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Egg morphology, larval development and description of the oncomiracidium of *Heterobothrium ecuadori* (Monogenea: Diclidophoridae) parasitising the bullseye pufferfish, *Sphoeroides annulatus*

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Summary

The present study is the first description of the egg morphology, embryonic development, and time required for hatching, and longevity of the oncomiracidium of *Heterobothrium ecuadori* (Meserve, 1938) Sproston, 1946. Experiments found that hatching time fluctuated between 7 and 10 days with a mean of 7.5 ± 1 days at 23 ± 1° C and 35 ‰. Eggs were provided with a polar filamentous appendage. The body of the oncomiracidium was flattened dorso-ventrally, 156 ± 9 μm long and 65 ± 8 μm wide. A full description of the egg development and morphology of the oncomiracidium is provided. The longevity of the oncomiracidia was 4 – 7 days at 21 ± 1°C, with a mean survival time of 121.8h. The ability to rear diclidophorids like *H. ecuadori* and to record precise information on their development provides valuable data for further studies.

Keywords: oncomiracidia; *Heterobothrium ecuadori*; Diclidophoridae; bullseye puffer fish; *Sphoeroides annulatus*; Mexico

Introduction

The genus *Heterobothrium* Cerfontaine, 1895 family Diclidophoridae, is a monogenean parasite of tetraodontid fish. The free-swimming larvae, oncomiracidia, invade a fish host; this direct life cycle can contribute to population explosions in aquaculture systems, resulting in clinical disease. Outbreaks of disease due to diclidophorids have been reported in several Asian countries, including Japan and have caused severe economic losses (Ogawa 2002). In Mexico along the Pacific coast, *Heterobothrium ecuadori* (Meserve, 1938) Sproston, 1946 infects the bullseye puffer fish *Sphoeroides annulatus* Jenyns, 1842 from Oaxaca state (Lamothe-Argumedo, 1967) to Sinaloa state (Fajer-Avila *et al*., 2004). In the Gulf of Mexico, *Heterobothrium lamothei* Vidal-Martinez & Mendoza-Franco 2008 has been detected on the gills of *Sphoeroides testudineus* (Linnaeus, 1758). All these diclidophorids are blood feeders that, depending on the parasite species, may infect the gills, branchial or buccal cavities of the host fish. Severe parasitic infections of *H. ecuadori* can result in significant mortality of cultured bullseye puffer fish (Fajer-Avila *et al*., 2003), which is currently ready to be cultured on a larger scale to test advances in hatchery technology (Abdo de la Parra *et al*., 2010). Knowledge of the biology and life span of the oncomiracidia contributes to the development of contingency plans for effective management and control of this parasite.

Materials and methods

Ten wild bullseye puffer fish adults from Mazatlan Bay, Sinaloa, Mexico (23° 13' N; 106° 25' W) were caught with hook and line by local fishermen and transported alive to CIAD's laboratory facilities. Holding facilities consisted of ten independent 450 L circular tanks with individual mesh-filtered (20 μm) flow-through seawater supply and constant aeration. Once the fish were placed into the tanks (1 fish per tank), cotton thread (15 cm length) was attached to the aeration tubes for one hour to collect parasite eggs used as a source of *Heterobothrium ecuadori* oncomiracidia. Water conditions during the experiment were maintained at 23 ± 1ºC and 35 ‰ . Eggs attached to the cotton thread were incubated in Petri dishes under natural photoperiod 12 h : 12 h with sterilized, aerated seawater until active larvae hatched. Eggs were observed daily using a stereomicroscope (LEICA MZ 9.5) and an optical microscope (LEICA DMLB 10) connected to a high resolution video camera (Sony CCD Iris) to record the development of the

larvae. Measurements of the length and width of 30 eggs are given in µm ± SD.

To estimate hatching time and lifespan of oncomiracidia, 10 eggs from 5 individual fish were obtained using the protocol described above. Petri dishes were incubated at 23 ± 1°C and 21 ± 1°C to determine, respectively, the hatching time and longevity of oncomiracidia. Eggs were monitored using a stereomicroscope each day to count the number of hatched eggs (empty egg shells). The hatching rates were calculated using the number of empty egg shells found each day. Final hatching rate was expressed as the accumulated percentage of empty egg shells until the 15th day relative to the total number of eggs incubated (n=50).

The mean hatching time $H_{50\%}$ (time at which 50% of the eggs were empty) and the mean survival time $S_{50\%}$ (time at which 50 % of the larvae remained alive) were estimated using a logistic model shown below:

$$P_i = \left(\frac{1}{1 + e^{-k(X_i - C)}} \right)$$

where P_i is the proportion of hatched eggs or larvae alive at day X_i, C is the $H_{50\%}$ or $S_{50\%}$, and k is the parameter representing the instantaneous rate of hatched eggs or larvae remaining alive. The model parameters were estimated by a non-linear least-square technique using Marquardt's algorithm (Neter et al. 1996). Mobility and active free swimming oncomiracidia were observed under a stereomicroscope every 15 minutes for the first hour and then at 2, 4, 8, 12, 14, 24, 33, and 48 h; after 48 h, they were observed every 24 h until they died. The parasites were considered to be dead if they were immobile and repeatedly failed to respond to small nudges with a needle.

The newly hatched swimming larvae were transferred to a glass slide to be observed alive under an optical microscope. A drop of neutral red (0.05 %) was added to each slide to observe the parasites internal structures (Lázaro-Chavez, 1984). For the morphological descriptions, oncomiracidia were first observed alive under a phase-contrast and optical microscope and the drawings were made with the aid of a camera lucida (1000X).Taxonomic characters were measured according to the methodology of Ogawa (1998, 2000). Measurements of the parasites are presented as µm ± SD (n=30).

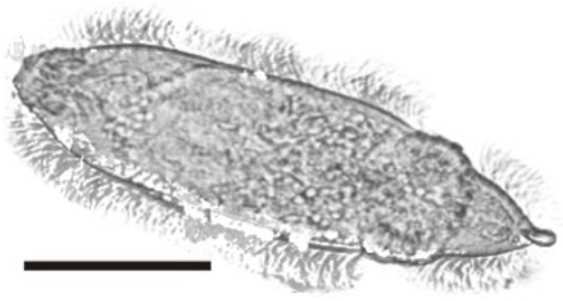

Fig.1. Oncomiracidium of *Heterobothrium ecuadori* after hatching. Scale bar: 100 µm.

Results

Description of the egg and embryonic development

Eggs oval (211 ± 7 x 75 ± 6), operculated (23 ± 1 x 52 ± 0.5) and ectolecyte with a long filament opposite to the operculum. Viable eggs were light brown and infertile eggs were transparent or colourless. Three hours after the egg was laid a mass in the centre of the shell and vitelline cells around the embryo were observed. At 48 h after the embryo consumed numerous vitelline cells the mouth and pharynx'cells were differentiated. At 72 h the larval shape was clearly defined, the pharynx and larval hooks were visible and the contractile movements of the embryo were more regular, indicating muscle development. At 96 h, the border of the body became delimited, the haptor and the ciliature were visible and larval movement inside the shell occurred more frequently. At 120 h, the larva filled the space within the shell and vitelline cells were no longer visible, the adhesive glands were formed and movements in the ciliature increased considerably. At 144 h, strong muscular contractions in the larva and dynamic ciliature movements were observed, indicating that the larva was ready to hatch. The oncomiracidia dislodged the operculum mechanically and emerged head first through the opercular opening, until the larva was free-swimming (Fig. 1).

Hatching time ranged between 7 and 10 days at 23 ± 1° C, with a mean hatching time ($H_{50\%}$) of 7.5 ± 0.6 days. The range defined by the lower quartile ($H_{25\%}$) and the upper quartile ($H_{75\%}$) indicated that 50% of the eggs hatched between 7 to 8 days; a large k (2.41) implied that hatching occurred during a short span of time (Fig. 2) .

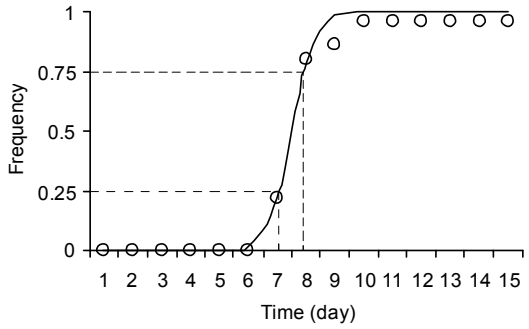

Fig. 2. Hatching time of *Heterobothrium ecuadori*. Regression coefficients and constants of the logistic model fitted to plots of the proportion of hatching eggs (y) against time (t). Observed mean proportions of hatching eggs (o), predicted proportions according to age-dependent hatching model (—), lower quartile $L_{25\%}$ and upper quartile $L_{75\%}$ (- - -).

Description of the oncomiracidium

Body flattened dorso-ventrally 156 ± 9 long, maximum width over the hind-part of the body behind the haptor (65 ± 7). Epidermis thickened and covered extensively by cilia (18 ± 1 long). Anteriorly to the haptor a strip of ciliated epithelium ran forward, confined to the lateral margin of the body except for the ventral surface just in front of the haptor. Five pairs of marginal and flexible hooks of the same shape and size (18 ± 1 long) with a recurved handle

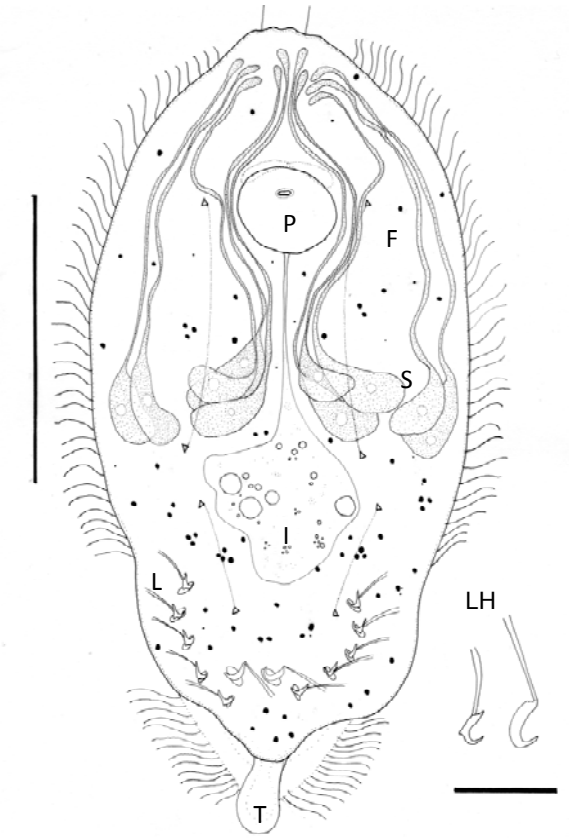

Fig. 3. Oncomiracidium of *Heterobothrium ecuadori*. LH - Larval hooks Pair I-V, which rest in a domus; P - pharynx; SG - Secretion Glands; F - flame cells; I - intestine; T - terminal projection. Scale bar 100 μm. Hamuli (scale bar: 20 μm).

(13 ± 1 long) and a domus. A pair of hamuli, larger than the marginal hooks located at the posterior end of the haptor, between the first pair of hooks. Each hamulus 25 ± 1 long consisted of a straight handle 15 ± 1 long located at 45° position with a curved thick blade. No pigmented eyespots. A pair of sensillum 8 ± 0.4 long at the anterior part of the body next to the mouth. Mouth opened terminally and a muscular pharynx almost spherical (23 ± 1 long and 25 ± 2 wide). Alimentary tract or gut sack-shaped dilated posterior ending in the anterior part of the haptor and containing numerous rounded globules that stained easily with neutral red. A group of five pairs of gland cells; extended from the anterior to the end of the first half of the body in the middle of the body. The gland cells were divided into two outer and three inner gland cells on each side of the body, with each cell having a duct that opened independently and released a secretion to the apical zone. The five ducts were distributed with three outer ducts originating from the two outer and one from one of the three inner cells, and two inner ducts from two of the three inner cells. Four pairs of flame cells as excretory system were observed. The first anterior pair was lateral to the pharynx and the second anterior pair lateral to the gland cells. The third pair was located posterior-lateral to the gut sac, the fourth pair external to the median hooks. No ending pore or collecting tubules were observed. Presence of a terminal projection at the end of the haptor. No nervous system was detected (Fig. 3).

Longevity of the oncomiracidia

The life span of the oncomiracidia of *H. ecuadori* was 4 – 7 days long at 21 ± 1º C and 35 ‰. The mean survival time ($S_{50\%}$) was 121.8 h (5 days). The range defined by the lower quartile ($S_{25\%}$) and the upper quartile ($S_{75\%}$) indicated that 50 % of the larvae died between 100 to 136 h (4 to 5 days); a large k (0.076) implied that larvae died during a short time interval (1.5 days) (Fig. 4). Mobility decreased with time and the larvae were considered dead when they did not show a physical response to an external stimulus.

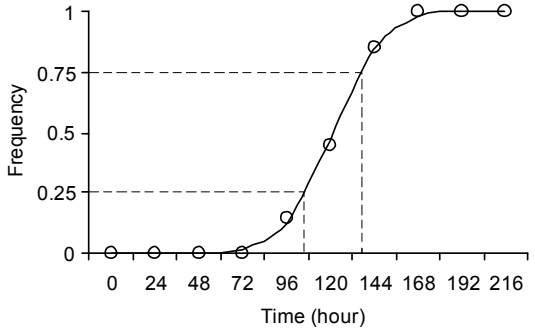

Fig. 4. The influence of time on larval survival of *Heterobothrium ecuadori*. Regression coefficients and constants of the logistic model fitted to plots of the proportion of dead larvae (y) against time (t). Observed mean proportions of larval survival (o), predicted proportions according to age-dependent survival model (—), lower quartile $L_{25\%}$ and higher quartile $L_{75\%}$ (- - -).

Discussion

This study is the first description of the embryonic development, oncomiracidium morphology, hatching period and longevity of *Heterobothrium ecuadori*. The egg morphology of *H. ecuadori* was similar to other species in the genus, even though some aspects of egg capsule morphology are species-specific, as in the case of *H. okamotoi* which possess two filaments attached to the capsule (Ogawa, 1997). The eggs of *H. ecuadori* had a single appendix that allowed clusters or egg chains accumulated in the mucus or organic matter of the host fish, similar to eggs of *Diclidophora luscae* and *D. denticulada* (Macdonald, 1977). This egg distribution pattern was similar to the long chains (up to 2.83 m) observed for *H. okamotoi* (Ogawa, 1997). There are few detailed descriptions of embryonic development of monogeneans and none available for any diclidophorid. Nevertheless, information on other monogeneans, *Entobdella soleae* (Kearn, 1963) and *Merizocotyle* sp. (Kearn, 1968), suggests that *H. ecuadori* follows a similar pattern of development.

The morphological features of *H. ecuadori* oncomiracidia (e.g. ciliation pattern, hook distribution, unpigmented eyes, haptoral armatures, flame cell distribution and the presence of droplets in the body) were similar to other diclidophorids described previously, including *H. okamotoi* (Ogawa, 1998) and *Neoheterobothrium hirame* (Ogawa, 2000). The pair of sensilla cilia found in the oral part of *H. ecuadori* oncomiracidia suggests a sensory function. The gland cells resemble Macdonald's (1977) description for *Diclidophora* spp. and presumably these cells have a sticky function such that larvae can attach to the bottom of the Petri dish; this function perhaps facilitates attachment to skin and movement around the body or gills of the host, similar to Ogawa (1998) observations. The main morphological features that distinguish *H. ecuadori* oncomiracida from the oncomiracidia of other diclidophorids were total length and the shape of the hamuli. The oncomiracidia of *H. ecuadori* were smaller (156 ± 9) than *H. okamotoi* (200 – 300 µm) and *Neoheterobothrium hirame* (250 – 320 µm) oncomiracidia, which are related with the different size of these monogeneans. The handle's position on the hamulus of *H ecuadori* oncomiracidum and its less curved blade can be distinguished from the hamulus of *H. okamotoi.*

The distribution of the lipid droplets of *H. ecuadori* oncomiracidia tended to be located in the posterior of the body and the haptor as reported for *H. okamotoi* (Ogawa, 1998). In contrast, *Heteraxine heterocerca* revealed a random distribution of droplets within the whole body whereas in *D. merlangi* they were distributed much more densely in the haptor than in the body proper (Macdonald, 1977; Kearn *et al*., 1992).

Successful host location and attachment are vital for oncomiracidia survival. The results estimated that oncomiracidia of *H. ecuadori* had a lifespan of 4 – 7 days at 21 ± 1°C, which may indicate a high infectivity for a long period of time. This is also the case for other monogenean larvae belonging to the same family, including the oncomiracidia of *Heterobotrium okamotoi* with the longevity of 7.3 days at 20°C (Ogawa, 1998).

The present study provides precise information on the basic biology of *H. ecuadori* that could be useful for further researches on the effective control of this monogenean infection.

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