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Original paper

Description of the life cycle of *Dolops discoidalis* (Bouvier, 1899) (Branchiura: Argulidae), a parasite of the fish species *Rhytiodus argenteofuscus* (Kner, 1858) from the Brazilian Amazon

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ABSTRACT. The genus *Dolops* (Audouin, 1837) is endemic among ectoparasites present in fish, with nine species described in Brazil, five of which are from the Amazon region. Among the genus, the species *Dolops discoidalis* (Bouvier, 1899) stands out due to its high prevalence of parasitism in Amazonian fish. The present study aims to characterize the development phases of *D. discoidalis*. The parasites were obtained from infected fish species *Rhytiodus argenteofuscus* from a natural environment, kept in polyethylene experimental boxes (500 liters). The physical-chemical parameters of the water (pH, electrical conductivity, dissolved oxygen and temperature) were monitored daily. A total of 30 egg layings were analyzed. After identification of the oviposition, the eggs were transferred to another container containing water (0.5 liters). The number of eggs was recorded, with observations made in stereomicroscopic and photographic records until hatching occurred. The egg layings exhibited differences in colour throughout their development. On the seventh day, the embryos exhibited visible ocelli and on the 17th day the hatching period began. Hatching occurred in batches, with around 5–10 hatches/day/laying. However, peak hatching was recorded on the 19th day of the cycle. Therefore, the cycle of *D. discoidalis* comprised a total period of 17–22 days at 28ºC, with hatching in batches and pigmentation of the eggs observed close to hatching. Newly hatched parasites did not survive for more than 24 hours in the absence of the host. This study contributes to knowledge of the biology of *D. discoidalis*, and adds to information regarding possible studies into its control.

Keywords: freshwater ectoparasite, crustaceans, brachyurans, ontogeny

Introduction

Crustaceans are important parasites of freshwater fish, and are therefore one of the main causes of damage to fish farming [1,2]. Notable among these are, ectoparasites of the Argulidae. Brachyurans are important due to the damage they cause to natural fish populations, weakening adult hosts and generally killing younger fish [3].

The freshwater brachyurans are constitute for four valid genera *Argulus* (Muller, 1785), *Chonopeltis* (Thiele, 1900), *Dipteropeltis* (Calman, 1912) and *Dolops* (Audouin, 1837) and currently comprises 155 valid species [4,5]. The group consists of ectoparasitic species that generally live on fish. The greatest diversity of species occurs in the Afrotropical and Neotropical regions [4]. In the Neotropical region there are 33 species, 13 of which are of the genus *Dolops* [6], for Brazil nine species of this genus are mentioned. In the Amazon, five species of the genus were registered [7,8].

Specimens of *Dolops* present hooks in their mouth apparatus that is used for apparatus, for fixation and feeding, which pierce the host's skin, sucking the blood and the epithelial cells [5,9]. The hematophagous habits of these parasites cause anemia, while histophagia causes inflammation of the cutaneous integument, allowing secondary infections to become established [5,10,11].

The following forms of damage caused by brachyurans can occur in natural environments or in fish farms: reduction of weight, fat and growth; metabolic and respiratory disorders; blood loss, hemoglobin reduction; delay of gonadal development; abnormal behavior (disordered swimming). The parceled out reproduction, high fertility and a direct life cycle, which can be completed in a few days, make it possible for the brachyurans population to increase exponentially, becoming real threats to fish farming [3,12].

Among the *D. discoidalis* stands out due to its highest intensity rate of parasitism in Amazonian fish. It occurs seasonally, with the highest intensity of infestation in high waters and lower infestation in low waters [3,13–15]. *Dolops discoidalis* exhibits low parasitic specificity, with a preference for scaleless fish (Siluriformes), where it has been found parasitizing the outer surface of the host [7,13,14].

The population size and growth of *D. discoidalis* determines the extent of the infestation and damage to the host fish, as is evident from the patterns of outbreaks on fish farms [16–18]. Information on life cycle characteristics is a prerequisite for successful regulation of parasitic populations of species of the genus *Dolops* [9,19].

Problems related to *D. discoidalis* infestations are evident both in the natural and in the cultivation environment as they cause damage to their host in a short period of time [3]. However, knowing the biology of this parasite is important for future studies aimed at its control. In this sense, the objective of this study was to describe the life cycle of *D. discoidalis*, with an emphasis on the characterization of the stages of development and the time of hatching and post-hatch survival without the host in a controlled environment.

Materials and Methods

Collection of fish in a natural environment, and maintenance in a controlled environment

Populations of *D. discoidalis* were maintained in infested freshwater fish of the species *Rhytiodus argenteofuscus* (Kner, 1858), which has the popular name of Aracu. The fish were collected in Lago do Maicá, a lake located to the east of the municipal region of Santarém in the state of Pará, beginning at the Amazon River and extending to the Paraná do Ituqui River. It is connected to smaller lakes and contains several species of flora and fauna typical of the lower Amazon. The fish were collected using nets with different mesh sizes. These were placed at five points (P) along Lake Maicá (P1 2°28'25"S 54°39'23.7"W; P2 2°28'23.7" S 54°39'23.7"W; P3 2°28'24.4"S 54°39'16.4"W; P4 2°28'24.9"S 54°39'10.8"W and P5 2°28'23,4"S 54°39'09.0" W) and inspected every four hours. The *D. discoidalis* were collected from the surface of *R. argenteofuscus* (Fig. 1 a). The identification of the species *D. discoidalis* (Fig. 1 b, e, c) was in accordance with the taxonomic keys of [5,20,21].

The infested fish were collected in May 2018, and were transported to the laboratory of the Universidade Federal do Oeste do Pará (the Federal University of Western Pará) (UFOPA) and kept in

Table 1. Physical-chemical parameters of water with *Rhytiodus argenteofuscus* fish in boxes and with *Dolops discoidalis* larvae in a plastic container, in a controlled environment

	Box with fish (n=60)	Plastic container with larvae (n=10)
Parameters	Mean ± SD	Mean ± SD
Temperature °C	28.00 ± 3.38*	28.00 ± 0.05
pH	7.3 ± 1.11*	7.2 ± 0.01
Dissolved oxygen mg/l	6.7 ± 2.04*	9.03 ± 0.07
Electrical conductivity µSm/cm	92.45 ± 7.25*	82.26 ± 0.03

* Differences in the physical-chemical parameters of the water, when compared with the amount of eggs laid and hatched using Tukey's Test ($p < 0.05$)

experimental polyethylene boxes (500 liters) equipped with a mechanical filter recirculation system. The physical and chemical parameters of the water (pH, electrical conductivity, dissolved oxygen and temperature) were monitored daily with the aid of a multipara meter (Extech EC500 model) and with a light: dark 12:12 photoperiod. Water (non-chlorinated) was used in all systems. Infestation intensities typically ranged from 10 to 30 *D. discoidalis* per fish. To obtain the egg layings of *D. discoidalis*, the boxes were lined with transparent plastic, which functioned as a removable substratum. The host fish were monitored daily and the parasite's eggs were removed to control the number of parasites when the infestation intensities appeared high, as indicated by the condition of the host and behavioural changes, such as lethargy and loss of appetite. All the parasite populations were established under these conditions for several generations.

Observing the life cycle of Dolops discoidalis

To avoid washing the substratum, thow box, each box was covered with a mesh cover which did not prevent the passage of light. Some layings occurred in the boxes. For the egg layings of *D. discoidalis*, the transparent plastics that functioned as removable substrata were removed, and the laying sites were cut out and transferred to another container containing water (0.5 liters). Some samples were examined with a dissecting microscope and the remainder were kept in the boxes under daylight. Both tests found that all eggs were viable and hatched.

All the phases of *D. discoidalis* (adults (males and females), eggs and the larval stage) were examined by stereomicroscopy (2× and 4× magnification), photographed with a Samsung PL120 digital camera and examined by light microscopy with magnification from 100 to 400× at the Microscopy and Sample Collection Laboratory of the Universidade Federal do Oeste do Pará (UFOPA). The parasite phases were photographed using a Zeiss Axioplan optical microscope with an Axiocam ERc 5s camera, and the measurements of the organs of the reproductive system were taken with the aid of the Blue Zen edition 2 software package. A biological cycle was prepared using a modified version of that proposed by [12,22,23].

Post-hatch survival of newly hatched larvae of Dolops discoidalis

The survival characteristics of *D. discoidalis* larvae outside the host were examined using the same physical-chemical parameters of the water (pH, electrical conductivity, dissolved oxygen and temperature) every two hours and with a 12:12 light: dark photoperiod. The experiment was carried out in plastic containers (0.5 liters) with the same water as the experimental polyethylene boxes (500 liters) where the hosts (fish) were kept, each containing ten newly hatched larvae. The mortality of these larvae was monitored by removing and counting the number of individuals killed that did not exhibit motility and swimming ability. The death of the larvae was confirmed by gently touching those which did not exhibit motility with a pipette.

Data analysis

Quantitative data were expressed as mean ± standard deviation, mainly of the physical-chemical parameters of the water. The data were first analyzed and evaluated with a One-Way ANOVA test with the physical-chemical parameters of the water and the amount of egg laying and hatching as factors that may or may not influence the viability of the newly hatched larvae of *D. discoidalis*. The data were further subdivided by stage of development (egg and larval) and evaluated with One-Way ANOVAs. When these tests indicated significant differences in the data set, the Tukey test was used. To ensure that the data met the premises of statistical tests (ANOVA, Tukey's test) significance was set at $P<0.05$.

The hatching index during cultivation was analyzed according to an adapted version of [24], where (E_t) corresponds to the hatching index, (O_i) the number of eggs deposited on the substratum and (O_m) the number of eggs that did not hatch.

$$E_t = \frac{O_i - O_m}{O_i} \times 100$$

Compliance with ethical standards

The collection of fish was authorized by IBAMA /ICMBio (Nº46202-3/2017). The procedures for collecting and maintaining the fish were approved by the Animal Research Ethics Committee of the Universidade Federal do Oeste do Pará (N°12008-2017-CEUA/UFOPA) and were in accordance with the Ethical Principles in Animal Experimentation adopted by the National Council for the Control of Animal Experimentation (CONCEA).

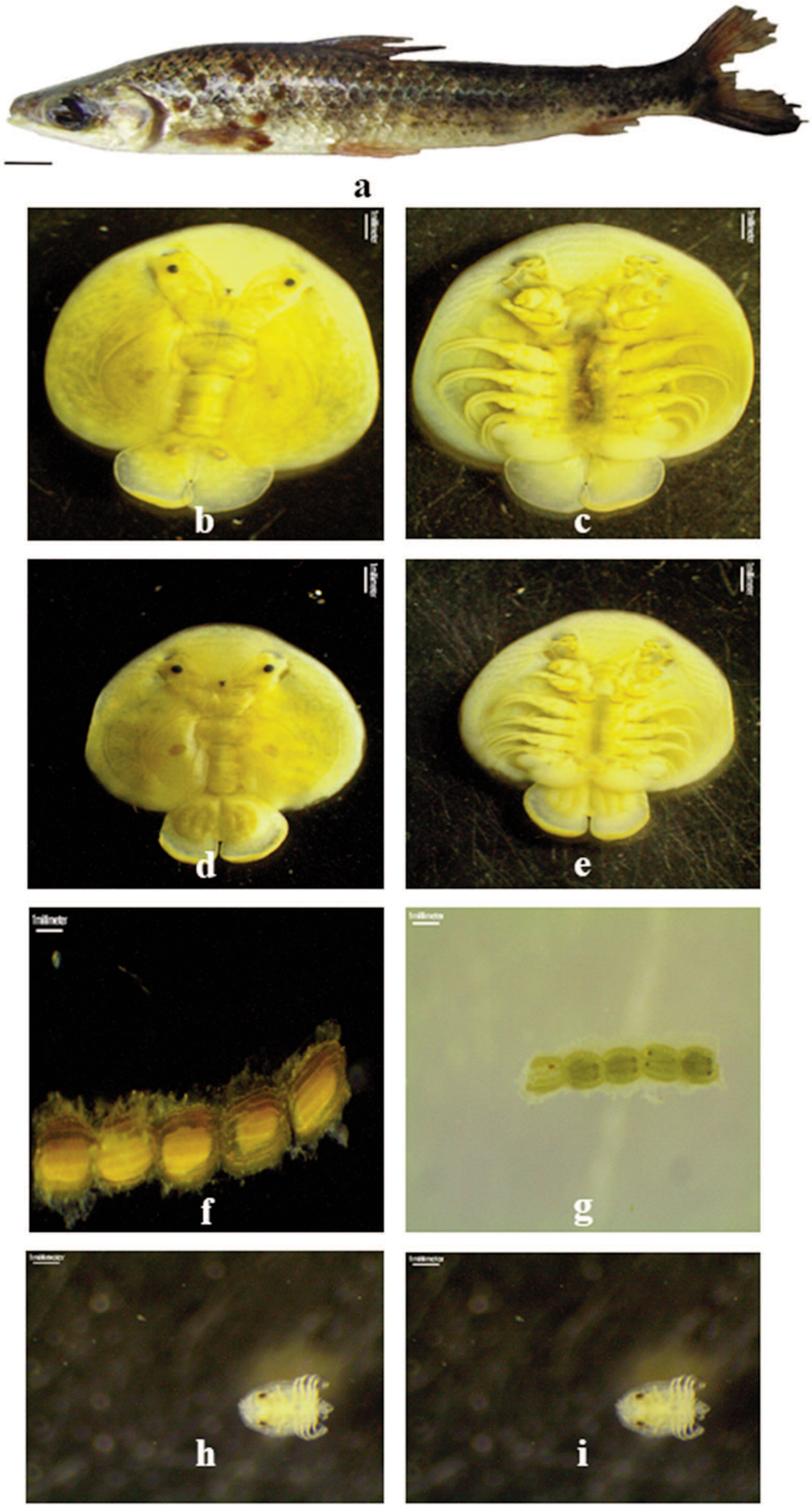

Figure 1. a: *Rhytiodus argenteofuscus* infested with *Dolops discoidalis*; b: Dorsal view of adulthood *Dolops discoidalis*; c: Ventral view of adult *Dolops discoidalis*; d: Dorsal view of young *Dolops discoidalis*; e: Ventral view of young *Dolops discoidalis*; f: Eggs with laying of eggs with an ellipsoid shape and a gelatinous layer; g: Eggs with embryos presented macroscopically visible ocelli; h, i: post-hatch larval (epimorphic) stage of *Dolops discoidalis*. Scale bar: 1 mm.

Results

A total of sixty fish (n=60) were used to maintain the life cycle of the parasites. The parasitized fish were kept for six months in a controlled environment and the physical-chemical parameters of the water were monitored (Tab. 1). During the experiment, there was a period of oscillation in the energy supply, which interfered with the functioning of the mechanical-filter recirculation system. Consequently, when the data were analyzed with the ANOVA test, the physical-chemical parameters of the water affected the amount of egg laying and hatching. These factors therefore influenced the analysis of these variables (Tab. 1), but did not have an effect when the stage of larval development was compared.

In general the parasites were fixed on the external surface of the body, the dorsum and the side close to the head and tail of the hosts (Fig. 1a). When young (Fig. 1d,e), the parasites clustered near the base of the pectoral fins and the caudal peduncle.

When analyzing the shape and structure of the layings, an oviposition process was observed, where the females moved forward on the substratum, placing one egg at a time, forming successive rows an established distance from the first row (Fig. 1f,g).

Thirty layings were observed over the course of 22 days. The total number of eggs observed was 1,342, with the minimum number of eggs observed (n=10) and the maximum number (n=177) (Fig. 2). For all layings, a white colour and ellipsoid shape were initially observed, with a gelatinous cover layer, protecting and securely attaching the eggs to the substratum (Fig. 1f). On the second and third day of observation, the colour of the layings exhibited a light yellow hue, while from the fourth day on, a brown colouring could be seen, which augmented until the first hatchings. The eggs that did not hatch were black in colour. The embryos developed ocelli between the 5th and 7th day, which was the first structure to be viewed macroscopically inside the egg, while the structure of the appendages was also observed (Fig. 1g).

Hatching occurred in batches (5 to 10 hatches/day/laying) between the 17th and the 22nd day, with the majority of hatchings on the 19th day. The hatch rates of *D. discoidalis* ranged from 55.5% to 96%, with an average hatch rate 80±11.53% (Fig. 3).

The larval stage of *D. discoidalis* is of the epimorphic type, where the larvae develop completely within the eggs and hatch as miniature adults, with all their functional appendages and few morphological differences from adults (Fig. 1 h,i). It was not possible to determine the survival time of the larvae outside the host, as access to the laboratories was restricted to the morning and afternoon, although it was observed that the larvae died in the night and did not survive 24 hours after hatching.

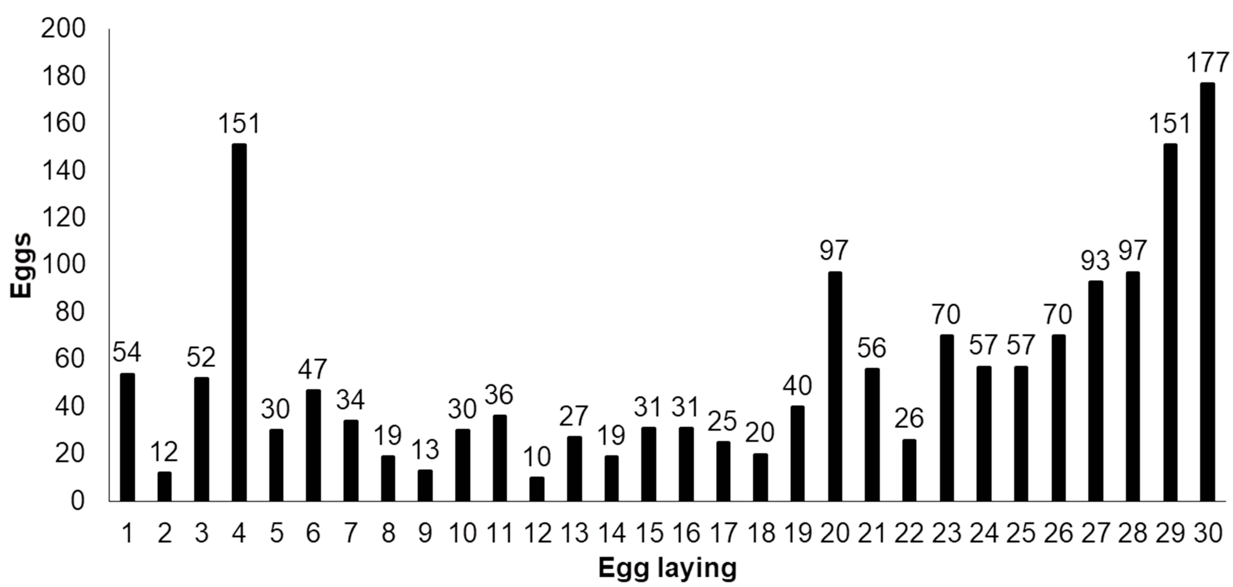

Figure 2. Number of eggs per laying of *Dolops discoidalis* in a controlled laboratory environment

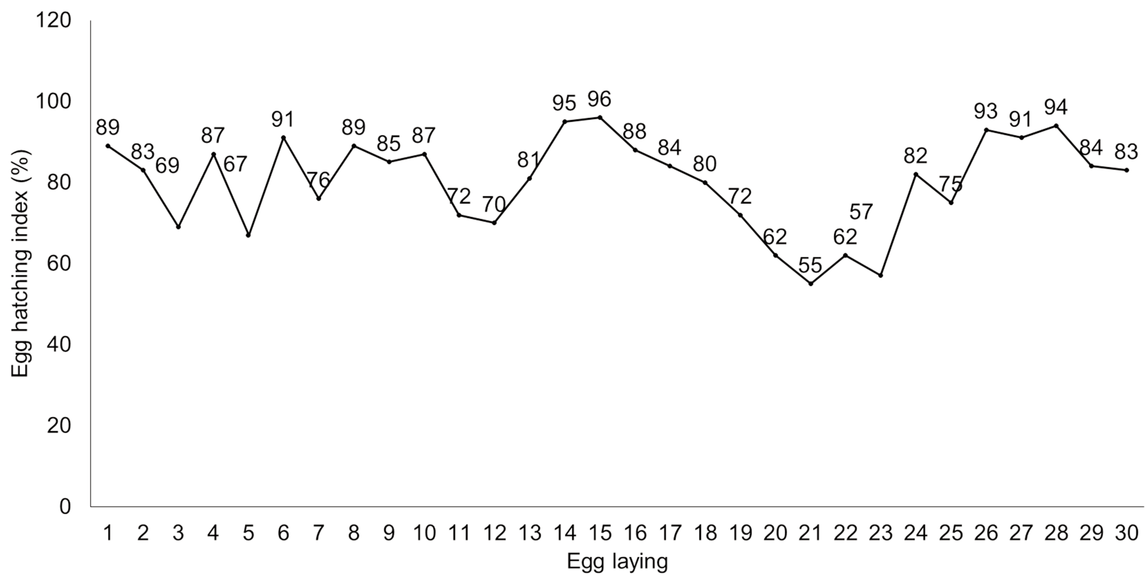

Figure 3. Averages of hatching indexes of *Dolops discoidalis* in a controlled laboratory environment

The species *D. discoidalis* has a direct life cycle (monoxenic cycle) which facilitated its cultivation in an artificial environment (Figure 4). In this case, it is possible to infer that the parasites need to feed soon after hatching. Behavioural changes in the host tanks were identified, such as the stress these parasites caused in their host, especially when the parasites were in the adult stage (Figure 1 b,c). The fish exhibited erratic swimming, beat against the edges of the box, suffered anorexia and injuries to the body, and most died during the six months the parasite life cycle was maintained. Of the fish that began the experiment (n=60), only seven (n=7) survived, with one death due to being very weak and the many injuries caused by the high levels of *D. discoidalis* infestation.

Discussion

According to [6], little is known about the genus *Dolops*, and the information is fragmented, irregular and inconsistent. Some species are known to be relatively widely distributed, especially *D. ranarum* (Stuhlmann, 1891) [25]. In the present study, we provide an improved description hatching of *D. discoidalis*. For [19] the reproduction described in the literature was most likely due to the fact that this genus is abundant in large parts of Europe and Asia, while the species *D. ranarum* is widespread in Africa. Some of the first information describing the genus *Dolops* the Brazilian Amazon was provided by [15], who described *D. discoidalis* parasitizing eight species of fish, three of which were scaled, and reported a high incidence of infestation in Amazonian fish.

As described by [22], only 20 branchiuran life cycles have been described, many of which involved *Argulus* (Müller, 1785), and few of which were of species of *Dolops* (Audouin, 1837). For the majority of *Argulus* males, sperm is transferred directly to the females, using a variety of modified structures on the third and fourth thoracic legs. In *Dolops*, however, sperm are transferred into chitinous spermatophores [15,26,27]. It is worth mentioning that only the *Argulus* cycle is well known, and there is no knowledge of any marine species life cycle [4,25]. This study is therefore the first to describe in detail all the life forms of this ectoparasite of fish from the Brazilian Amazon, as well as aspects of the life cycle of *D. discoidalis*.

Behavioral changes in fish of the species *R. argenteofuscus* were observed in this experiment, especially when highly infested by *D. discoidalis*. Studies of parasite aggregation, an extremely important process, especially during the reproduction period, when it can occur on different scales in the host and the host parasites, can include a comparative analysis of how monoxenic and heteroxenic ectoparasites alter the behaviour of the host [28–30].

In the present study, it was observed that the physical-chemical parameters of the water negatively influenced the number of eggs layed and hatching, confirmed both through statistical analysis and as result of the reported electricity fluctuation in

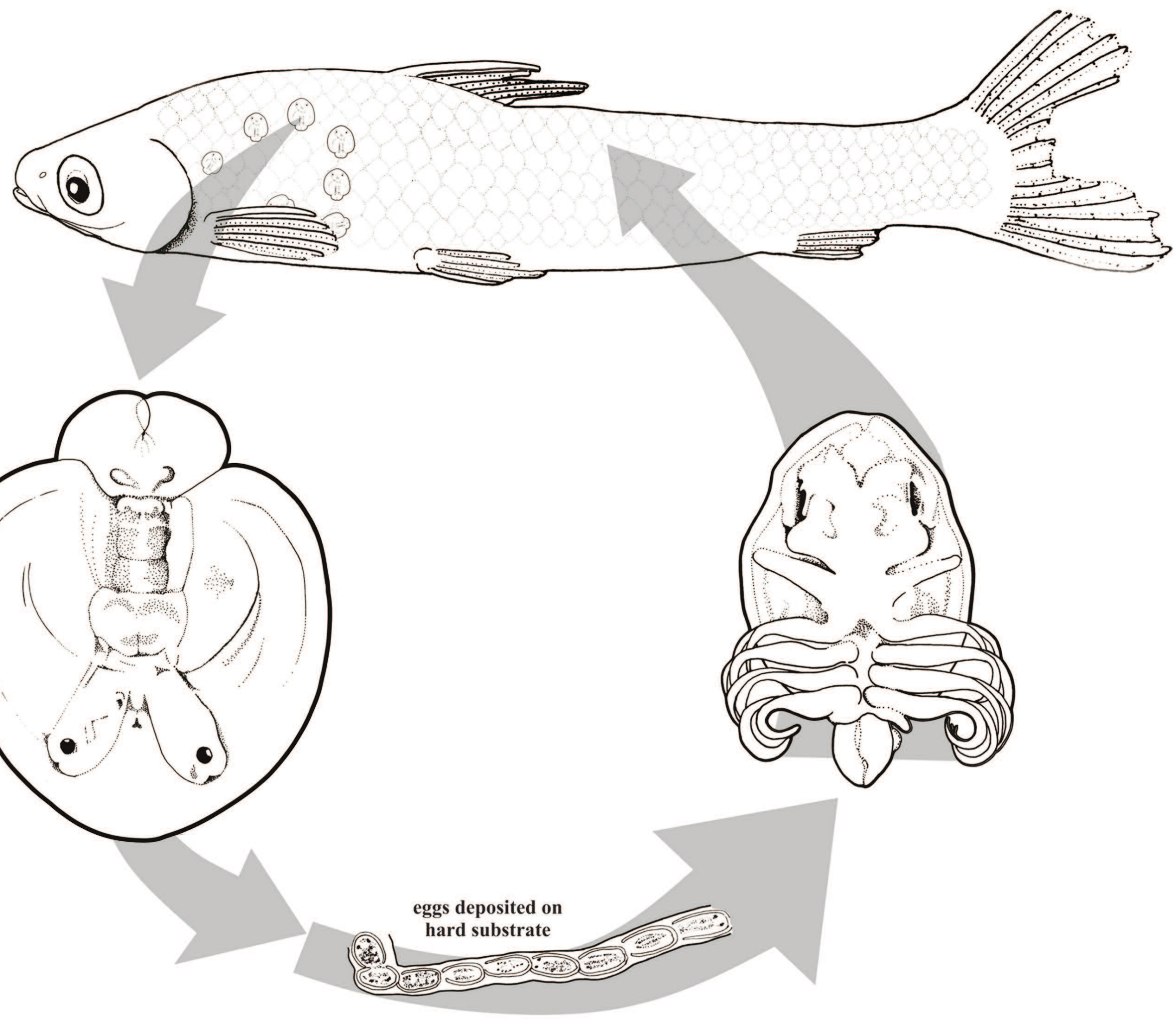

Figure 4. The constructed life cycle of *Dolops discoidalis* (Bouvier, 1899), an ectoparasite widely distributed in the Amazon, was studied by parasitizing fish of the species *Rhytiodus argenteofuscus* (Kner, 1858) in a controlled environment. A biological cycle was prepared as proposed and modified from [12,22,23]. The cycle is of the monoxenic type where mature adults copulate on the outside of the host, pregnant females loosen themselves from the host and swim until they find objects placed in the boxes to deposit their eggs. The hatching peak was recorded on the 19th day of the cycle in a controlled environment at 28ºC. After hatching, the larvae (epimorphic) seek a host and pass through a seedling phase before reaching maturity.

the laboratory. The days that the oscillations occurred provided the lowest egg laying by *D. discoidalis* females, which consequently interfered in the hatching of the eggs. This characteristic corroborates the descriptions of the studies of [17] and [31], which describe how temperature variation and dissolved oxygen significantly interfere in the life cycle of species *Argulus*. The description in this study, referring to the variation in the colour of eggs until hatching, is similar to that described by [32], [7,15] for *Argulus* and by [33] for *D. ranarum*.

In the present study, adult parasites of the *D. discoidalis* species were found attached to the external surface of the body, on the back and side close to the head and tail. When young, the parasites clustered near the base of the pectoral fins and the caudal peduncle. The difference in the infestation sites is related to the life span of the parasite in the host, and is probably due to fixation capacity in the young and the reproductive period (copulation) of the adults. These differences in infestation site in relation to the parasite's life span significantly corroborate the findings of [31,34], which describe and analyze aggregated generations of *Argulus* in their hosts.

In a study of *D. carvalhoi* (Lemos De Castro, 1949) by [7], it was observed that the strings were very similar to those of *D. ranarum,* including the curled shape of the rows [33]. Egg laying (oviposition) in the present study corroborated the number of eggs was within the range reported by [7], although the eggs were arranged in simple rows, unlike in the case of *D. carvalhoi*. The shapes and structures of the egg layings of *D. discoidalis* were similar to those described by [7] in their study with *D. carvalhoi,* and with [26] in their study of *D. bidentata* (Bouvier, 1899). It can be suggested and confirmed that oviposition is a specific characteristic of the Branchiura, however the arrangement of eggs in some species within the *Dolops* genus differs [25].

The mean hatching rate per laying was 80%, but this may be an underestimate, with the hatching rate equal to or greater than 90% if the interference in the variables of the physical-chemical parameters of the water had not occurred. A deeper and more comprehensive comparison of the hatching index of this study with those of other works could not be undertaken, due to the methodology adopted for the calculation of the hatching index in the life cycle of *D. discoidalis*. However, in the present study, the successful hatching index and population increase in the life cycle of *D. discoidalis* led to a discussion about of the exponential increase of this monoxenic ectoparasite when compared with heteroxenic ectoparasites, such as those described by [30,35].

D. discoidalis is a freshwater parasite, with wide distribution in South America, and is frequently found in the rivers and lakes of the Amazon region, generally preying on Siluriformes or scaly fish, with no host specificity [3,13–15]. This species presents a monoxenic life cycle, which is promising for the study and analysis of its life cycle in an artificial environment. The present study showed that *D. discoidalis* had a short life cycle of 22 days, and that larvae did not survive 24 hours post-hatching in the absence of the host. In this case, it can be inferred that this ectoparasite needs to feed immediately after hatching, a factor which contributes to the lack of host specificity of this ectoparasite.

The death of almost all the fish during this experiment was due to the high levels of infestation, caused by the exponential growth of the *D. discoidalis* population in the recirculation system, related to the success of the direct life cycle this was also observed in artificial systems, confirming low parasitic specificity. Care should be taken to prevent introduction of *D. discoidalis* during introduction and reintroduction of fish species, transportation, restocking, and the cultivation of natural or exotic species, as the damage caused by this parasite can be incalculable for both natural stocks and fish farms.

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