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Effect of environmental variables and their interaction on gordiid hairworm larvae (Nematomorpha)

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Abstract

The different stages of the life cycle of parasites are important components of ecosystems. Changes in environmental conditions may affect free-living stages, host–parasite interactions and ecosystem functioning. The larvae of *Chordodes nobilii*, which belongs to the parasitic phylum Nematomorpha, are susceptible to extreme temperatures and different pollutants, but the effects of pH and moderate temperature variations have not been evaluated yet. Our objective was to assess the effect of temperature, pH and their interaction on the infectivity of *C. nobilii* larvae to *Aedes aegypti* larvae over time. Larvae were treated with factorial combinations of temperature (18, 23 and 28°C), pH (7, 8 and 9) and time periods (24 and 48 h). Results show a highly significant interaction among all variables. The highest infectivity was recorded at 18°C and pH 7 at 24 and 48 h, and the lowest one at 28°C and pH 8 at 24 and 48 h. Infectivity differed significantly among the three temperatures only at pH 8 and 48 h, decreasing with increasing temperature. Our study is the first report of the effect of pH on a Nematomorpha species and suggests that the infectivity of *C. nobilii* larvae may be affected negatively by an increase in temperature and its interaction with pH and time. Since parasites must be considered for a better understanding of the effects of stressors on freshwater ecosystems, our results may help in the design and analysis of studies of anthropogenic impact.

Introduction

Parasites constitute the most abundant and diverse group of animals, occupying a wide variety of environments (Hudson, 2006). Parasite–host systems are part of complex networks of interacting species. Moreover, parasites play an important role in many ecosystems because they are specialist consumers and prey, exert a major influence on biodiversity and represent a large amount of biomass (Hudson, 2006; Lagrue & Poulin, 2015; Williams, 2019).

Due to the importance of parasites in the environment, their use as indicators of anthropogenic change has opened up a new field of research: Environmental Parasitology. This discipline, which deals with the responses of parasites to pollutants and environmental disturbances, has gained increasing importance in recent years. Studies in this discipline contribute to identify taxa serving as indicators of environmental health and anthropogenic impacts (Vidal-Martínez *et al.*, 2010; Sures *et al.*, 2017; among others). In this regard, Sures *et al.* (2017) consider that the use of parasites as effect indicators at the level of individual organisms, populations and communities is a promising line of investigation. However, most of the experiments have been performed with free-living stages of digeneans (Thieltges *et al.*, 2008; Sures *et al.*, 2017). In contrast, there is little information on the effect of pollution and abiotic factors on the infectivity of free-living stages of other parasitic groups.

The effect of stressors on parasite developmental stages has been poorly studied in some groups, such as the freshwater nematomorphs (Nematomorpha: Gordiida), commonly known as hairworms. The free-living adults live in freshwater water bodies. Females produce from thousands to millions of eggs in strings, which become darker as embryonic development progresses. After hatching, larvae remain very close to the egg strings and are viable and infective from some days to a few weeks, until they find a paratenic host such as a copepod, fish, amphipod, mollusc or aquatic insect larva, among others (Schmidt-Rhaesa, 2012; Bolek *et al.*, 2015). Hanelt *et al.* (2005) stated that gordiid cysts can also be transmitted between paratenic hosts through paratenesis. The most accepted hypothesis is that terrestrial definitive hosts (mainly orthopterans, praying mantids and cockroaches) are infected upon the consumption of paratenic hosts. Gordiids manipulate host behaviour, inducing insects to jump into water, where adults emerge (Bolek *et al.*, 2015). Moreover, it has been hypothesized that the manipulation of terrestrial arthropod hosts by hairworms can drastically change energy flow in aquatic ecosystems (Sato *et al.*, 2011).

The free-living larvae of gordiids are in direct contact with the environment and need to find a host as soon as possible, before their energy reserves are depleted (Tinsley, 2006). It is expected that environmental conditions affect the survival, infectivity and lifespan of parasite infective stages, thus conditioning transmission and infection success. This may be particularly important for gordiid larvae because their reduced mobility prevents them from escaping to multiple stressors. Moreover, environmental variables may influence the spatial and temporal dynamics of host–parasite interactions, which may have a profound impact on the ecosystem (Holt & Boulinier, 2006; Thieltges *et al.*, 2008).

Temperature and pH are among the most important abiotic variables affecting aquatic organisms in freshwater bodies. Temperature may fluctuate daily, yearly and/or seasonally, and variations in diurnal temperature are more pronounced in shallow than in deep waters. The pH is unstable and depends on the nature of the substrate and biotic factors (Pietrock & Marcogliese, 2003; Tinsley, 2006; Thieltges *et al.*, 2008). The influence of these abiotic factors on the transmission success of free-living endohelminth stages has been thoroughly studied in trematodes, which showed a positive correlation between temperature and production and shedding of cercariae (Thieltges *et al.*, 2008). However, there is little information on gordiid larvae. The infectivity of *Chordodes nobilii* larvae showed a significant decrease after being kept at −3°C for 48 h, compared to the control group (at 23°C), and it was almost null at 40.5°C; while *Paragoridus varius* larvae remained viable after being frozen at −80 ± 2°C for seven months (Achiorno *et al.*, 2008a; Bolek *et al.*, 2013). However, no data are available about the effect of moderate temperature variations on this stage. Free-living stages of *C. nobilii* have shown to be susceptible to different pollutants (Achiorno *et al.*, 2008b, 2009, 2010, 2015). These results suggest that *C. nobilii* larvae could serve as an effect indicator of contamination. Moreover, the fact that their susceptibility to pesticides was also affected by the dilution medium (reconstituted water or natural freshwater) (Achiorno *et al.*, 2018) makes it necessary to assess the responses of this species to environmental factors.

Based on the considerations mentioned above, the objective of the present study is to determine the effect of temperature, pH and their interaction over time on the infective capacity of *C. nobilii* larvae, using *Aedes aegypti* larvae as host. Considering reference values of 23°C for temperature and 8 for pH based on a previously validated bioassay protocol (Achiorno *et al.*, 2010), we hypothesize that a variation of ±5°C and ±1 from the reference values of temperature and pH, respectively, will affect the infectivity of *C. nobilii* larvae.

Materials and methods

Test organisms

Adults of *C. nobilii* were collected from streams of the Sauce Grande basin in Sierra de La Ventana (38°9′0″S, 61°48′0″W), Buenos Aires, Argentina, in late summer. The pH and temperature values measured during the samplings ranged from 7.73 to 8.74 and from 19 to 26°C, respectively. In the laboratory, individuals of both sexes from each stream were placed in separate containers with water from the collection site, which was gradually replaced by aerated dechlorinated tap water, and kept in a rearing chamber at 23 ± 1°C, in the dark. After mating, females were held individually in containers until oviposition. Egg strings were maintained under the same conditions as adults. They were periodically checked by microscope for the presence of free larvae until the amount of free larvae in the egg strings was >50%.

Study design

The experimental design was based on previous studies made with *C. nobilii* (Achiorno *et al.*, 2008a, b, 2010; Achiorno, 2011). The bioassays were performed with a factorial combination, by treating larvae with different temperature–pH–time combinations. We evaluated three different temperatures (18, 23 and 28°C) at three different pH values (7, 8 and 9), and determined the infective capacity of larvae at two time periods, 24 and 48 h. In one bioassay, larvae were kept at 18°C ± 1°C and pH values of 7, 8 or 9 for 24 or 48 h. In another bioassay, they were kept at 28°C ± 1°C and at the same pH values and time periods. For both bioassays, larvae were simultaneously maintained at the reference temperature (23°C) at the same pH values and time periods. Each variable was tested in triplicate, under semi-static conditions.

Figure 1 shows a flow diagram of the experimental protocol, which consisted of two consecutive periods as follows:

(1) Experimental treatments. Egg strings were cut into segments of about 3 mm long, each one containing approximately 4000 individuals, with ≥50% of free larvae. Each segment was randomly taken with a Pasteur pipette and transferred into a 1.5-ml Eppendorf tube, to be immediately used in the experiment. The experimental medium was reconstituted hard water, with the following chemical composition (mg/L): sodium bicarbonate 192, calcium sulphate dihydrate 120, magnesium sulphate 120, potassium chloride, 8 (Weber, 1993), and adjusted to the pH values assayed by the addition of 0.1N sodium hydroxide or 0.01M sulphuric acid. Each experimental series was performed in three 1-l glass beakers filled with 800 ml of reconstituted hard water at pH 7, 8 or 9. Six Eppendorf tubes containing one egg string segment each were held in racks and submerged in the experimental medium of each beaker. Half of them were treated for 24 h and the other half for 48 h. Then, beakers were kept in a rearing chamber at 23 ± 1°C or in a room at 18 ± 1°C or 28 ± 1°C, in darkness. Temperature and pH were periodically checked. The pH was monitored throughout the treatment period using a digital pH meter calibrated daily using standard buffer solutions.

(2) Exposure of *A. aegypti* (Diptera) larvae to *C. nobilii* larvae. The viability of *C. nobilii* larvae was determined by evaluating their infective capacity. To this aim, 30 s- and third-instar larvae of *A. aegypti* reared in the laboratory were placed in containers with 12 ml of dechlorinated tap water and wheat germ as food, and maintained at 23°C. After 24 or 48 h, each sample from each treatment was added to each container. After being exposed for 72 h, mosquito larvae were fixed in 70% ethanol and then dissected under light microscope to search for *C. nobilii* larvae in the body cavity. This procedure was repeated for all replicates.

Data analysis

Eighteen experimental conditions resulted from combining pH values (7, 8 and 9), temperatures (18, 23 and 28°C) and time periods (24 and 48 h). The end point was the assessment of the number of *C. nobilii* larvae that infected *A. aegypti* larvae. We considered 23°C, pH 8 and an exposure time of 48 h as reference values for each variable evaluated.

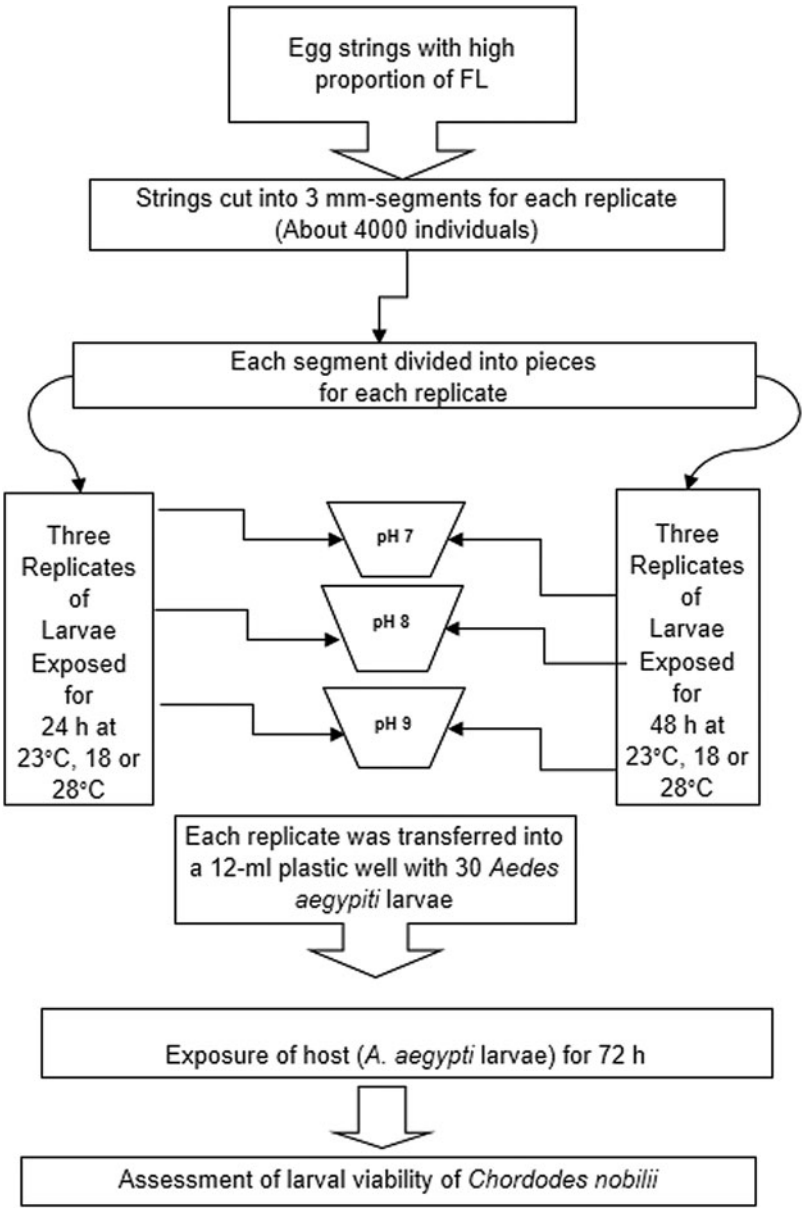

Fig. 1. Flow diagram of the experimental procedure for two assays at 18 or 28°C, and the respective control at 23°C. FL, free larvae.

The modelling strategy was to obtain the best possible model with fixed effects and then add the relevant random effects. The initial model with fixed effects for the number of *C. nobilii* is a Poisson regression with pH values, temperatures and time periods as covariates and all possible interactions. Since the number of *A. aegypti* larvae differs for each replicate, it is necessary to include it as an offset in the model. So the fixed effects model is $\text{Log}(\mu) = \text{Temperature} + \text{pH} + \text{Time} + \text{Temperature} : \text{pH} + \text{Temperature} : \text{Time} + \text{Time} : \text{pH} + \text{Temperature} : \text{pH} : \text{Time} + \text{offset}(\log(\text{NLA})) + \epsilon$, where, if NCn = number of *C. nobilii* larvae, then NCn follows a distribution Po(μ), and NLA = number of *A. aegypti* larvae (Zuur *et al.*, 2009; Faraway, 2016).

Moreover, we added an observation-level random factor to account for overdispersion and, based on the experimental design, a random factor was also included to account for a possible correlation with the beaker used.

For the goodness of fit, test scaled deviance and deviance were used and both residuals, Pearson and Deviance, were analysed. Simultaneous inference procedures have to be used, which adjust for multiplicity and, thus, control the overall type I error rate. The global null hypothesis considers all the contrasts of interest. A suitable scalar test statistic for testing the global hypothesis is to consider the maximum of the individual test statistics that can be used for each individual test. This procedure leads to a max-t type test statistic (Hothorn et al., 2008).

Data were analysed with R software, version 4.0.3 (R Core Team, 2020) using packages *lmer4* to construct a mixed Poisson Regression model and *multcomp* to perform simultaneous comparisons.

Results

The most complete model considers the random effects of both the beaker and the individual observations (to remedy overdispersion). The comparison between this model and the model without the beaker random effect showed no evidence of improvement ($\chi^2 = 0.4651$, df = 1), and, therefore, it was discarded. Hence, the model is described by the following equation: logNCn ~ Temperature *

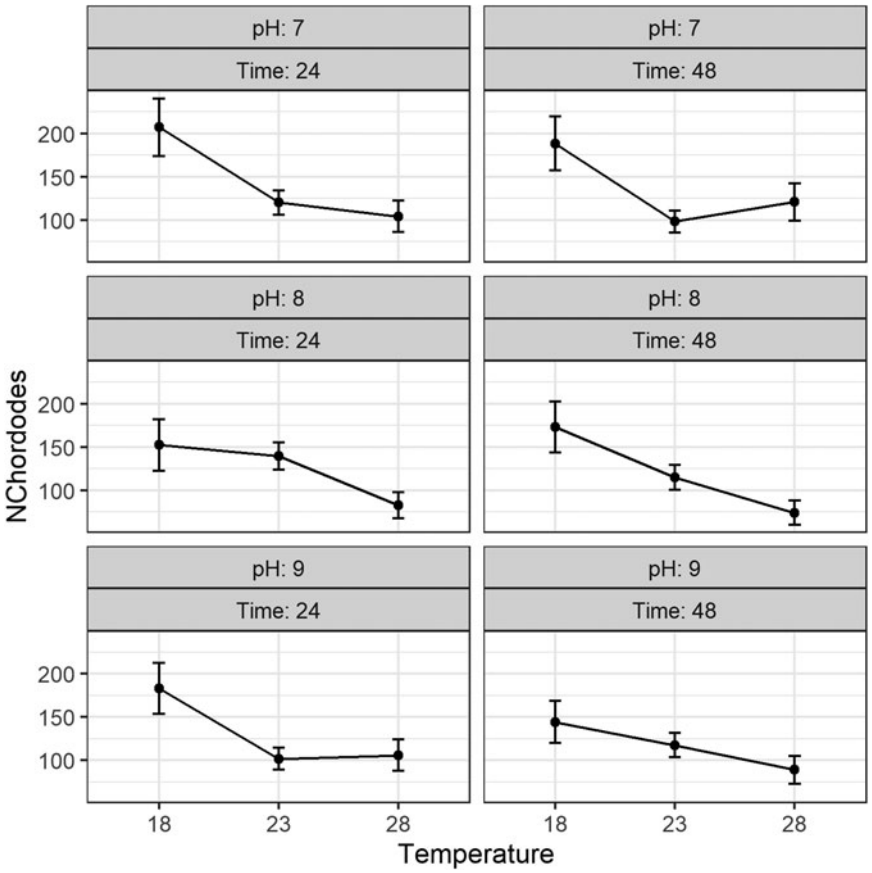

Fig. 2. Effect of temperature on the infectivity of *Chordodes nobilii* larvae at pH 7, 8 and 9 after 24 and 48 h of exposure. X-axis, evaluated temperatures (18, 23 and 28°C; Y-axis, number of infective ***Chordodes nobilii*** **larvae (NChordodes).**

pH * Time + offset(log(N.L.A)) + (1 | ID_obs). The estimates are shown in the table of fixed effects (see supplementary material).

It is worth noting that the model predicts the existence of a triple interaction among temperature, pH and time period, which explains the results obtained (fixed effects, see supplementary material).

Although no pattern of infectivity could be determined with respect to the variables evaluated in this study (see fig. 2 and results of the statistical analysis in table 1), there were significant differences in the number of infective *C. nobilii* larvae between treatments. At pH 7, after 24 and 48 h, infectivity values were highest at 18°C, showed a significant decrease at 23°C, and a significant difference was found between 18 and 28°C. At pH 8 and after 24 h, infectivity showed significant differences between all temperatures except between 18 and 23°C, while it decreased significantly between all temperatures at 48 h. At pH 9, infectivity decreased significantly between 18 and 23°C and between 18 and 28°C after 24 h, while it decreased significantly between 18 and 28°C after 48 h.

Finally, the highest infectivity values were obtained at 18°C, regardless of the pH, reaching a peak at pH 7, and the lowest infectivity value was found at 28°C and pH 8 (fig. 2). Although infectivity showed no clear pattern of variation, the data suggest that it decreases with increasing temperature, this effect being more evident between 18 and 23°C than between 23 and 28°C.

Discussion

This study expands the knowledge of the effect of temperature on *C. nobilii* larvae (Achiorno *et al.*, 2008a) and represents the first report of the effect of pH on a Nematomorpha species.

Our results showed that the variables analysed had an effect on the infectivity of *C. nobilii* larvae, and indicated that the interaction of temperature, pH and time affected the infective capacity of *C. nobilii* larvae. Although caution must be exercised when extrapolating the results of laboratory-based studies to natural environments, they may enable a better understanding and prediction of how and under what conditions abiotic factors affect *C. nobilii* larvae. The temperatures assayed (i.e. 18, 23 and 28°C) represent the air temperatures of the Sauce Grande basin, located in the south-western region of the province of Buenos Aires, Argentina. In this region, the average temperature is 7°C in the coldest month (July) and 23.5°C in the warmest month (January), with an annual average of 15°C. The absolute maximum temperature was recorded in January with a value of 41.5°C, while the absolute minimum was recorded in July with −10°C (Scian, 2010). With respect to pH variations in freshwater bodies, Berezina (2001) stated that water pH is seasonally unstable in the same water body. According to this author, an increase in water pH is observed in summer due to a remarkable reduction in the concentration of hydrogen ions, while in winter organic matter decomposition and an increase in the concentration of carbon dioxide and acidic products lead to a drop in pH (water acidification).

Based on the considerations mentioned above, the worst scenario for the infectivity of *C. nobilii* larvae would occur in a water body at pH 8 and 28°C, while at pH 7 and 18°C they are likely to hatch and successfully parasitize hosts, thus continuing the life cycle. Our results are in agreement with observations made in streams of the Sauce Grande basin while collecting adults for this and previous studies performed in our laboratory. The

Table 1. Multiple comparisons.

Contrast	Estimate	SE	*z*-value	*P*-value
T24:pH7:Tp18 – T24:pH7:Tp23 = 0	**0.544**	0.0948	5.74	**< 1e-05**
T24:pH7:Tp28 – T24:pH7:Tp23 = 0	−0.143	0.0997	−1.44	0.8943
T24:pH7:Tp18 – T24:pH7:Tp28 = 0	**0.687**	0.1121	6.13	**< 1e-05**
T24:pH8:Tp18 – T24:pH8:Tp23 = 0	0.089	0.1062	0.84	0.9987
T24:pH8:Tp28 – T24:pH8:Tp23 = 0	**−0.524**	0.1026	−5.11	**< 1e-05**
T24:pH8:Tp18 – T24:pH8:Tp28 = 0	**0.613**	0.1256	4.88	**1.78E-05**
T24:pH9:Tp18 – T24:pH9:Tp23 = 0	**0.586**	0.0974	6.02	**< 1e-05**
T24:pH9:Tp28 – T24:pH9:Tp23 = 0	0.040	0.1014	0.39	1,0000
T24:pH9:Tp18 – T24:pH9:Tp28 = 0	**0.547**	0.1110	4.93	**1.41E-05**
T48:pH7:Tp18 – T48:pH7:Tp23 = 0	**0.652**	0.0992	6.57	**< 1e-05**
T48:pH7:Tp28 – T48:pH7:Tp23 = 0	0.208	0.1032	2.01	0.4838
T48:pH7:Tp18 – T48:pH7:Tp28 = 0	**0.444**	0.1144	3.89	**0.0017**
T48:pH8:Tp18 – T48:pH8:Tp23 = 0	**0.412**	0.1001	4.12	**0.0006**
T48:pH8:Tp28 – T48:pH8:Tp23 = 0	**−0.437**	0.1066	−4.11	**0.0007**
T48:pH8:Tp18 – T48:pH8:Tp28 = 0	**0.849**	0.1183	7.18	**< 1e-05**
T48:pH9:Tp18 – T48:pH9:Tp23 = 0	0.203	0.0989	2.05	0.4522
T48:pH9:Tp28 – T48:pH9:Tp23 = 0	−0.278	0.1023	−2.72	0.0974
T48:pH9:Tp18 – T48:pH9:Tp28 = 0	**0.481**	**0.1157**	**4.16**	**0.0006**

= 0, difference of parameters equal to zero. SE, standard error.
Significantly different comparisons are shown in bold.

number of adults was higher during summer samplings, when temperatures may exceed 28°C, than in early autumn, when temperature periodically reaches 18°C. However, egg strings were observed in autumn and many of them were at an advanced stage of development, identifiable by the dark colour (personal observation).

In addition, we only found significant differences in the infective capacity of larvae among the three assayed temperatures at pH 8, with infectivity decreasing as temperature increased. The pH of water bodies in the Cuenca Sauce Grande has an average value of 8 and it has been classified as neutral to slightly alkaline (Álvarez *et al.*, 2018). This suggests that the infective capacity *C. nobilii* larvae inhabiting the Sauce Grande basin may decrease with an increase in temperature.

Bearing in mind that parasites must be considered for a better understanding of the effects of different stressors on freshwater ecosystems, our results may help in the design, analysis and interpretation of studies of anthropogenic impact. In particular, gordiids are parasites of terrestrial insects potentially capable of altering energy flow in aquatic environments. Within this group, eco-toxicological studies have only been conducted in *C. nobilii*, which proved to be sensitive to different anthropogenic stressors (Achiorno *et al.*, 2008b, 2009, 2010, 2015, 2018). The present study showed that pH and temperature affected larvae of this species. All these results suggest that *C. nobilii* may serve as a promising effect indicator, and further studies are needed to confirm this possibility.

The limitation of the experimental design of our study is worth special mention. We found practical difficulties in placing each Eppendorf tube in an individual beaker to assess changes in the infectivity of *C. nobilii* under different conditions of temperature and pH over time. It became unfeasible to measure the pH of each microtube and to maintain constant the pH due to the large number of beakers required. Taking account of *in situ* studies (e.g. Barata *et al.*, 2007; Salmelin *et al.*, 2016), the Eppendorf tubes assigned to a given treatment were placed in the same beaker. However, they are pseudoreplicates, making it impossible to distinguish between the effect of the microtube and the effect of the beaker. To overcome this drawback, the microtube to which each group belonged was implemented as a random factor (nested for each beaker) in the generalised linear model, and the in-between variation of the groups of hairworm larvae in each microtube could be incorporated into the model. Notwithstanding this, our results revealed that the beaker effect can be discarded.

Another issue to be considered is the negative impact of global climate change on environmental factors (e.g. rising temperatures), which can adversely affect the performance of individuals and alter the dynamics of populations, communities and host–parasite interactions. This would lead to changes in habitat productivity and in the geographical distribution of species. The effect of environmental factors influenced by climate change on parasite infectivity is gaining increasing attention in the last years (Holt & Boulinier, 2006; Lafferty & Kuris, 2006; Poulin, 2006; Williams, 2019; Prasopdee *et al.*, 2020). Our study is within the framework of this approach, suggesting that increasing temperatures interacting with pH and time may reduce the infective capacity of *C. nobilii* larvae. Moreover, this work is in line with that of Williams (2019), who emphasizes the importance of studying the effects of global climate change on parasites from an ecological perspective, instead of solely focusing on parasites of public health and economic importance. Indeed, we showed that moderate changes in important abiotic parameters may affect *C. nobilii* infectivity, leading to further changes in community structure.

To summarize, the information provided in the present study contributes to a better understanding of the effect of some environmental variables on the survival and infectivity of *C. nobilii* larvae. The multivariable approach used in this work allowed a more realistic evaluation of results compared to each factor separately, and indicates that the infectivity of *C. nobilii* was affected by temperature and its interaction with pH and time. Such information advances our knowledge of its biology and is a key step for studying the relationship between *C. nobilii* susceptibility and anthropogenic stressors, and the possibility of using this species as an effect indicator.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X21000420

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

Data availability statement. Data that support the findings of this study are available in the supplementary material. If more information is still needed, data are available from the authors upon reasonable request.

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