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# Delayed hatching of *Neobenedenia* sp. parasite of almaco jack *Seriola rivoliana* in La Paz, Baja California Sur, México.

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## Abstract

*Neobenedenia* species are parasites that caused outbreaks in aquaculture of marine fishes around the world like *Seriola spp*. Monogenea eggs require specific environmental conditions for larval development and hatching. Several works have evaluated the effect of temperature and salinity, reporting the optimal ranges of hatching at 5-7 dpl (days post laying) with more than 90% of hatched eggs, and normal morphology of larvae. We observed delayed hatching of *Neobenedenia* eggs in laboratory condition and in fishponds. This situation leads to a reinfection of fishes in those systems. The objective of the present work was to evaluate for how long hatching could extend, and the viability of the larva along the experiment. Small masses of eggs were incubated in laboratory for three months of winter 2018-2019; daily routine was established for counting larvae and evaluating morphology. We perform five trials of infection in juveniles of *Seriola rivoliana*. Hatching was observed, from day 5 to day 105 dpl with decreasing number of larvae observed each day. At the end of the experiment over 98% of egg hatched, sparse developing eggs or ocellated were observed. Along the experiment, a subsample of 364 alive larvae were observed and categorized by morphology in 4 types: type A, 35% had normal morphology, Type B, 29% had both deformed opistohaptor and eyespots, type C, 23% had only deformed eyespot and type D, 13% only deformed opistohaptor. In trials, infection success was obtained with larvae from eggs of 46 dpl to 77 dpl, and failed infection with larvae from 92 dpl. This work gives the period for hatching and the infectivity of larvae to infect, important information for the control of the parasite in captivity and set a correct period of quarantine for tanks and ponds for marine fishes.

**Key words:** *Neobenedenia*, Seriola rivoliana, delayed hatching.

## Introduction

*Neobenedenia* species are recorded from over 100 species from wild, aquarium and farmed teleost worldwide (Whittington and Horton 1996). These parasites cause major epidemics in marine aquaculture like greater amberjack *Seriola dumerili*, almaco jack *Seriola rivoliana*, and Japanese amberjack *Seriola quinqueradiata* in Japan (Whittington 2012). *Seriola* spp farming in the world are important as a candidates for diversification in different areas of the world and one of the main bottlenecks for further expansion appear to be disease impact (Sicuro and Luzzana 2016).

The infection of *Seriola* by capsalids like *Neobenedenia girellae* can cause hemorrhage, inflammation, and mucus hyperproduction (Leong and Colorni 2002, Valles *et al* 2019) (Fig 2). These monogeneans are ectoparasite and have a single host in their life cycle (Fig 3) with an adult, egg and a free-swimming ciliated larvae emerging from the egg.

Monogenea eggs require specific environmental conditions for larval development and hatching. Several works have evaluated the effect of temperature and salinity mainly, reporting the optimal ranges. After 5 to 7 day (dpl) hatching finish to occurs in short periods of 1 or 2 days with more than 90% of hatched eggs (Valles *et al* 2019).

However, we began to see that when the *Neobenedenia* eggs were incubated in the laboratory, we observed that in general they were hatching on days 7 and 8, but some hatched until day 15. On the other hand, after the fish treatment cycles to eliminate the parasite considering a hatching of 7 to 8 days, we continued to have reinfection. Therefore, the objective of the work was to evaluate if the conditions of large numbers of eggs, small masses could delay their hatching, so we needed to determine first how long it could be prolonged, second in what state the larvae would be, normal or with alterations and third if these larvae are still infectious to fish.

## Methodology

Source of the parasites

A specimen of the leopard grouper, *Mycteroperca rosacea*, from the Cibnor facilities. (Fig.1) Broodstock with 4 years in captivity, showed lethargic behavior and opaque eyes. A severe monogenean infestation was detected. Only the eggs produced in 24 hours were used, threads in the hoseair, at the air-water interface and in collectors (nylon threads), where placed to catch them (Fig 4).

The specimen received a freshwater immersion treatment for 5 min, from which adult monogenean samples were recovered for morphological and molecular identification.

Egg Incubation Assay

The masses egg were washed with sea water to remove adhering material. Wet weight was obtained on an analytical balance and placed in 6-well plate (w1 to w5) and in small petri dish of 60 mm (p1) and 120mm (p3). As control, a low quantity of eggs was used on a substrate, nylon thread in well plate and petri dish (w6-s, p2-s). They were incubated in the laboratory at room temperature, 18 to 20 °C, and natural photoperiod from December 2019 to March 2020. They were examined 3 times a week and a change in filtered seawater was made at 35 ppt. At the end of the trial, the masses were weighed and the percentage of hatched, ocellated or developing eggs was quantified. The number of eggs per gram was estimated.

Oncomiradium observations

In each observation day, the number of active larvae was quantified at surface and in the water column, and a subsample (average 10 larvae) was observed at the microscope, 100 and 400 times magnification, bright field without staining. The shape of the body, opistohaptor and the eyespots were considered as they are important characteristics. The shape of the body, the condition of the opistohaptor shape, the number and shape of the sclerites, the hamuli, the number, shape and arrangement of both the pigmented part and the lenses were considered.

Samples were fixed in 10% formalin for subsequent analysis**.**

Infestation trial

The fish available for testing were juvenile's amberjack *Seriola rivoliana,* 20 cm total length average. Therefore, the first trial was carried out to verify that they were susceptible hosts. It was performed with the first larvae obtained, 7 days after laying, and as the infestation was positive, 4 infestation trials were carried out throughout the experiment. The methodology consisted, as described by Valles *et al* 2019. Briefly, larvae were gently harvested by pipetting into a beaker. A clean tank (500 L) with the fish, water flow (seawater from well) and aeration were stopped, the larvae were added. In 10 minutes the aeration was slightly reactivated, one hour later the flow of water and aeration resumed.

Collectors, white multifilament nylon threads 30 cm long by 1cm diameter, were used as substrates placed in the water daily to detect the presence of eggs and thus the presence of adult parasites in the fish and after a few days the absence of eggs would indicate that the fish are no longer infected with adults.

The fish were sedated with 1ml/100L eugenol to give a 4-minute immersion in fresh water and to hand gently remove parasites from the skin of the fish. The parasites were counted, measured, and preserved in 10% formalin for their subsequent staining and mounting.

## Results

About hatching egg along the trials

The monthly environmental temperature from December to March (23.1, 20.7, 19.4, 21.2) with an average of 21.10 °C ± 1.56.

Hatching was observed, from day 5 in all cases to day 105 dpl (day post laying) in one case (Fig 5) . Regarding the activity of the larvae, more than 75% were in water column in all experimental units along the experiment.

In the experimental units with scattered eggs, it was observed that 50% accumulated hatching at 5 and 6 dpl. In contrast, mass condition was variable from 7 to 28 dpl.

90% of the larvae hatched at 7 and 12 dpl in scattered eggs, in contrast in mass it was obtained at 34 and 69 dpl.

Only 10% of the larvae hatch after 7 and 12 dpl under dispersed conditions, in contrast, more than 50% under mass conditions.

At the end of the experiment, over 98% of egg hatched in all cases, sparse developing eggs or ocellated were observed.

About morphology of larvae

The observed larvae were classified into 4 types, according to the normality or deformity presented in the opistohaptor and in the eyes.

Classification Larvae types (Fig. 6)

Type A, normal morphology larvae, elongated body with 3 bands of cilia, disc-shaped opistohaptor with 2 sclerites, 2 hamulus anterior and 2 hamulus posterior in the midline of characteristic shape and size, and 14 marginal larval hooks. Present 4 eyes with hemispherical pigmented cells and spherical lenses.

Type D: deformed opistohaptor, changes position, number, shape, size and orientation of the sclerites, hamulus or larval hooks. From slight affectations such as reduction in size of one sclerites to severe condition such as disorganization of pieces, aberrant shapes and missing sclerites.

Type C deformed eyes. Alterations in position, number, shape, size, and orientation of pigmented cells and lenses. From slight affectations such less spherical lens to aberrant lens in shape, absence of one or all eyes or duplicated eyes.

Type B: deformed opistohaptor and eyes. Present at least one characteristic of Type B and Type C in same the larva.

From January 9 (35 dpl) to March 18 (dpl 103), 31 days of observations were made to a total of 364 alive larvae. Overall, the following were observed: type A 35% normal morphology, Type B 29% both deformed opistohaptor and eyes, type C 23% only deformed eyes and type D 13% only deformed opistohaptor.

A trend towards the end of the trial of a decrease in the percentage of normal larvae and consequently an increase in deformed larvae was observed (Fig.7).

Fig. 1 Ponds for marine fishes at Cibnor. *Seriola rivoliana* and *Mycteroperca rosacea*

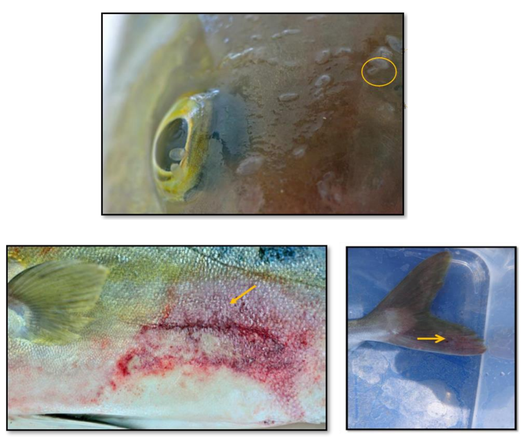

Fig. 2 *Neobenedenia* sp. (circle) on skin of amberjack and skin damage (arrow) from Valles *et al.,* 2019)

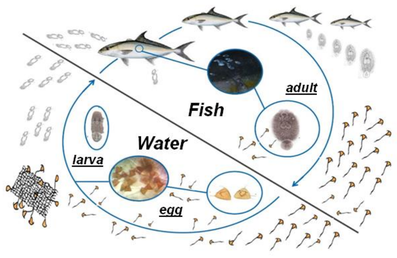


Fig. 3. Schematic direct life cycle of *Neobenedenia* sp. Adult, egg and larva modified from Valles *et al.,* 2019.

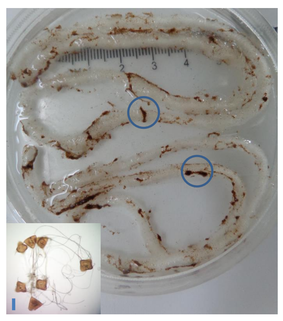

Fig. 4 Masses (circles) and scattered eggs of *Neobenedenia* sp. on a multifilament nylon thread used as substrate for collecting. Scale in cm. Insert detail of the eggs, pyramid form and long filament. Scale bar 120 µm.

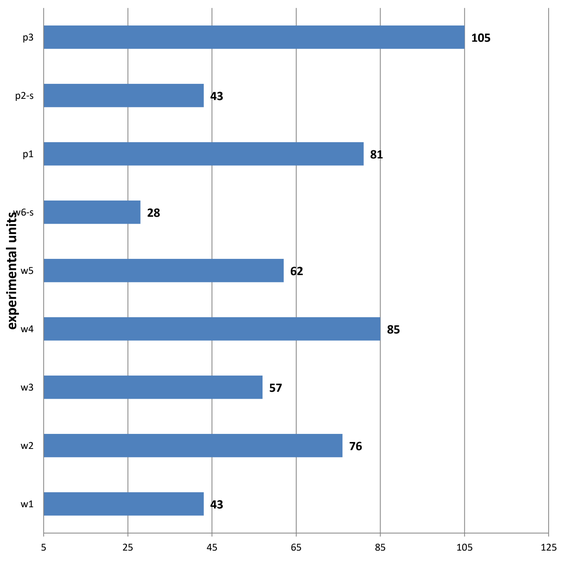


Fig. 5 Period of hatching observed in each experimental unite along the experiment. Hatching observed from 5 to 105 days after laying.

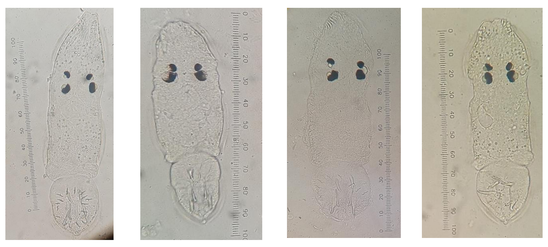

Fig. 6 Larva types, left to right, Type A Normal, Type B Eyespots and opistohaptor deformed, Type C Eyespots deformed, and Type D Opistohaptor deformed. Bar scale 256 µm.

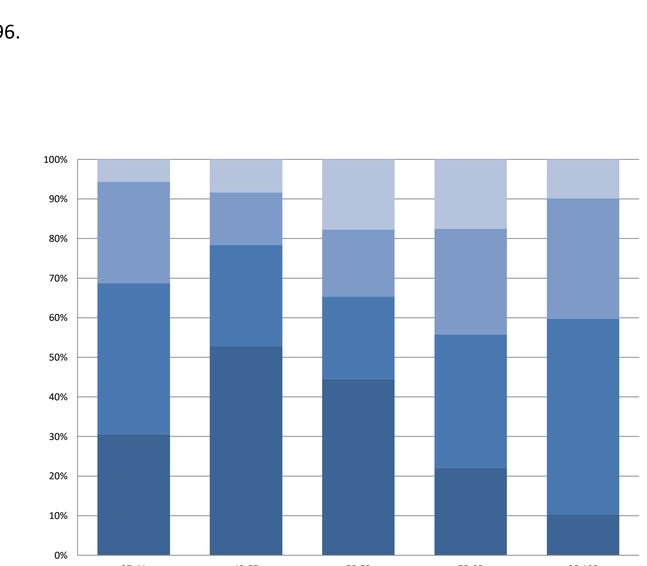


Fig. 7 Average percentage of larvae *Neobenedenia* sp. observed of each morphological type (A,B,C,D) throughout the experiment, from day 27 to 103 post-laying (December 2019 to March 2020)

About infection trials (Table I)

In trials 2 and 4, infestation success was obtained when using larvae of eggs laid after 46 dpl and from 74 to 77 dpl. In both, the presence in the tank collector of eggs produced by the adult parasites in the fish was observed. Adults and juveniles parasites of different sizes were recovered in the freshwater baths, the latter indicating that reinfection occurred in the tank.

In trials 3 and 5, failed infestation, with larvae from 63 to 65 dpp and from 90 to 92 dpp, no eggs were observed in the collectors, nor parasites were recovered in the baths.

In trial 3, the number of larvae for infection was actually good. In trial 5, larvae from 90 to 92 days, very few only 36 and of them only 4 larvae of type A, apparently normal.

The percentage of infection success was low if total larvae are considered, but if adjustment is made to normal type A larvae according to the percentages observed on the corresponding day.

Table I. Trials of infestation on fish juveniles, *Seriola rivoliana*, with larvae *Neobenedenia* sp. obtained of the experiment of delayed development under high density incubation.

| **Trial** | **1** | **2** | **3** | **4** | **5** |
|---|---|---|---|---|---|
| **Infection conditions** | | | | | |
| Number of fishes | 3 | 2 | 1 | 1 | 1 |
| Fish condition | naive | naive | non naive | non naive | naive |
| Number of infections | 1 | 1 | 3 | 4 | 3 |
| Larvae obtained of egg at day after laying (dpl) | 7 | 46 | 63, 64, 65 | **74, 75, 76,77** | 90, 91, 92 |
| Total number of larvae | 500 | 400 | 680 | 220 | 36 |
| Number of larva normal, Type A | 500 | 98 | 218 | 76 | 4 |
| **Parasites obtained** | | | | | |
| Adult parasites | 0 | 21 | 0 | 0 | 0 |
| Juveniles parasites | 14 | 0 | 0 | **39** | 0 |

## Discussion

The hatching of monogenean eggs is determined by multiple factors, endogenous and environmental. Hatching respond to cues like shadows, chemical and mechanical signals (Whittington and Kearn 2011). The effect of temperature and salinity on development and hatching has been studied in *Neobenedenia* (Brazenor and Hutson 2015, Valles et al 2019). They recorded that the time elapsed until hatching did not exceed 15 days. In the present study, hatching were observed since 5 to 105 days post laying and the larva were infective to the 74 days, this record is unusual. Long hatching periods can increase the likelihood of a successful of monogenean infestation, as reported by Whittington and Kearn 2011 in the case of *Entobdella soleae* of sole.
Further research is required to understand factors that lead to delayed hatching.

This information is of great importance to establish treatment protocols for this parasite both in tanks and ponds, so that an adequate treatment or rest period is given to the tanks or ponds so that when new batches of fish enter these systems don't get infected.

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