in optimal breeding grounds. According to these two considerations
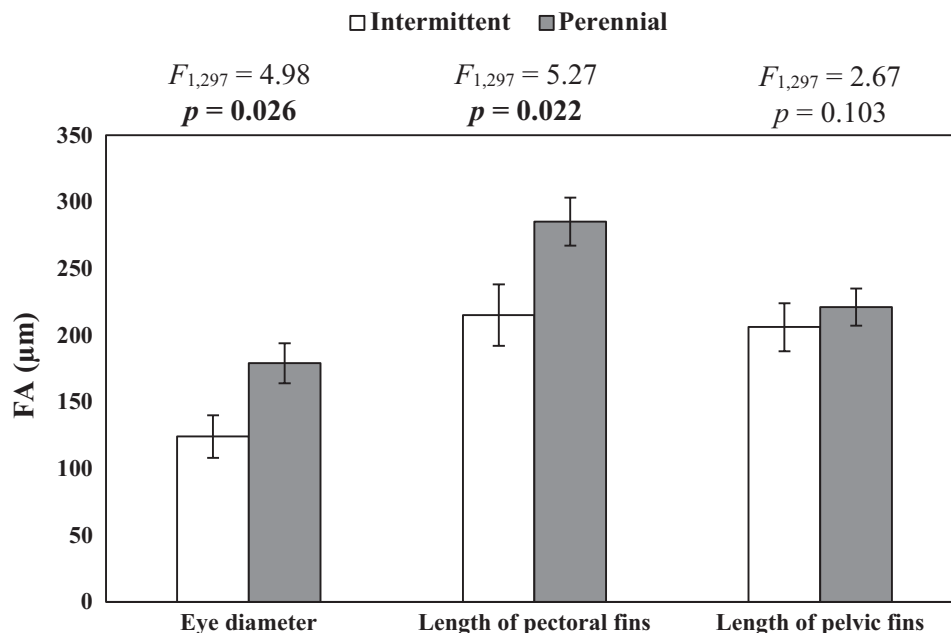

FIGURE 2 Fluctuating asymmetry (FA) levels (μm) of three bilateral and morphometric characters in Languedoc minnows *Phoxinus septimaniae* from Tordera stream. Results are (adjusted) means ± SEM, per reach type. *F*-ratios, degrees of freedom, and significance levels (*p*-values) of the ANCOVA factors are presented (covariate: character size, see Section 2.7 for details). Significant results are highlighted in bold

TABLE 2 Parasite list found in Languedoc minnows *Phoxinus septimaniae* from Tordera stream

Parasite (taxon)	Parasite (family/genus/species)	Intermittent	Perennial
Ciliophora	*Trichodina* sp.	0.9	1.3
Cestoda	*Ligula intestinalis*	–	0.9
	Proteocephalus exiguus	0.6	0.8
Monogenea	*Dactylogyrus phoxini*	1.8	1.6
	Paradiplozoon homoion	0.8	0.7
	Gyrodactylus macronychus	6.4	8.9
	Gyrodactylus phoxini	3.3	2.8
Digenea	*Allocreadium transversale*	0.3	0.4
	Diplostomum commutatum	–	0.7
	Diplostomum phoxini	–	0.4
	Hysteromorpha triloba	0.4	1.2
	Ichthyocotylurus platycephalus	0.7	1.6
	Posthodiplostomum cuticola	2.8	3.9
	Tylodelphys clavata	–	0.6
Nematoda	*Camallanus lacustris*	–	0.3
	Raphidascaris acus	1.1	0.6
Acanthocephala	*Neoechinorhynchus rutili*	0.7	0.2
Mollusca	Unionidae (glochidium larvae)	–	0.1
Crustacea	*Argulus foliaceus*	–	0.5
	Ergasilus sieboldi	0.2	0.9

Note: Mean parasite loads are shown per family, genus or species and reach type. Dashes indicate undetected parasites on examined minnow individuals ($n = 62$ for the intermittent reach and 142 for the perennial reach).

(i.e. intermittent reach as a sub-optimal habitat and minnow displaying partial migration), the morphological findings found in Tordera Stream were puzzling and contrary to our expectations (see first and second hypotheses in the Section 1), as migrating fishes tend to involve less competitive individuals (i.e. poor physical status) that move to areas with sub-optimal conditions. These individuals can be forced to pay the costs of migration in order to seek new niche opportunities, which is known as the *competitive release hypothesis* (Chapman et al., 2012). Although it is true that the study fish population is non-native in Tordera Stream, the same pattern of intra-specific competition (i.e. migrants displaying low body condition) has been also observed for the dispersal of invasive fishes (Grabowska et al., 2019). Therefore, an overall potential explanation for the present results consists of considering that body condition, health index, and asymmetry level are highlighting individuals with higher colonisation capacity. This has been also observed by Brodersen et al. (2008) in the common roach *Rutilus rutilus*, another seasonal and partly migrant leuciscid fish. Specifically, roach with better body condition migrated to streams from a lake during winter to avoid the predation risk. Nonetheless, the lake (as *source* of migrants) was the sub-optimal habitat in this case. Merciai et al. (2017) observed contrasting responses for Cypriniformes fish assemblages in Tordera Stream. In particular, Mediterranean barbel displayed higher body condition in the intermittent reach (as per minnows), whereas this parameter was lower for the Catalan chub. These results suggest species-specific strategies to the same

environmental constraints. Similarly to our results, Alexandre et al. (2014) analysed the eco-morphology of the Iberian barbel *Luciobarbus bocagei* and found a lower body condition in perennial reaches when compared with the population from the temporary water course. The explanation consisted of a relationship between body condition/shape and hydro-dynamics for this large Cypriniformes species (up to 500 mm of total length). However, the effect of hydraulic conditions may be profoundly different on the small-bodied study species (genus *Phoxinus*; Tan et al., 2018). Despite the ecological interpretation mentioned above (i.e. individuals with higher colonisation capacity in the intermittent reach), habitat suitability must be properly assessed in terms of biological fitness to reveal the adaptive significance of the observed migration pattern. Thus, colonisation capacity may be a proximal factor explaining partial migration, but an increased reproductive success should be also detected as a *selective payoff* for this reproductive behaviour to persist in the intermittent reach. More specifically for FA, pelvic fin was not found as a bilateral character showing differences between stream reaches. This could be related to the selected trait, according to its functional importance, which is a general pattern previously observed in aquatic tetrapods. Thus, anurans (Didde & Rivera, 2019) or freshwater turtles (Rivera & Neely, 2020) display lower FA in hind-limbs (more important than fore-limbs for aquatic propulsion), whereas the use of pectoral fins is higher in Cypriniformes fishes for manoeuvrability, displaying a lower FA level (Almeida et al., 2008).

With respect to health and parasitology, hosts can protect themselves against parasites by actively reducing parasite load (i.e. resistance) or by limiting the damages caused by parasites (i.e. tolerance; Blanchet et al., 2009, 2010; Råberg et al., 2007). Both parasitological levels (i.e. resistance and tolerance) were higher in the intermittent reach, with minnow displaying a lower parasite load and better health index, respectively. A potential explanation could be related to a higher immuno-competence (see an example for common carp in Rohlenová et al., 2011). As per previous results, colonising individuals appeared to be well-equipped (e.g. immune response) to spread in a harsher environment, relative to a more stable perennial reach. Evidence of a genetic–immune mechanism appear to exist in animal populations (Råberg et al., 2007), including invasive birds (Prüter et al., 2020) and mammals (Biedrzycka et al., 2020), although less support has been found for non-native fish (but see a possible example for an invasive goby in Gendron & Marcogliese, 2017). Thus, more research is needed to reveal the particular regulatory mechanisms at the genetic level and the components of the immune response that are acting at the molecular/cellular levels (Rohlenová et al., 2011). Alternative to this immunological interpretation, health and parasitological results may simply be a direct consequence of the environmental context. Given the similarities between the artificial flow alteration in the intermittent reach and natural temporary rivers in Mediterranean ecosystems, the structure of aquatic ecosystem must be much simpler than downstream biological communities. As mentioned before, this is due to the need for a sequence of re-colonisation events in the intermittent reach (Larned et al., 2010). Accordingly, higher parasite taxonomic richness and diversity were found in the perennial reach. More specifically, several parasite taxa on minnows display complex life-cycles (e.g. Cestoda, Digenea, Nematoda), with up to four potential different host species (e.g. copepods, freshwater snails, fishes, birds). Representative examples were *Ligula*, *Camallanus*, *Diplostomum*, and *Tylodelphys* species, which were only found in minnows from the perennial reach. In particular for Digenea flukes (i.e. the two latter genera), these parasites have a larval stage, so-called metacercaria, which often infect the eyes or brain of fish (intermediate host) and alter its behaviour to be better detected by piscivorous birds (e.g. herons or gulls as definitive host species), where the parasites then reproduce. Since these parasite species require habitats with a certain degree of ecological and taxonomic diversity, our study demonstrates that: (1) fish infra-communities may rapidly change in colonising individuals (few months; Faltýnková et al., 2011); and (2) parasites can be used as a surrogate of biotic relationships in the wider environment (Lafferty, 2008; Marcogliese, 2005), according to our expectation (see third hypothesis above). Thus, a combination of low fish density (see data in the description of Section 2.3) and low biological complexity may help colonising minnows to avoid parasites (and improve health) in the intermittent reach.

For genetic traits, this minnow population is the result of few and relatively recent introductions in Tordera Stream (*c.* 20 years; Corral-Lou et al., 2019), with the original diversity being presumably low. Thus, the deeply contrasting environmental conditions between stream reaches have been able to induce such intra-population phenotypic differences (e.g. morphology), probably also including their genetic bases. In accordance with the fourth hypothesis (see Section 1), genetic diversity was lower in resident minnow, similarly to the result found by Nevado et al. (2013) when comparing fish within the same population between contrasting habitat types. In the Tordera system, the intermittent reach was considered an environmentally more unstable habitat due to its particular seasonal dynamic. Specifically, this study showed that minnow colonising this fluctuating habitat to spawn were genetically more diverse. Given that this reproductive strategy is repeated annually, such conditions could result in micro-evolutionary changes at the intra-population level. Consequently, further extensive studies on population genetics are needed to reveal particular structures between stream reaches, accounting for a wide range of ages/sizes (i.e. reproductive adults and YOY fish) in May–June. In particular, differences of allelic richness, SNPs or other specific molecular markers (see an example for North American sub-populations of rainbow trout *Oncorhynchus mykiss* in Deiner et al., 2007) may reveal evidence of *rapid evolution* within this non-native minnow population; a typical feature of many bio-invaders (Whitney & Gabler, 2008), including fish (Costa-Pierce, 2003). In this respect, Blanchet et al. (2009, 2010) analysed the relationship between parasite load and IR in the leuciscid dace *Leuciscus leuciscus*, inhabiting the River Viaur (south-west France). The authors found a unimodal (curvilinear) response, with higher load for intermediate IR values, which was explained as a micro-evolutionary process of intra-population disruptive selection. In our study, a linear relationship was found instead. Two main differences exist between the studies: (1) Blanchet et al. (2009, 2010) analysed only one ecto-parasite species (*Tracheliastes polycolpus*, Copepoda), whereas a more comprehensive parasitological study (up to 20 taxa, both ecto- and endo-parasites) was carried out in the present work; (2) dace is native to the River Viaur, whereas the study minnow population is non-native, which may imply a different evolutionary process to rapidly adapt to the novel habitat conditions (see a more detailed explanation above). Moreover, the positive linear relationship between parasite load and homozigosity (i.e. low genetic diversity) may represent a support for the previously mentioned genetic immunocompetence explanation. Thus, more genetic diversity may imply a better immune response and a lower parasite load (Gösser et al., 2019). Indeed, the observed higher developmental stability (low FA level) in colonising individuals was also related to an elevated genetic diversity (Almeida et al., 2008; Parsons, 1992), which may broaden their environmental tolerance.

In conclusion, our results show that colonising minnows appear to display a particular profile at the morphological, parasitological, and genetic levels that allow these individuals to increase their spread capacity in harsher environmental conditions. This will probably facilitate the persistence and subsequent invasion in other typical Mediterranean and temporary water courses across the Iberian Peninsula. To test for potential limitations of the present study, future research perspectives on biological fitness, immunology, and population genetics have been suggested above. Even though this study focuses on one fish species in one stream system, these data complement those from other native species within this

stream, which are also exposed to the same colonisation dynamics, such as Mediterranean barbel and Catalan chub (see these fish examples above and originally in Merciai et al., 2017). For these native species, data on parasites, health, asymmetry, and genetic diversity would provide a more complete *picture* in relation to the environmental mechanisms structuring fish assemblages in the Tordera system. Apart from the Iberian eco-region, this study species and stream system could be used as a model for ecological/evolutionary processes in a broader taxonomic range of colonising non-native fish world-wide. More specifically, the biological/environmental model presented here could provide insights into the complex ecological process of *invasion front*, as *founder* populations can largely display contrasting biological traits than *resident* populations. As one example in the Danube River, round goby *Neogobius melanostomus* exhibited an upstream-directed range expansion, which was not caused by out-migrating weak individuals, similarly to the present study. However, greater abundances of particular endo-parasites were observed at the invasion front, contrary to our results (Brandner et al., 2013). Also, in the John Day River (Oregon, USA), Rubenson and Olden (2017) analysed the *leading edge* for an upstream expansion of invasive smallmouth bass *Micropterus dolomieu*, providing critical information on morphological growth potential to predict future range shifts. Thus, there is a need for more research on colonising non-native fishes along fluvial ecosystems. All these overall findings will surely assist environmental managers to identify vulnerable areas (according to habitat conditions), where monitoring programmes can be established for early detection. This would contribute towards reducing the spread of non-native fish populations.

AUTHOR CONTRIBUTIONS

A.C., P.A., E.G-B., and D.A. conceived the ideas, and designed the research. E.G-B., D.H.F., and D.A. collected samples, and performed the field work. A.C., C.L., I.M-B., G.C-G., P.A., and D.A. performed the laboratory work. E.G-B., D.H.F., and D.A. performed the statistical analyses. A.C., C.L., and D.A. led the writing, with assistance from E.G-B., and D.H.F.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ORCID

Alejandra Cruz https://orcid.org/0000-0002-5128-7035
Carla Llinares https://orcid.org/0000-0003-0028-9564
Inés Martín-Barrio https://orcid.org/0000-0001-6282-6578
Gema Castillo-García https://orcid.org/0000-0003-1034-2138
Pilar Arana https://orcid.org/0000-0002-7828-5945
Emili García-Berthou https://orcid.org/0000-0001-8412-741X
David H. Fletcher https://orcid.org/0000-0001-5029-7453
David Almeida https://orcid.org/0000-0003-0592-7174

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