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Infectivity of *Chordodes nobilii* larvae (Gordiida: Nematomorpha)

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Abstract: The gordiids are freshwater representatives of the parasite phylum Nematomorpha that function as a link between aquatic and terrestrial ecosystems. In recent years, different ecotoxicologic studies have been made with the South-American gordiid species, *Chordodes nobilii,* that have demonstrated the capacity of this group to act as a bioindicator of contamination. Despite the Gordiida's ecologic relevance, further studies are still needed to elucidate different aspects of the biology of the class, and among those, the infective capacity, a parameter that can be evaluated by the infection index mean abundance (IIMA). A knowledge of the intrinsic variability in the infective capacity of *C. nobilii* would warrant priority in order to establish, the range of acceptable responses for normal or standard conditions in the laboratory, and, to compare the criteria among different assays. The objective of this study was to establish a baseline value for the infective capacity for *C. nobilii* larvae, under controlled laboratory conditions, by employing the IIMA as the evaluation parameter. To that end, we analyzed the infective capacity of *C. nobilii* larvae that had hatched from different strings of eggs laid in the laboratory by a total of 12 females. The *C. nobilii* adults were collected from streams within the Argentina Sauce Grande basin, between 2006 and 2009. Once in the laboratory, after mating, the females were placed in individual containers for oviposition. The egg strings obtained from each female were cut in 3 mm long segments; and when free larvae were observed, the segments (N= 90) were placed together with 30 *Aedes aegypti* larvae for evaluation of the gordiids' infective capacity. After 72 h, the mosquito larvae were observed by microscopy in order to quantify the *C. nobilii* larvae in body cavities. The IIMAs were calculated as the total number of *C. nobilii* larvae present divided by total number of *A. aegypti* larvae examined. For analysis of the IIMAs obtained, the data were grouped according to the female who made the original ovoposition. Our results enabled the corroboration of an ample range of responses in the infective capacity of this species, a characteristic that would normally be linked to the progenitors originating the hatch. Because this relationship prevents the establishment of a baseline for making comparisons among assays with gordiids, through the IIMA as a response parameter, we recommend expressing the IIMA values in each assay relative to their respective controls. These findings also provide evidence for the greater success in infections by certain members of the progeny over others. Finally, on the basis of the results obtained from this study, we stress the relevance of the use of the IIMA as a decisive aspect to be considered in different studies on the biology of Gordiida. Rev. Biol. Trop. 65 (1): 1-8. Epub 2017 March 01.

Key words: parasite, Nematomorpha, infective capacity, infection index mean abundance, female.

Within aquatic environments, the gordiids –freshwater representatives of the parasite phylum Nematomorpha– are of great ecologic value because of their life-cycle characteristics. These features enable them to function as a link between aquatic and terrestrial ecosystems. The Gordiida –in its worldwide distribution– comprises 19 extant genera with approximately

350 species. Studies on domesticated members of these so-called *hairworms* have indicated that the life cycle of gordiids involves five different life stages: (1) egg strings, (2) free-living larvae, (3) parasitic cysts, (4) parasitic juveniles, and (5) dioecious or parthenogenetic free-living adults. The larvae of the Gordiida –being benthic, microscopic, and scarcely motile– survive from three days to a few weeks after hatching, and during that time have the capacity to infect hosts that are either paratenic or definitive (Bolek, Schmidt-Rhaesa, de Villalobos, & Hanelt, 2015; Schmidt-Rhaesa, 2012). Thus, the viability of the larval stage directly after hatching will determine the success in infection and overall survival. The parasitic juveniles choose mainly terrestrial arthropods as the final host, in which the parasitism can cause severe injury or even death. Therefore, the potential of these helminthes as biologic-control agents still needs to be tested. A significant aspect of their mode of parasitism is that they induce an altered behavior in the host, causing in those that are terrestrial, a need to search for and enter water, in an attempt to release the parasites. This immersion can result in drowning (Bolek, Schmidt-Rhaesa, de Villalobos, & Hanelt, 2015; Cochran, Kinziger, & Poly, 1999; de Villalobos, Ortiz-Sandoval, & Habit, 2008; Hanelt, Thomas, & Schmidt-Rhaesa, 2005; Kinziger, Cochran, & Cochran, 2002; Ponton et al., 2006; Schmidt-Rhaesa, 1997; Schmidt-Rhaesa & Ehrmann, 2001).

In spite of the obvious ecologic significance of the Gordiida, an essential detail is still lacking regarding certain key aspects of their biology - namely their infective capacity. This property, in turn, would permit inferences regarding other aspects of the gordiid biology, such as their reproductive success. The infective capacity accordingly can be evaluated by means of the quantitative descriptor of parasite populations "infection index mean abundance" or IIMA (Bush, Lafferty, Lotsg, & Shotsak, 1997). That index has been used to evaluate the susceptibility of the gordiid species *Chordodes nobilii* Camerano, 1901 (Gordiida, Nematomorpha) to environmental contamination (Achiorno, de Villalobos, & Ferrari, 2008a, 2009, 2010, 2015) and to variations in temperature (Achiorno, de Villalobos, & Ferrari, 2008b), for which purpose assay protocols specific for this group have been developed. The ecotoxicologic assays devised up to the present have demonstrated that the preparasitic stages of *C. nobilii* are susceptible to different toxic agents –*i. e.*, the three pesticides glyphosate, malathion, and carbendazim; the two metals, chromium (Cr^{6+}) and cadmium (Cd^{2+}) and the detergent sodium dodecyl sulfate. Mean abundance has also been used by Bolek et al. (2013) in order to determine if after being exposed to a freezing temperature, the larvae and/or cysts could successfully infect. Those studies provided evidence in favor of the possibility that the gordiids could be utilized as bioindicators of contamination.

For this reason, a knowledge of the intrinsic variability in the infective capacity of the species under investigation here –as a measurement of gordiid viability– would warrant priority in order to establish the range of acceptable responses for normal or standard conditions in the laboratory, and to compare the criteria among different ecotoxicologic assays.

The objective of the present study was therefore to establish a baseline for the infective capacity of the South-American species *C. nobilii* under controlled laboratory conditions through the use of the IIMA as the parameter of evaluation.

MATERIALS AND METHODS

Collection of gordiids and generation of larvae in the laboratory: For conducting this study, adults from both sexes of *Chordodes nobilii* were collected from streams within the Sauce Grande basin in the locality of Sierra de La Ventana, Buenos Aires, Argentina (38°09" S - 61°48" W). Samplings were made in various field trips during the spring and summer seasons from 2006 through 2009, those being the reproductive seasons of the species. Once in the laboratory, individuals were kept in containers with aerated natural freshwater at a controlled room temperature of 23 ± 1 °C. This water was gradually replaced with reconstituted hard water (pH 7.6-8.0, hardness 160-180 mg/L as CO_3Ca), which was exchanged each week. Because adults do not feed, providing food was unnecessary. After mating, all the females identified –in this study a total of 12– were placed in individual containers for oviposition. Subsequently, the egg strings obtained in the laboratory –those being *ca*. 0.5 mm width– were separated and thereafter maintained in running dechlorinated water under the same temperature conditions and in the ambient photoperiod until reaching the free-larva stage. Egg maturity was judged by the color of the egg strings, which shade became darker as the larvae developed (Hanelt & Janovy, 1999). At that stage the egg-string samples were scrutinized microscopically. If in the sample 50 % of the individuals appeared to be free larvae, we proceeded to evaluate the infectivity. The taxonomic determination of the *C. nobilii* adults was made according to de Villalobos and Zanca (2001). The females were identified after the completion of oviposition and before the eggs developed into free larvae.

Determination of viability of *Chordodes nobilii* individuals under study: Figure 1 is a schematic diagram of the methodology used. For evaluation of the gordiids' infective capacity– and therefore their survival –*Aedes aegypti* larvae, reared in the laboratory, were used as hosts. These larvae were placed in containers (N= 30 *A. aegyptii* larvae per container, with 12 mL of dechlorinated tap water and wheat germ as food) along with 3-mm-long *C. nobilii* egg-string segments containing *ca*. 4000 *C. nobilii* individuals. Those egg-string segments first had been checked to confirm that ≥ 50 % were in the free-larval stage. In this way, we could assume a similar exposure rate for each sample. For the entire study, a total of 90 egg-string segments were used. After a 72 h exposure to the *C. nobilii* larvae, the *A. aegypti* larvae were fixed in 70 % (v/v) aqueous ethanol. The mosquito larvae were then dissected under the light microscope in order to quantify the *C. nobilii* larvae in the body cavities. The IIMAs were calculated as the total number of *C. nobilii* larvae present divided by the total number of *A. aegypti* larvae examined (Bush et al., 1997).

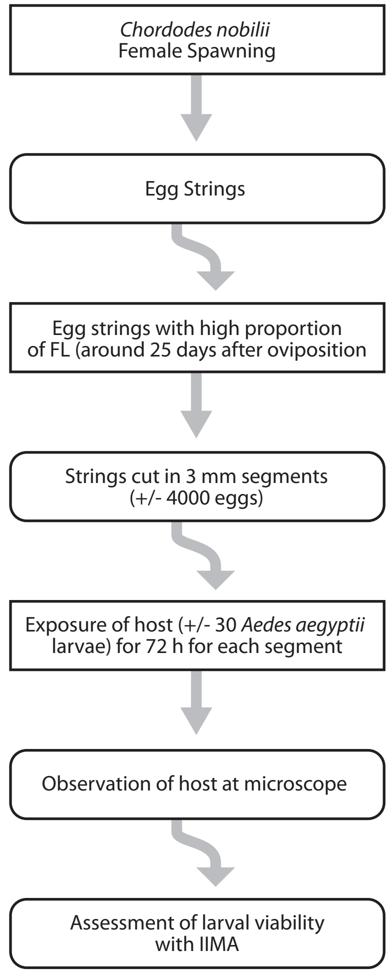

Fig. 1. Flow diagram of experimental procedure. FL: free-living larvae.

For analysis of the IIMAs obtained, the data were grouped according to the female who made the ovoposition. Descriptive statistical analyses were performed, as well as analyses of frequency along with the Kruskal-Wallis test, at a significance level of < 0.05 (Zar, 2010). According to Hanelt (2009), the length of the female gordiid worm is an accurate indicator of the egg output over a lifetime and moreover the variation in reproductive success is extremely low in this population; in order to discard the possibility that the size of the egg string influenced the infective capacity of the *C. nobilii* progeny, we analyzed the correlation between those two parameters by means of the coefficient of Spearman. All of the statistical analyses were performed by the Infostat statistical program (www.infostat.com.ar).

RESULTS

The present study –undertaken in order to determine the viability of *C. nobilii* larvae through their ability to infect *A. aegypti* larvae - indicated a great inconsistency in their infectivity. The first IIMA analysis (N= 90) indicated that a wide range and variability existed among the values, with a minimum of 1.56, a maximum of 21.4, a mean ± SD of 6.84 ± 4.67, and a variance of 21.5. Nevertheless, the analysis of frequency indicated that almost 50 % of the values fell in the class between 1.56 and 4.87 at a mean of 3.21, while the remaining 50 % was divided among five classes (Table 1).

The grouping of the IIMA values according to the female progenitor (Fig. 2) demonstrated that the infective capacity of the larvae originating from females J, K, and L was significantly greater than those from the rest of the females (H= 71; P <0.0001), and the females A and B were those individuals whose larvae were the least infective. This differential infective capability among members of the cohort originating from the different females, would infer that the variability in the infectivity of

TABLE 1
Frequency analysis for *Chordodes nobilii* infectivity

Class	LL	UL	M	AF	RF
1	1.56	4.87	3.21	49	0.52
2	4.87	8.18	6.52	22	0.23
3	8.18	11.49	9.83	7	0.07
4	11.49	14.79	13.14	7	0.07
5	14.79	18.1	16.45	8	0.08
6	18.1	21.41	19.76	2	0.02

Frequency analysis for indexes IIMA (N = 90). The table shows for each class, lower limit (LL), upper limit (UL), mean (M), absolute frequency (AF) and relative frequency (RF).

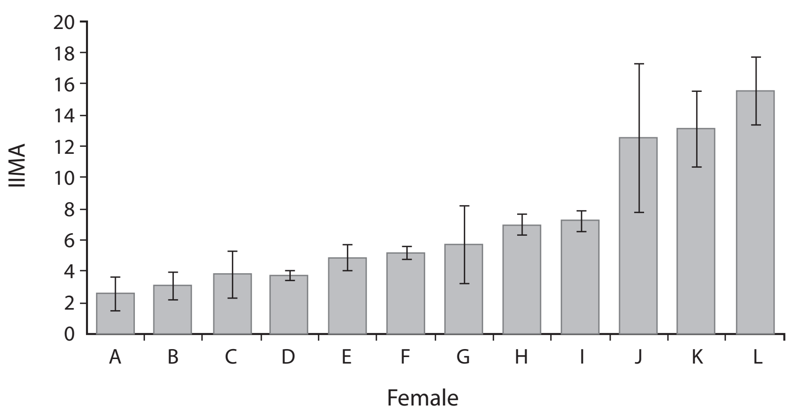

Fig. 2. Infective capacity of *Chordodes nobilii* larvae for *Aedes aegypti* larvae with respect to the female that made the oviposition. In the figure, the average IIMA on the ordinate for each of the *C. nobilii* progenitor females indicated on the abscissa. The error bars represent the SDs.

C. nobilii larvae could result from differing characteristics of the progenitors.

The analysis by the Spearman coefficient (at - 0.04) indicated that no correlation existed between the IIMA values and the total lengths of the egg strings. Therefore, under the experimental conditions used here, the length of the egg string bears no relationship to the infectivity of the resulting larvae.

DISCUSSION

The results presented here are the first providing information on the infective variability of a South-American species of Gordiida, *Chordodes nobilii*, on the basis of laboratory experiments. These findings enable the wide range in that infective capacity of *C. nobilii* to be attributed to the particular progenitors of those larvae. Thus, the evaluation of infectivity on the basis of the IIMA assay, demonstrated that the progeny of certain specific females were more successful in infecting *Aedes aegypti* larvae than others. A very relevant and crucial result that stems from the analysis of infective capacity, as evaluated by the IIMA, is related to the potentiality of the gordiids to be used as bioindicators of contamination. In such an application of *C. nobilii*, comparisons among different assays would be required to establish a baseline set of IIMA values. The infectivity of these larvae, however, proved to be extremely variable; moreover, according to the findings presented here, that capacity was attributable to the progenitors. This characteristic of the species results in a considerable and highly significant variability in the baseline level of infectivity. These findings therefore constitute a warning to investigators alerting them to the necessity of normalizing comparisons among assays conducted with different populations of *C. nobilii*. As a consequence, in order to make comparisons among different bioassays with these gordiid larvae through the use of the IIMA as the form of measurement, the data obtained would always need to be referred to the respective control values for each assay.

If we consider the difference in successful infection among the larvae originating from different progenitors, a significant point that results from the analysis of our findings is that the data enable us to make inferences on the reproductive success of the group. If we consider that “reproductive success can be defined as the passing of genes on to the next generation in a way that they too can pass those genes on” (Pagel, 2009) —that is, we must not only consider the reproductive success of an individual or population but also the probability passing on that capacity to the progeny - then “the genetic contribution of a subpopulation to the next generation depends not only on the number of propagules produced but also critically on the fitness of these propagules” (Therese & Bashey, 2012). In this regard, only certain fraction of a female’s eggs hatch into larvae, and only a portion of those larvae will have an adequate infective capacity, in order to pass on those genes to the next generation. Therefore, we must consider the possibility that the reproductive success of a species of parasite such as the gordiid *C. nobilii* is related to both the numbers of offspring and the latter’s individual and collective infective capacity.

In order to evaluate the reproductive successfulness of a given population of these gordiids, the influence of the progenitors must be taken into account. Hanelt (2009) proposed that “within gordiid populations the offspring of the next generation are contributed nearly equally by females” –in other words, the variation in reproductive success is low for the group. The findings of our study strengthen the point of view that these populations of gordiids exhibit a high variation in reproductive success that has been described as a normal characteristic of the helminths (Hanelt, 2009), such that certain progenitors contribute more greatly to the next generation than others.

These results accordingly argue for the necessity of performing new studies, since the gordiids have a low variation in reproductive success, upon consideration of the numbers of offspring produced (Hanelt, 2009), but exhibit a significant variability in infective capacity among different ovopositions. Such variation can be thought not to be a property definitively influenced by the females, consistent with the asseveration of Levitan (1991) that "estimates of reproductive output based on body size or gamete production alone can be misleading. The assumption that large body size and high gamete production translate into high reproductive success may be not right when the influence of fertilization success is ignored." Thus, the fecundation modality of the group needs to receive attention because, when fertilization occurs, the females and males form Gordian knots that are fraught, with the possibility that a given female may be inseminated by various males at the same time (Bolek et al., 2015; Schmidt-Rhaesa, 1997). Such multiple fecundations could result in a major degree of genetic variability in the progeny and as a consequence affect the reproductive success. Furthermore, in the example of the gordiids, what must also be kept in mind is "the importance of the host environment to the fitness of parasites and the potential for trans-hosts [*sic*] effects to alter the ecological and evolutionary dynamics of parasite populations" (Therese & Bashey, 2012).

To conclude, we wish to emphasize that the data from the present study not only contributed with information on the gordiids of South America, but also enabled inferences to be made that clarified doubts regarding different aspects of the biology of this group. Moreover, the conclusions drawn from the results of these experiments will be highly useful when performing ecotoxicological studies with the gordiids. Furthermore, these findings would be relevant to the standardization of bioassays involving a species from any given parasitic group. Finally, these results would indicate as well the necessity to use the parameter IIMA as a benchmark for consideration in different studies on the biology of the Gordiida in particular. A highly relevant point stemming from the present investigation concerns the conclusions drawn regarding the evaluation of reproductive success. Although an analysis of reproductive success was not the principal objective of these experiments, the data we obtained reinforce the notion previously proposed by other investigators that to determine the reproductive success of a given species one must take into account the significance of fertilization and the fitness of the progeny. In the *C. nobilii* under investigation here, the progeny's fitness is tantamount to that generation's infective capacity. From this conclusion arises the necessity of studying parasites utilizing, whenever possible, the property of infectivity for the evaluation of different parameters.

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RESUMEN

Infectividad de las larvas de *Chordodes nobilii* (Gordiida: Nematomorpha). Los gordiidos son representantes dulceacuícolas del Phylum parásito Nematomorpha que actúan como un enlace entre ecosistemas acuáticos y terrestres. En años recientes, diferentes estudios ecotoxicológicos se han desarrollado con una especie sudamericana de gordiido, *C. nobilii*, que ha demostrado la capacidad de este grupo de actuar como bioindicador de contaminación. A pesar de su evidente importancia ecológica, aún se necesitan realizar estudios para dilucidar distintos aspectos de su biología, entre estos, la capacidad infectiva, un parámetro que puede evaluarse utilizando el Índice de Infección Abundancia Media (IIMA). El conocimiento de la variabilidad intrínseca en la capacidad infectiva de *C. nobilii* merece prioridad con el objeto de establecer el ámbito de respuesta aceptable para condiciones normales o estándar en el laboratorio, y que permita comparar los resultados entre distintos ensayos. El objetivo de este estudio es establecer la línea de base de la capacidad infectiva del gordiido *C. nobilii* en condiciones controladas de laboratorio, empleando el IIMA como parámetro de evaluación. Con este fin, se analizó la capacidad infectiva de larvas de *C. nobilii* que eclosionaron de diferentes cordones de huevos depositados por un total de 12 hembras, mantenidas en laboratorio. Los adultos de *C. nobilii* fueron recolectados de arroyos de la cuenca argentina Sauce Grande, entre 2006 y 2009. Una vez en el laboratorio, después de la cópula, las hembras se ubicaron en recipientes individuales a la espera de la oviposición. Los cordones de huevos obtenidos de cada hembra se cortaron en segmentos de 3 mm de longitud; y cuando las larvas libres fueron observadas al microscopio, los segmentos (N= 90) fueron ubicados junto con 30 larvas de *Aedes aegypti* para evaluar la capacidad infectiva del gordiido. Después de 72 h, las larvas del mosquito fueron observadas al microscopio para contabilizar las larvas de *C. nobilii* en las cavidades corporales. El IIMA fue calculado como el número total de larvas de *C. nobilii* presentes dividido entre el número total de larvas de *A. aegypti* examinadas. Para el análisis de los IIMAs obtenidos, los datos fueron agrupados de acuerdo a la hembra que hizo la oviposición. Nuestros resultados permiten corroborar un amplio rango de respuesta en la capacidad infectiva de esta especie, que estaría vinculada al origen de la camada. Debido a que no se pudo establecer una línea de base para realizar comparaciones entre estudios en los gordiidos utilizando el IIMA como parámetro de respuesta, se aconseja relativizar los valores de los IIMAs a sus respectivos controles. Estos resultados también pusieron en evidencia la ventaja en el éxito de infección de algunas progenies sobre las restantes. Finalmente, con base en los resultados obtenidos a partir de este estudio se plantea la importancia del uso del IIMA como punto final a considerar en distintos estudios sobre la biología de los gordiida.

Palabras clave: parásitos, Nematomorpha, capacidad infectiva, índice de infección, abundancia media, hembra.

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