



# Major determinants of the occurrence of a globally invasive parasite in riverine fish over large-scale environmental gradients

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## ABSTRACT

The increased rate of outbreaks of infectious diseases in ecosystems is a dramatic consequence of global change, particularly when outbreaks affect important resources such as freshwater fish. However, the links between disease-inducing epizootics and widespread human impacts, including nutrient pollution and high water conductivity, in freshwater organisms are largely unexplored. We used data from extensive surveys in northeastern Spain (99,700 km<sup>2</sup>, 15 river catchments,  $n = 530$  sites) to explore the environmental factors that singly, or in combination, are likely to influence the occurrence of the invasive parasite, *Lernaea cyprinacea*, after accounting for host fish characteristics. Smaller fish, lower altitudes, higher water conductivity and nutrient pollution were associated with higher probabilities of infection in 19 endemic and widely distributed fish species. We found no evidence that interactive effects among riverine stressors related to water and physical habitat quality better explained the probability of occurrence of *L. cyprinacea* in fish than did additive-stressor combinations. Nutrient pollution and high water conductivity were two of the major factors contributing to the increased occurrence of *L. cyprinacea*. Therefore, the improvement of wastewater treatment processes and agricultural practices probably would help to reduce the occurrence of this parasite among native fish.

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## 1. Introduction

Infectious diseases are emerging at unprecedented rates and scales, raising concerns among conservationists, ecologists, and public health agencies (Jones et al., 2008; Smith et al., 2014). Although the causes of the emergence of many diseases are uncertain, human actions probably contribute substantially to many epidemics (Rogalski et al., 2017). Human activities such as intensive agriculture (Ponisio et al., 2016), fishing (Kaiser et al., 2015), and dam construction (Poff et al., 2007) are homogenising aquatic ecosystems around the world, and disease outbreaks often arise when ecosystems are simplified (Elton, 1958). Ecosystems are altered further by the accumulation of alien species, which continues unabated (Seebens et al., 2017). Given that the global human footprint is expected to increase even further (Hoekstra and

Wiedmann, 2014), understanding how human impacts affect infection dynamics is a priority for disease ecology under global change.

Global change factors consist of a variety of biotic and abiotic alterations induced by human actions (hereafter referred to as stressors), which may interact in complex ways (see Piggott et al., 2015; Côté et al., 2016). Native hosts are often more vulnerable to alien parasites than to native parasites (Dunn and Hatcher, 2015). Other global change processes such as aquatic nutrient pollution (Sinha et al., 2017) can further increase host vulnerability if such processes increase the virulence of a disease (Budria, 2017). However, the impacts of pollution on host-parasite relationships are difficult to predict due to context (Marcogliese and Pietrock, 2011). While high concentrations of pollutants, including nutrients, can kill both parasites and hosts (Vidal-Martínez et al., 2010; Smallbone et al., 2016), nutrients sometimes may improve host body condition and allow hosts to better cope with the effects of pollutants and parasites (Marcogliese and Pietrock, 2011; Budria, 2017). Nutrient pollution also is expected to promote the spread of horizontally transmitted parasites (e.g. ectoparasites) because nutrients increase host population density (McKenzie

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and Townsend, 2007). External parasites often are more sensitive to environmental fluctuations in aquatic systems than are internal parasites (Overstreet, 1993). Riverine ecosystems are among the most dynamic and human-affected ecosystems in the world (Williamson et al., 2008), yet the ways in which multiple natural and anthropogenic factors affect interactions and epidemiology in host–parasite systems remain little studied (Blasco-Costa et al., 2013; Hofmann et al., 2016; but see individual stressors reviewed in Sures et al., 2017).

The Asiatic anchor worm *Lernaea cyprinacea* Linnaeus 1758 is a parasitic copepod introduced, incidentally, widely into temperate and tropical regions with fish stocking (Hoffman, 1999; Hassan et al., 2008; Welicky et al., 2017). The anchor worm is capable of infecting fish and amphibians (Hoffman, 1999), and high mortalities have been reported for fish suffering from anchor worm infections (Noga, 2011; Raissy et al., 2013). The anchor worm is expected to spread further with global warming (Bulow et al., 1979; Marcogliese, 1991; Sánchez-Hernández, 2017) and when fish are forced into high concentrations during drought (Medeiros and Maltchik, 1999; but see Welicky et al., 2017). Infections by parasitic copepods including *L. cyprinacea* are influenced by fish size (Poulin et al., 1991), water quality stressors such as nutrient pollution, and other contaminants which can kill the parasites (Noga, 2011; Palm, 2011). However, rivers are subject to other stressors such as alterations in river morphology and riparian areas than can reduce body condition in host fish (Maceda-Veiga et al., 2014), potentially increasing fishes' susceptibility to disease. However, interactions among stressors may be advantageous to the fish if sewage effluents contain chlorides, which are strongly correlated with river–water conductivity (Fernandez-Turiel et al., 2003) and reduce the toxicity of some nutrients such as nitrite (Noga, 2011).

Determining the spatial location of disease outbreaks, the individuals that are most vulnerable, and the environmental conditions that promote infection, are central thinking in disease ecology (Stephens et al., 2016). The limited spatial extent of datasets often hinders the identification of relationships between parasitic infections and large-scale environmental gradients (Stephens et al., 2016). While the effects of multiple factors on parasite transmission are testable experimentally (e.g. Lenihan et al., 1999; Studer and Poulin, 2013), manipulations rarely can be conducted at the spatial and temporal scales at which biota actually experience the stressors (Mac Nally, 1997). Smaller scale experiments may not well represent patterns of spatial and temporal variation in stressors and hence the consequent biotic effects (Englund and Cooper, 2003). Experiments exploring the effects of multiple factors also require many animals as experimental units, which is ethically unacceptable for studies on vertebrates, especially threatened species, under animal welfare legislation (e.g. European Union (EU) Directive 2010/63/UE5). Therefore, extensive field surveys typically are used to identify the factors that, alone or in combination, are harmful to biota (e.g. Dahm et al., 2013; Gieswein et al., 2017; Kath et al., 2018), including the causes of parasite presence.

Here, we explored the host and environmental factors that singly, or in combination, might influence the presence of the invasive parasite *L. cyprinacea* in 19 fish species in an extensive area of northeastern Spain (99,700 km<sup>2</sup>, 15 catchments). These rivers are subject to major environmental stressors that are common in rivers worldwide (e.g. impeded water flows, riparian removal, nutrient pollution, and high water conductivity) (Tockner et al., 2009), and such rivers are highly prone to biological invasions (Leprieur et al., 2008), including by *L. cyprinacea* (Sánchez-Hernández, 2017). The wide range of orographic and river conditions in this Spanish region (Sabater et al., 2009; Maceda-Veiga et al., 2013) allowed us to explore how combinations of different stressors

might affect a host–parasite system along upstream–downstream gradients. Fish species included endemic taxa (e.g. *Barbus haasi* Mertens 1925, *Parachondrostoma miegii* Steindachner, 1866) and widely distributed European native (e.g. *Anguilla anguilla* Linnaeus, 1758, *Salmo trutta* Linnaeus, 1758) and alien fish species (e.g. *Cyprinus carpio* Linnaeus, 1758, *Gambusia holbrooki* Girard, 1859) (Froese, R., Pauly, D., 2017. FishBase. World Wide Web electronic publication. [www.fishbase.org](http://www.fishbase.org). (Last accessed 03/02/2019)). Alien fish often replace native fish in degraded rivers (Maceda-Veiga et al., 2017), but the anchor worm has low host specificity (Hoffman, 1999; Noga, 2011) and can exploit most host fish.

Anchor worm infections were expected to occur mostly in low-land rivers because the copepod prefers warmer waters, and infections may be more limited by the riverine environment than by host availability given the parasite's lack of host specificity. We expected the probability of parasite presence to be the highest in degraded rivers because pollution often induces immunosuppression in fish and promotes disease (Yada and Tort, 2016; Bly et al., 1997; but see Biagianti-Risbourg et al., 2013; Smallbone et al., 2016). We explored the occurrence of interactions among some of the major natural and anthropogenic stressors that may govern parasite dynamics. Given that such stressor combinations are common around the world, the identification of these interactions will help to project the distribution of the parasite well beyond our study system.

## 2. Materials and methods

### 2.1. Study area

We gathered data on river environmental conditions and fish from surveys performed in northeastern Spain from 2002 to 2009 (e.g. Maceda-Veiga et al., 2013, 2017). This data set consisted of 530 sampled sites that involved all Catalanian catchments from the Muga to Riudecanyes basins, and the whole River Ebro and part of the Garonne basin (Fig. 1). We surveyed most river typologies in this region for hydrological alterations, riparian characteristics, geology, water quality, river size and elevation. Most of these rivers are small and have a typical Mediterranean hydrological regime, with droughts in summer and potentially torrential floods in autumn. Large rivers peak in flow in spring from snowmelt. We surveyed in low-flow conditions when fish populations are more stable and can be sampled most effectively using electrofishing (see Section 2.2). Both native and alien fish species were present in a wide range of geographic, hydro-morphological, and water conditions (see Supplementary Table S1 and Maceda-Veiga et al., 2017 for further details). The conservation status of riparian areas ranges from relatively well preserved riparian forests, grasslands, extensive agriculture areas, to weedy areas (Sabater et al., 2009).

### 2.2. Fish surveys and host factors

We used an international standardised fish sampling method (European Committee for Standardization (CEN) standards EN 14962 and EN 14011), in accordance with the European Water Framework Directive (EU Directive 2000/60/EC). Fish were sampled by single-pass electrofishing using a portable unit that generated up to 200 V and 3 A pulsed DC in an upstream direction, covering the whole wetted width of the 100 m long reaches at each location (e.g. Maceda-Veiga et al., 2017). We selected the location of each sampling site based on representativeness, and included a variety of habitat types (pools, riffles and runs). The same fishing equipment was used at all sites to avoid potential gear-related bias in fish captures. All fish were maintained in buckets provided with air pumps until the end of the survey. Fish captures were expressed

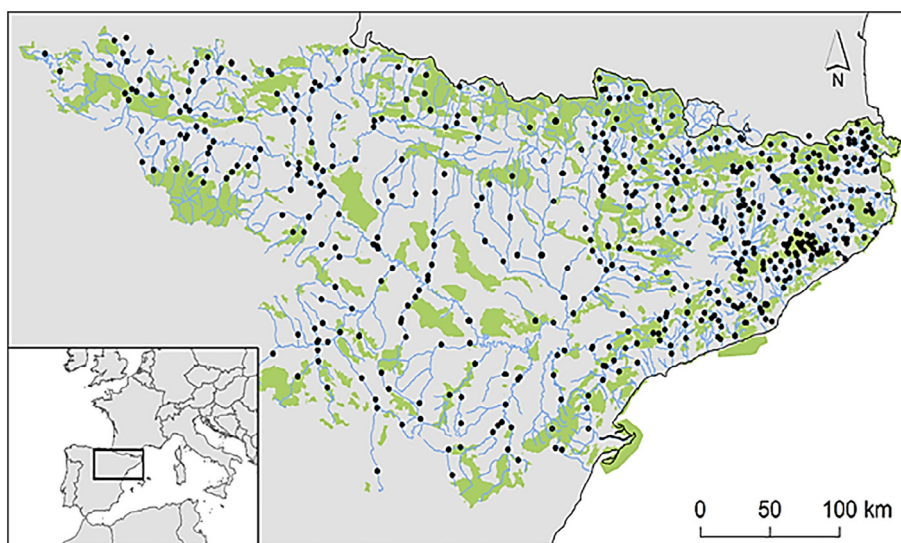


Fig. 1. Location of the 530 sampling sites surveyed in rivers of north-eastern Spain with protected areas highlighted in green (medium grey).

as captures per unit of effort (CPUE, fish caught divided by fishing time in min and the area surveyed in m<sup>2</sup>). A pilot study in our study area, comparing estimates of richness and abundance between single- and four-pass electrofishing, showed that estimates from a single pass were reasonably high with 80–100% of the species detected and 60–90% of the individuals captured (Sostoa, unpublished data).

Fish were identified to species, counted, and a haphazard set of individuals ( $\geq 40$  if possible) from each species in each sampling site was measured (total length, mm) and weighed (mg) after being anaesthetised in a buffered MS222<sup>®</sup> solution (0.02%, Tricaine methane-sulfonate, Sigma<sup>®</sup>). The entire external surface of measured fish was inspected for infections of *L. cyprinacea*. The copepod is easy to detect visually (see Noga, 2011). All fish were allowed to recover in buckets provided with air pumps and released at the site of capture. The procedures used in this study

were approved by the University of Barcelona (Spain) Ethical Committee (C.E.E.A. 510/14) and fish captures were authorised by the Autonomous Government of Catalonia, Spain (AP/003).

The taxonomic status of four fish taxa (*Barbatula* sp., *Phoxinus* sp., *Gobio* sp., and *Carassius* sp.) was uncertain due to the description of new species after we completed our surveys and the presence of morphologically cryptic species of the genus *Carassius*. For the new species, a straightforward translation of the nomenclature of the former is not possible because there are several candidate species in each catchment (e.g. *Gobio occitaniae*, *Gobio lozanoi*; Aparicio et al., 2013). The native status of fish was defined as follows (Table 1): (i) native, if the fish historically occurred in the basin where captured according to regional faunal lists (Doadrio, 2011), and (ii) alien, if the fish species did not historically occur in the basin of capture or elsewhere in the Iberian Peninsula (IP). Each fish species was assigned to a trophic guild (mostly 'omnivo-

Table 1

Mean ( $\pm$ S.E.) host fish size, status in Spain, and prevalence of the alien parasitic copepod *Lernaea cyprinacea* and minimum–maximum range (min–max) for each of the frequently captured fish species ( $\geq 10$  sites) in the 15 catchments of northeastern Spain ( $n$  = number of sites where the species occurred, out of 530 sites).

| Scientific name                    | Status | N   | <i>L. cyprinacea</i> prevalence <sup>b</sup> |         | Fish length (mm) |         |
|------------------------------------|--------|-----|--|---------|------------------|---------|
|                                    |        |     | Mean $\pm$ S.E.                              | Min–Max | Mean $\pm$ S.E.  | Min–Max |
| <i>Achondrostoma arcasii</i>       | Native | 25  | 0  | 0       | 63 $\pm$ 1       | 8–133   |
| <i>Anguilla anguilla</i>           | Native | 61  | 0.10 $\pm$ 0.03                              | 0–4     | 287 $\pm$ 11     | 75–810  |
| <i>Barbatula</i> sp. <sup>a</sup>  | Native | 61  | 0.07 $\pm$ 0.03                              | 0–4     | 65 $\pm$ 0.5     | 16–108  |
| <i>Barbus haasi</i>                | Native | 141 | 3.25 $\pm$ 0.56                              | 0–100   | 93 $\pm$ 0.8     | 10–253  |
| <i>Barbus meridionalis</i>         | Native | 83  | 3.89 $\pm$ 0.45                              | 0–68    | 74 $\pm$ 0.7     | 20–305  |
| <i>Cobitis calderoni</i>           | Native | 13  | 0  | 0       | 45 $\pm$ 0.5     | 5–85    |
| <i>Gobio</i> sp. <sup>a</sup>      | Native | 100 | 1.45 $\pm$ 0.49                              | 0–100   | 73 $\pm$ 0.5     | 6–150   |
| <i>Luciobarbus graellsii</i>       | Native | 149 | 7.70 $\pm$ 0.49                              | 0–100   | 160 $\pm$ 12     | 6–529   |
| <i>Parachondrostoma miegii</i>     | Native | 116 | 4.04 $\pm$ 0.76                              | 0–100   | 88 $\pm$ 0.7     | 17–453  |
| <i>Phoxinus</i> sp. <sup>a</sup>   | Native | 128 | 0.06 $\pm$ 0.02                              | 0–6     | 54 $\pm$ 4       | 5–153   |
| <i>Salaria fluviatilis</i>         | Native | 24  | 0.74 $\pm$ 0.18                              | 0–18    | 72 $\pm$ 0.6     | 9–128   |
| <i>Salmo trutta</i>                | Native | 182 | 0.34 $\pm$ 0.18                              | 0–50    | 141 $\pm$ 1.1    | 8–932   |
| <i>Squalius laietanus</i>          | Native | 86  | 6.59 $\pm$ 0.94                              | 0–100   | 99 $\pm$ 1.5     | 5–438   |
| <i>Alburnus alburnus</i>           | Alien  | 48  | 0.55 $\pm$ 0.13                              | 0–18    | 34 $\pm$ 1       | 8–133   |
| <i>Cyprinus carpio</i>             | Alien  | 79  | 8.82 $\pm$ 1.18                              | 0–100   | 187 $\pm$ 6      | 20–735  |
| <i>Gambusia holbrooki</i>          | Alien  | 13  | 0  | 0       | 30 $\pm$ 0.5     | 10–65   |
| <i>Lepomis gibbosus</i>            | Alien  | 22  | 5.59 $\pm$ 1.02                              | 0–100   | 46 $\pm$ 1       | 20–156  |
| <i>Oncorhynchus mykiss</i>         | Alien  | 10  | 1.25 $\pm$ 0.19                              | 0–13    | 299 $\pm$ 6      | 234–377 |
| <i>Scardinius erythrophthalmus</i> | Alien  | 18  | 0.45 $\pm$ 0.07                              | 0–6     | 53 $\pm$ 4       | 24–259  |

<sup>a</sup> Species identity requires genetic confirmation in some catchments.

<sup>b</sup> Percentage of infected individuals out of the number of individuals inspected of each fish species in each site.

rous' or 'invertivorous'), a swimming guild ('benthic' or 'water column') and a migratory-behaviour guild ('almost sedentary' or 'migratory') based on regional fish atlases (Doadrio, 2011), scientific articles (e.g. Colin et al., 2016a,b) and our experience because the ecology of some of these species remains poorly reported in the literature. In our study area, only the European eel (*A. anguilla*) migrates between marine and freshwater habitats, while the other migratory species (e.g. *S. trutta*, *P. miegii*, *Luciobarbus graellsii* Steindachner, 1866) typically perform within-river upstream migrations for spawning (Doadrio, 2011).

### 2.3. Environmental stressors

We used 12 variables related to geography, water and physical habitat quality to identify some environmental factors that might be managed to control the invasion of *L. cyprinacea* after accounting for the effects of the six host fish factors detailed in Section 2.2 (see the hypotheses for all explanatory variables in Supplementary Table S2). Geographical factors were used as covariates and riverine water and physical habitat conditions were defined broadly as potential stressors because changes in these environmental conditions have the potential to induce a stress response in host fish or in the parasite *L. cyprinacea* and are factors in which management actions can act to control *L. cyprinacea* invasion.

As geographical variables we recorded the basin name and elevation (metres above sea level (m.a.s.l.) using Google Earth®. Elevation was used as a surrogate for the position of the sampling site in the river, and we calculated the Strahler stream order number (Strahler, 1964) using a map (1:50,000) as a measure of river size. Rivers were ranked from small, first order tributaries to the largest main river based on a hierarchy of tributaries.

We measured seven water-quality variables in situ prior to each fish survey. A digital multiparametric YSI® probe was used for temperature (°C), conductivity ( $\mu\text{S}/\text{cm}$ ) and pH, and the colorimetric test kit VISOCOLOR® for ammonium ( $\text{NH}_4^+$ , mg/l; detection limit (dl) = 0.2 mg/l), nitrite ( $\text{NO}_2^-$ , mg/l; dl = 0.02 mg/l), nitrate ( $\text{NO}_3^-$ , mg/l; dl = 1 mg/l) and phosphate ( $\text{PO}_4^{3-}\text{-P}$ , mg/l; dl = 0.2 mg/l) concentrations. These water quality variables measure nutrient pollution, acidity and changes in the overall ionic composition of water (i.e. conductivity), all of which alter the physiology of freshwater organisms directly (e.g. toxic effects) or indirectly through profound alterations in ecosystems (e.g. food availability, toxic algal blooms) (e.g. Kaushal et al., 2005; Johnson et al., 2010; Noga, 2011). Many toxicants in sewage from industries and urban and agricultural areas (e.g. chlorides, sodium, heavy metals, pesticides and drugs) alter nutrients, pH and conductivity in rivers (e.g. textile industry in Colin et al., 2016a,b), so that these variables are stressors that are widely used as indicators of chronic water pollution. Nutrient concentrations were aggregated based on their toxic severity (Camargo and Alonso, 2006; Noga, 2011), such as ammonium and nitrite (TN), or nitrate and phosphate-P (NP). TN is highly toxic to fish even at low concentrations, whereas NP is regarded as relatively benign apart from the effects of eutrophication (but see Camargo and Alonso, 2006; Smallbone et al., 2016). Clean waters in the study region have nutrient levels close to the lower detection limits in our tests and conductivity values  $\leq 600 \mu\text{S}/\text{cm}$  (Sánchez-Montoya et al., 2012), being particularly low in upstream compared with downstream clean river reaches. We estimated silting as the percentage of the streambed covered by sediment <1 mm diameter (Townsend et al., 2008). Silting is associated with high levels of eutrophication, agricultural runoff, and the release of detritus from sewage treatment plant outflows.

Physical habitat quality (i.e. river hydro-morphology and the quality of riparian vegetation) were characterised with the index QBR ('Qualitat del Bosc de Ribera' in Catalan, Munné et al., 2003), which is widely used by water agencies in Spain. Riparian vegeta-

tion can affect host-parasite relationships either directly by altering ecosystem productivity or indirectly by altering a river's ability to degrade pollutants (Gurr and Reinhard, 2006; Maceda-Veiga et al., 2018). QBR ranks the total riparian cover (the presence of riparian areas and their connectivity among them and with adjacent woodlands), cover structure (percentage of riparian trees, shrubs and emergent aquatic plants), cover quality (presence of alien riparian species), and river channel naturalness (e.g. presence of weirs, channels, and walls) on a scale from 0 to 100.

Last, we calculated the mean current speed (m/s) in each sampling site from three values measured along transects set perpendicular to the water flow at 20 m intervals because water velocity may alter the capacity of the free-swimming stage of *L. cyprinacea* life-cycle to attach to host fish (Supplementary Table S2).

### 2.4. Statistical analyses

#### 2.4.1. Overview of statistical analyses

Analyses were conducted in R (R Core Team, 2016. R: A language and environment for statistical computing (<https://www.R-project.org/>). R Foundation for Statistical Computing, Vienna, Austria) using the packages and functions referred to below. The response variable was the absence or presence of *L. cyprinacea* (0 or 1) for each individual fish. We constructed generalised linear mixed models (GLMM) with Bernoulli errors for the response to identify the environmental factors in which natural managers might act to control the invasion by *L. cyprinacea*, while accounting for the effects of host fish factors and random factors (collectively referred to as  $\rho$ ) for year, species, basin, and site. Outputs of GLMMs also were examined using hierarchical partitioning analysis, which identifies independent explained variation for sets of correlated explanatory variables (Mac Nally, 2000). Predictor variables always covary to some degree, which can bias regression estimates of GLMMs (Mac Nally, 2000; Freckleton, 2011), even when the most highly correlated predictor variables are excluded (Supplementary Table S3).

#### 2.4.2. Groups of explanatory variables and pair-wise correlations

For brevity, explanatory variables were collectively referred to as sets of predictors: *host* (host fish variables), *geo* (geography), *wv* (water velocity), *wq* (water quality), and *phq* (physical habitat quality) (Supplementary Table S1). However, all predictors within each set of predictors were included in the individual fish-based modelling as separate variables (e.g. *geo* = elevation + Strahler stream order number). Predictors (Supplementary Table S1) were standardised (0 mean, unit variance) so that all predictors were on the same scale (i.e. values of each predictor are expressed in units of the S.D. of the predictor) after continuous variables were log-transformed to reduce the influence of extreme values.

We used Spearman's  $\rho$  correlation coefficients to identify pairs of highly correlated predictor variables. For such pairs, we dropped the variable that was on average more highly correlated with all other predictor variables or for which there were logical grounds to favour the retained variable. Temperature was excluded due to its high correlation with elevation (Spearman's  $\rho > 0.70$ ). Elevation was highly correlated with the minimum and maximum annual air temperature in a subset of our study area ( $n = 325$  sampling sites; |Spearman's  $\rho| > 0.75$ ; Maceda-Veiga et al., 2013). The overall fit of a model including temperature was worse than that of a model including elevation, so that we retained elevation as the variable with the highest explanatory power. Elevation also represents better within-day and inter-seasonal variation in water temperature than a snapshot measure at the time of sampling fish. Spearman's  $\rho$  among the retained nine environmental predictors was  $\leq |0.56|$  (Supplementary Table S3). A previous study using this data-set also

indicated that collinearity among these environmental variables was of little statistical concern (Variation Inflation Factor (VIF) <2, Maceda-Veiga et al., 2018).

#### 2.4.3. Individual fish-based GLMMs: random and fixed factors

The modelling process was divided in three steps: (i) the selection of the best structure for random factors; (ii) the selection of the most influential host fish factors; and (iii) the selection of the most important environmental factors to explain the occurrence of *L. cyprinacea*. Candidate models in each step were ranked using the Akaike Information Criterion (AIC), which penalises model complexity (i.e. a balance between the level of likelihood and the number of variables). Following Grueber et al. (2011), we built a full model using the function *glmer* in the *lme4* package (Bates, D., Maechler, M., Bolker, B., Walker, S. 2015. *lme4*: Linear mixed-effects models using Eigen and S4. R package version 1.1-7. 2014) and candidate models were models with and without sets of predictors. The full model included all nine environmental predictors, five host predictors, and four random factors. Top ranked and similar ( $\Delta AIC \leq 2.0$ ) models were considered the most plausible descriptors of the data.

- (i) We used random intercept models (collectively referred to as  $\rho$ ) for year, species, basin, and site separately because when nested structures of these random factors (e.g. site nested in basin) or random slopes for species for all predictors were included in models, there was over-parameterisation and model complexity was not justified by the AIC criterion. Nonetheless, we checked that the direction of the effects of environmental variables did not differ among fish species (e.g. water conductivity was always positively associated with the occurrence of *L. cyprinacea*). The selected random intercept model accounted for multiple measurements for a unit and potential systematic differences among components of these factors (e.g. multiple sites for the same basin, multiple conspecifics for the same site, taxonomic relatedness).
- (ii) We then constructed nested models to explore whether the occurrence of *L. cyprinacea* was strongly associated with host fish abundance, body size, native status, trophic guild, migratory behaviour and swimming behaviour (Supplementary Table S4 and S5). This model included all four random factors and all nine environmental predictors. Host availability in a given site was the total combined abundance of all fish species recorded as hosts for *L. cyprinacea* in our region (e.g. Sánchez-Hernández, 2017; Maceda-Veiga et al., 2017). We used individual length as a measure of fish body size but outcomes were similar using fish body mass (results not shown).
- (iii) Using the most plausible combination of host fish predictors and random factors as a baseline, we built nested models to explore the potential additive effects of all four sets of environmental predictors (*geo*, *wv*, *wq*, and *phq*) to the probability of presence of *L. cyprinacea* (Supplementary Table S4). We then used the resultant, most plausible model to test for the likelihood of additive effects (+) of *geo*, *wv*, *wq*, or *phq* compared with five models including interactions (\*) among these four sets of stressors.

Interactive models for the probability (Pr) of presence of *L. cyprinacea* explored whether: (i) the effects of poor habitat and water quality interact with elevation ( $Pr = \alpha + host + geo + elevation * wv + elevation * wq + elevation * phq + \rho$ ); (ii) changes in water velocity interact with water or habitat quality degradation ( $Pr = \alpha + host + geo + wv * wq + wv * phq + \rho$ ); (iii) water and

physical habitat quality interact together ( $Pr = \alpha + host + geo + wv + wq * phq + \rho$ ); (iv) water conductivity interacts with nutrient pollution ( $Pr = \alpha + host + geo + wv + conductivity * TN + conductivity * NP + pH + phq + \rho$ ); and (v) pH interacts with nutrient pollution ( $Pr = \alpha + host + geo + wv + pH + pH * TN + pH * NP + phq + \rho$ ). Although the number of stressor combinations can be larger, we sought only to compare additive and interactive effects among some of the most prevalent stressors, which is a crucial first step in studies of multiple stressors (Piggott et al., 2015).

Additive models would be favoured over interactive models if additive models had the lower AIC values and if those values were at least two AIC units less than the AIC values of the corresponding interactive models. That is, the likelihood of environmental stressors having interactive effects on the probability of presence of *L. cyprinacea* would be low and the added model complexity of interactive models does not provide enough improvement to justify the inference that interactions are important. The AIC framework only compares among models but does not provide information about whether the 'best' of those models adequately accounts for variation in the response variables. Therefore, we assessed the overall adequacy of the most plausible model for the probability of presence of *L. cyprinacea* by using the function *auc* (Area Under Curve, AUC) in the *pROC* package (Robin et al., 2011). Predicted probabilities of occurrence of *L. cyprinacea* as a function of the selected host and environmental factors were inspected visually using the function *effects* in the *effects* package (Fox and Hong, 2009). The importance of the environmental descriptors selected by the AIC approach was further assessed by comparing data between sites with and without infected fish using Mann-Whitney tests and within the GLMMs using  $\chi^2$  tests (the function *Anova* in the *car* package, Fox and Weisberg, 2011) with  $\alpha = 0.05$ . The selection of environmental and host factors in the final best model resulting from model-averaging the most plausible candidate models was refined further by eliminating predictors that were not significant, or whose 95% confidence intervals included 0, to avoid over-parameterising the final model.

#### 2.4.4. Hierarchical partitioning analysis

Standardised coefficients of the identified fish host and environmental predictors from GLMMs showed the strength and direction of associations to explain the probability of the presence of *L. cyprinacea*. We computed the relative importance of predictors in GLMMs by using hierarchical partitioning analysis (HP; function *hier.part*, Walsh, C., Mac Nally, R., 2003. The *hier.part* package. Hierarchical Partitioning. R project for statistical computing; <http://cran.r-project.org>). A randomisation test for HP based on the upper 0.95 confidence limits (the function *rand.hp*) was used to assess whether the independent explained variation for each predictor was significant to explain the occurrence of *L. cyprinacea*.

### 3. Results

A total of 30,932 fish individuals from seven endemic and 12 widely distributed species was captured in 15 Mediterranean catchments. Infection was seen in all fish species, apart from the alien *G. holbrooki* and the natives *Cobitis calderoni* Bacescu, 1962 and *Achondrostoma arcasii* Steindachner, 1966 (Table 1). The highest prevalence of infection was in five native fish (*B. haasi*, *Gobio* sp., *L. graellsii*, *P. miegii*, and *Squalius laietanus* Doadrio et al. 2007) and in two alien species, the common carp *C. carpio* and the pumpkinseed *Lepomis gibbosus* Linnaeus, 1758 (Table 1).

### 3.1. Individual fish-based modelling

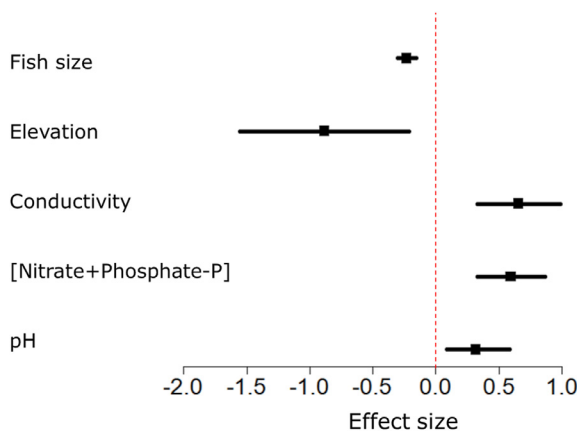
Among host predictors, fish length was more influential than total fish abundance, native fish status or fish species' ecology (trophic guild, swimming and migratory behaviour) in accounting for the presence of *L. cyprinacea*, after having controlled for potential systematic differences among years, species, basins, and sites (Supplementary Table S4). This was an adequate model based on the AUC value, and indicated that smaller fish were more vulnerable to *L. cyprinacea* infection than were larger fish (Table 2). However, water properties were as influential in accounting for infection as fish length (Table 2). Among the environmental factors, the regression estimates suggested that conductivity and nutrient pollution (i.e. nitrate and phosphate-P) made the largest contributions to variation in the probability of presence of *L. cyprinacea*, with an overall positive effect (Table 2; Fig. 2; Supplementary Fig. S1). The probability of presence of *L. cyprinacea* was negatively associated with elevation (Table 2; Fig. 2; Supplementary Fig. S1).

**Table 2**

Statistical tests for the host and environmental predictors identified as significant factors in the final best generalised linear mixed model to explain the occurrence of the invasive parasitic copepod *Lernaea cyprinacea* in fish in northeastern Spain. The significance of predictors was assessed using the function *Anova* within the package *car* at  $P < 0.05$ . The independent explained variance for each predictor is indicated in Section 3 using hierarchical partitioning analysis of variance. The Area Under the Curve (AUC) is a measure of the overall fit of the model. The variance and S.D. of random effects are indicated.

| Predictors                    | $\chi^2$ | <i>P</i> |
|-------------------------------|----------|----------|
| <b>Model fit (AUC = 0.80)</b> |          |          |
| <b>Host predictor</b>         |          |          |
| Fish length                   | 25.33    | <0.001   |
| <b>Geography (geo)</b>        |          |          |
| Elevation                     | 6.19     | 0.01     |
| <b>Water quality (wq)</b>     |          |          |
| Conductivity                  | 15.52    | <0.001   |
| pH                            | 5.31     | 0.02     |
| NP                            | 20.10    | <0.001   |
| <b>Random factors</b>         |          |          |
|                               | Variance | S.D.     |
| Basin                         | 0.03     | 0.18     |
| Site                          | 2.92     | 1.71     |
| Species                       | 2.02     | 1.42     |
| Year                          | 0.001    | 0.00     |

NP, the sum of nitrate and phosphate-P concentrations.



**Fig. 2.** Regression estimates (i.e. effect size on the logit scale) and 95% confidence limits for the generalised linear mixed model that best represents the probability of occurrence of *Lernaea cyprinacea* (i.e. a function of fish length, elevation, conductivity, pH, and nitrate and phosphate-P concentrations). Only those predictors significant at  $P < 0.05$  from Table 2 are shown.

The hierarchical partitioning analysis examined the independent contribution (as a percentage out of 100% explained variance) of the predictors identified by the GLMM analyses (fish size, water conductivity, nutrient pollution and pH) to variation in the presence of *L. cyprinacea*. The latter was significantly associated with fish length (53.3% of independently explained variance), although the collective effect of the set of environmental predictors also was significant and explained almost as much independent variation (46.7%). Of the latter, variation in the occurrence of *L. cyprinacea* was approximately twice as strongly related to conductivity (17.3%) than to the other three environmental predictors (elevation 9.2%, pH 9.3% and nutrient pollution 11.0%), but all four environmental predictors were significantly related to the presence of *L. cyprinacea* (HP randomisation test; Mac Nally, 2002).

Univariate tests also showed significant differences in elevation (Mann Whitney U-test:  $W = 3523$ ;  $P < 0.05$ ), conductivity ( $W = 1877$ ;  $P < 0.05$ ) and nutrient pollution ( $W = 1922$ ;  $P < 0.05$ ) but not in pH ( $W = 2299$ ;  $P > 0.05$ ) between sites with the presence or absence of *L. cyprinacea* (Fig. 3). Note that these tests do not include the effects of variation in other predictors and random effects of the multivariate models (c.f. GLMMs and HP) so that statistically important differences from the GLMMs or HP may be visually subtle when presented as individual stressors (Fig. 3).

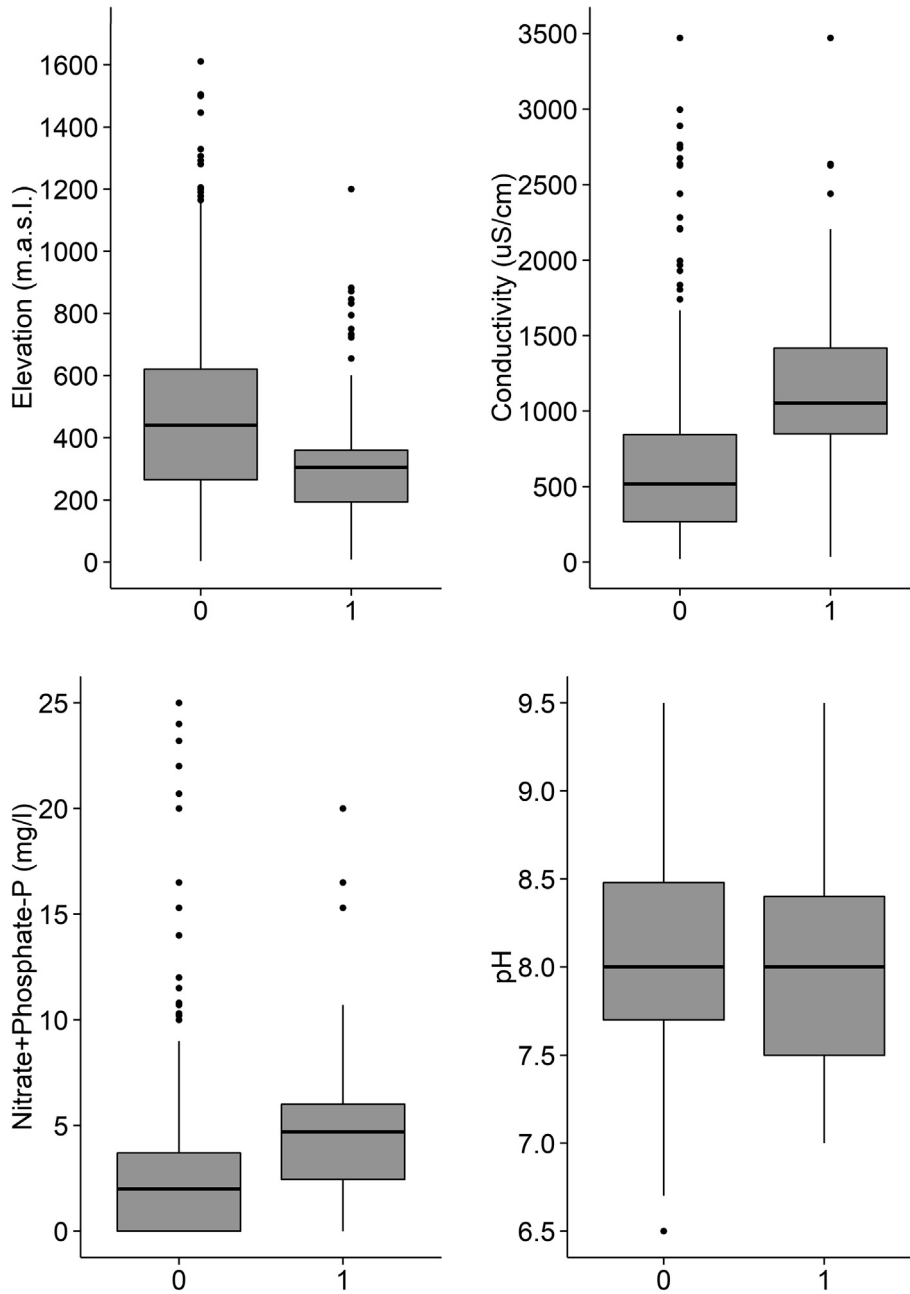
### 3.2. Do environmental stressors have additive or interactive effects?

The base additive model, consisting of geographical, hydro-morphological features, water properties and fish length, was more parsimonious (i.e. had the lowest AIC value) than the five interactive models (see AM in Table 3), so that including interactive effects among environmental stressors in the base additive model did not improve fit. Therefore, we found little evidence for an interaction among environmental stressor sets.

## 4. Discussion

The presence of a widespread alien parasitic copepod, the anchor worm *L. cyprinacea*, was explained mostly by fish host size and additive combinations of altitude and water quality degradation. Smaller fish and fish in lowland rivers had higher probabilities of infection as had fish living in waters with high conductivity, which is a general indicator of sewage discharges (e.g. Colin et al., 2016a,b). Nutrient concentrations in rivers passing through extensive agricultural areas, such as in northeastern Spain, also seemed to promote *L. cyprinacea* invasion. Given that this parasite is a host generalist (Hoffman, 1999), our results aid in identifying the determinants of the presence of *L. cyprinacea* in riverine fish.

Whereas interactions among stressors are frequently raised as a major contributor to the current biodiversity crisis (Piggott et al., 2015; Côté et al., 2016), little is known about stressor interactivity in freshwater fish (Jackson et al., 2016; Schinegger et al., 2016). We found no evidence that interactive effects among some major riverine stressors explained the presence of *L. cyprinacea* in fish better than did additive combinations, as occurred in other study systems (Gieswein et al., 2017; Maceda-Veiga et al., 2017, 2018; Kath et al., 2018). While definite causation cannot be determined from correlational studies, hierarchical partitioning analysis (HP) involves the calculation of the independent contributions of each predictor to variation in the response variable, and distinguishes these from the joint contribution with other predictors. The advantage of GLMMs is that they include random effects, which is not possible in HP analysis, so we used averaged fish size per sampling site to overcome this limitation. Therefore, the presence of *L. cyprinacea* in the study region seems to be determined largely by fish host and water quality factors.



**Fig. 3.** Differences in altitude, conductivity, nitrate and phosphate-P concentrations and pH at sites at which the alien parasitic copepod *Lernaea cyprinacea* was found infecting any of the 19 fish species examined (0 = absence, 1 = presence). We only showed the environment–parasite relationships with the strongest correlations from generalised linear mixed models in Table 2 (see full details on river environmental conditions in the presence or absence of *L. cyprinacea* in Supplementary Table S1). Note that untransformed data are used for these graphs to facilitate interpretation, although log-transformed data and linear combinations of predictors were used in the models and Fig. 2).

**Table 3**

Statistics for the comparison of an additive model including host–fish predictors and river descriptors (i.e. the most plausible model from Table 2 is the Additive Model here) compared with five models including interactive effects for relevant environmental combinations (see in Section 2.4.3 for model equations and further details) for the occurrence of the parasitic copepod *Lernaea cyprinacea* in rivers in northeastern Spain. The top ranked model defined by the lowest Akaike Information Criterion value is highlighted in bold, and its fit was assessed by using Area Under the Curve (AUC). We provide information on the differences in Akaike Information Criterion for each interactive model ( $\Delta AIC$ ) and the corresponding probability of support relative to the Additive Model A (Prob. =  $e^{-\Delta AIC/2}$ ; Wagenmakers and Farrell, 2004).

| Models   | AIC           | $\Delta AIC$ | Prob. | AUC         |
|--|---------------|--------------|-------|-------------|
| <b>Additive Model (AM)</b>                                 | <b>4261.7</b> | –            | –     | <b>0.80</b> |
| AM with interactive effects for elevation                  | 4274.5        | +12.8        | 0.002 |             |
| AM with interactive effects for water velocity             | 4273.3        | +11.6        | 0.003 |             |
| AM with interactive effects for <i>wq</i> and <i>phq</i>   | 4276.3        | +14.6        | 0.001 |             |
| AM with interactive effects for conductivity and nutrients | 4267.4        | +5.7         | 0.058 |             |
| AM with interactive effects for nutrients and pH           | 4271.0        | +9.3         | 0.01  |             |

*wq*, all water quality variables (sum of ammonium and nitrite, sum of nitrate and phosphate-P, pH, silt and conductivity); nutrients, ammonium, nitrite, nitrate and phosphate-P; *phq*, physical habitat quality as estimated by the index QBR (Qualitat del Bosc de Ribera, in Catalan).

Although the global decline of freshwater fish is due to many factors (Closs et al., 2016), there is growing concern about the role of parasites (e.g. Gozlan et al., 2005). Overall, the prevalence of *L. cyprinacea* was low in the studied fish, which typically occurs because outbreaks of infectious diseases are often abrupt (Noga, 2011). However, it is remarkable that we saw the highest prevalence of *L. cyprinacea* in native fish species (e.g. *P. miegii*, *S. laietanus*) that have experienced significant declines in northeastern Spain (Maceda-Veiga et al., 2010). Nonetheless, the lowest prevalence was in widespread, alien fish species, such as *G. holbrooki* and *A. alburnus* (Froese, R., Pauly, D., 2017. FishBase. World Wide Web electronic publication. [www.fishbase.org](http://www.fishbase.org). (Last accessed 03/02/2019)), apart from *C. carpio*, which probably was the source from which *L. cyprinacea* was introduced into Spanish waters as it has in other regions around the world (Hoffman, 1999; Hassan et al., 2008).

After controlling for the interspecific variability in the presence of *L. cyprinacea*, our individual fish-based models found that smaller fish had higher probabilities of infection than larger fish. This outcome differs from the works of Marcogliese (1991) on *L. cyprinacea*, who found no host size effect, and of Poulin et al. (1991), who found, in controlled exposure trials in clean laboratory water, that infection rates by another species of parasitic copepod increased with fish size. The difference could be due to immunosuppression of adult fish or pollution-induced immunosuppression in our systems (see below), which may affect juveniles more than adult fish (see Biagiante-Risbourg et al., 2013). Differences in the microhabitat used by each life stage also may contribute to our different findings. Juveniles generally occupy slow-flowing waters (Ribeiro et al., 2013), which facilitate the spread of *L. cyprinacea* (Medeiros and Maltchik, 1999). Spatial concentration of fish is expected to promote the spread of *L. cyprinacea* (Hoffman, 1999; Noga, 2011), but we did not find evidence for such an effect. Ecological traits of the fish hosts (e.g. migratory behaviour, trophic ecology) also seemed to have little influence in explaining the presence of *L. cyprinacea*, possibly because we lack detailed information on the ecology of these species, such as differences in habitat use (e.g. home-range), which is a major determinant of infection rate (Knudsen et al., 1996; MacColl, 2009).

Our results suggest that the presence of *L. cyprinacea* might be limited by high elevation and associated lower water temperatures (Marcogliese, 1991; Hoffman, 1999; Noga, 2011). However, even species that prefer cold water (<20 °C), such as the strictly freshwater populations of *S. trutta* in the Mediterranean (Doadrio, 2011), were infected, raising concerns about the spread of *L. cyprinacea* through entire vulnerable faunas as the climate continues to warm (Sánchez-Hernández, 2017). However, our results suggest that improving the quality of water chemistry might mitigate that effect. Our findings provide some support for the fact that a decrease in nitrate and phosphate concentrations would reduce the probability of *L. cyprinacea* being present, a conclusion differing from the reviews by Vidal-Martínez et al. (2010) and Palm (2011), who suggested that nutrients negatively affected crustacean parasites. Sewage discharges from urban areas and intensive farming are the major reasons for anthropogenic eutrophication in many aquatic ecosystems (Smith and Schindler, 2009). Given that human pressures on riverine ecosystems continue to grow (Vörösmarty et al., 2010; Sinha et al., 2017), it is crucial to make these by-products of human activity less environmentally damaging by optimising the application of agricultural fertilisers (Basso et al., 2016) and by regenerating riparian vegetation to act as 'green filters' (see Maceda-Veiga et al., 2018).

Improvement in sewage treatment and nutrient retention in riparian areas and reductions in water abstraction would reduce river conductivity (e.g. Basin Salinity Management Plan, 2015), for which we found a positive association with the occurrence of

*L. cyprinacea*. Conductivity has been widely used as a proxy for salinity (Kaushal et al., 2005) and as general indicator of water pollution, as we did here. Conductivity is altered by agricultural runoff, urban and industrial discharges (e.g. metal pollution, Colin et al., 2016a,b) and by common ions of clean sea water and potash mines (e.g. chlorides, potassium) (Fernandez-Turiel et al., 2003). The parasite was mostly found at conductivities >1000 µS/cm (Fig. 3 and Supplementary Table S1), and clean waters generally have values <600 µS/cm in our studied region (Sánchez-Montoya et al., 2012). Therefore, the contaminants occurring in insufficiently treated sewage discharges (traced by conductivity) probably caused immune-suppression in fish and explained the positive association between fish infection and water conductivity. Positive associations have been reported between water pollution and other directly transmitted ectoparasites (Marcogliese, 2005; Vidal-Martínez et al., 2010). This finding contrasts with endoparasites, which seem to reduce in abundance and diversity in polluted waters, possibly because endoparasites depend on trophic interactions for transmission and pollution typically alters the community structure of their final and intermediate hosts (Marcogliese, 2005; Vidal-Martínez et al., 2010).

Our results support the notion that the effects of pollution on host-parasite relationships are context-dependent (Marcogliese and Pietrock, 2011). The probability of presence of *L. cyprinacea* increased with nitrate and phosphate concentrations, although no infected fish were found in waters with concentrations of 15–25 mg/l. Infection might have been promoted because nutrients increase resource availability and boost host body condition, from which parasites may benefit (Johnson et al., 2008; Budria, 2017). Although fungal-like pathogens assimilate inorganic nutrients (Budria, 2017), this is unlikely to occur in the life-cycle of *L. cyprinacea*, especially for the non-feeding nauplius stage of the parasite (Shields, 1978). However, another free-swimming larval stage of *L. cyprinacea*, the copepodite, might benefit from eutrophic conditions (e.g. algae blooms) if these conditions promote non-parasitic copepods (see Budria, 2017). The positive association between parasite occurrence and nutrients might be inverted at higher concentrations, given that nutrients such as nitrates or eutrophication itself (e.g. oxygen depletion at night) are lethal to fish hosts and parasites (e.g. Johnson et al., 2008; Smallbone et al., 2016). Primary production increases pH in daylight, therefore the positive association we saw between pH and the occurrence of *L. cyprinacea* might be due to eutrophication. pH may not have been significant in Mann-Whitney U-tests because univariate tests such as the U-test do not account for covariates. However, bivariate plots showing raw data (see Fig. 3) may be more valuable for managers to guide actions on particular stressors than when the differences are shown as combinations in multivariate models.

Hydro-morphological alterations and riparian zone characteristics appeared to be of little importance in explaining the presence of *L. cyprinacea*, even though such alterations are known stressors for riverine biota (Tockner et al., 2009; Doadrio, 2011). The absence of a signal might be due to rivers being very dynamic so that snapshot samples of biota and abiotic conditions may not necessarily reveal potentially strong relationships (e.g. Heino et al., 2015; Maceda-Veiga et al., 2017). However, water velocity is likely to vary as much as nutrient concentrations and conductivity, and these two factors had discernible effects on the presence of *L. cyprinacea*. A plausible explanation for the lack of association with river hydro-morphology is that slow water velocity is only critical during the attachment stage of the parasite, and we found mostly mature *L. cyprinacea* individuals with egg clutches (Maceda-Veiga, personal observation). Therefore, although infection was not clearly related to water velocity, there may be negative relationships at an early stage of the infection, for which we have no data.



Understanding the host-specific and environmental governors of infection provides testable hypotheses for experimental studies and guidance for natural resource and conservation managers at relevant spatial scales. Improving water quality has long been advocated for its multiple benefits in biodiversity conservation, including the roles that rivers play in human society (Tockner et al., 2009; Hering et al., 2015). Our study shows that another benefit would be to reduce the probability of occurrence of a widespread alien parasite in fish. Nitrate, phosphate and high water conductivity remain major, widespread stressors in European rivers even 10 years after the enforcement of the EU Water Framework Directive (EC, 2000). Clearly, on-going effort is needed to improve water quality in European rivers for multiple ecological and societal gains.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpara.2019.03.002>.

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