

RESEARCH NOTE

Observations on the early development of the kelpfish *Myxodes viridis* (Clinidae) with the first description of their spawning habitat in subtidal kelp forests (*Lessonia trabeculata*)

Observaciones del desarrollo temprano de la doncellita, *Myxodes viridis* (Clinidae), y la primera descripción de su hábitat de desove en bosques de macroalgas pardas submareales (*Lessonia trabeculata*)

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Abstract.— The spawning sites, embryonic development, and initial larval stages were described for the Chilean kelpfish, *Myxodes viridis*. Six different egg clutches were found attached by an elastic filament to the fronds of the subtidal kelp, *Lessonia trabeculata*, at shallow depths (0-7 m). All egg clutches were cared for by one large (>15 cm TL) male, and egg color varied depending on the developmental stage. Embryonic development until hatching was categorized into 7 stages, which were completed within about 18 days in the laboratory. Recently hatched larvae presented fully pigmented eyes, a well-developed mouth with major shape changes in the head during the first week suggesting their ability to feed on plankton early in their larval development.

Key words: Kelpfish, spawning site, embryonic development, hatching, larvae

INTRODUCTION

Kelpfishes are a subgroup of species in the Clinidae family. Generally small in size, they live among the fronds of large brown macroalgae, turf algae, and seagrass in rocky substrates (Stepien 1990, 1992). Clinids belong to the monophyletic group Blennoidei, which share similar aspects of their reproductive biology and mating processes (Hastings & Petersen 2010). In general, most of these fishes use external fertilization, where females choose a spawning site and lay their eggs, then males fertilize and guard them (Hastings & Petersen 2010). The egg development success is determined, at least in part, by the oxygenation and temperature requirements suitable for embryonic development (Miller & Kendall 2009). Thus, site features (*i.e.*, water flow, substrate type, depth, refuge availability, Helfman *et al.* 2009) and the male parental care abilities to guard and aerate the nest are essential aspects that parents must fulfill (Gross 2005, Navarrete-Fernández *et al.* 2014).

In the southeastern Pacific, kelpfishes of the genus *Myxodes* are distributed from Bahía Independencia, Perú (14°6'S) to southern Chile (50°57.9'S) (Navarro & Pequeño 1979, Stepien 1990) and include *Myxodes viridis* (Valenciennes, 1836), *Myxodes cristatus* (Valenciennes, 1836) and *Myxodes ornatus* (Stephens & Springer, 1973). Specific information about larval development and feeding habits have been reported only for *M. viridis*, whose pelagic larvae shift from omnivorous to strictly carnivorous, coinciding with morphological shape changes during development (Muñoz & Ojeda 1998, Ochoa-Muñoz *et al.* 2013, Zavala-Muñoz *et al.* 2016). Benthic post-larval recruits and adults can be found in rocky intertidal habitats (Mansur *et al.* 2014), where they are considered important microcarnivores that feed on a wide variety of copepods, amphipods, isopods and snails (Stepien 1990, Quijada & Cáceres 2000). Juveniles and adults are also known to use *Lessonia trabeculata* and *Macrocystis pyrifera* kelp forests

in the shallow subtidal, where they have been particularly overlooked, probably due to their cryptic nature (Stepien 1990, Pérez-Matus *et al.* 2007, Reyes & Hüne 2012). However, no information about reproductive behavior and early life history stages (*i.e.*, eggs, embryos, settling larvae) of *M. viridis* have been described, as well as embryological development traits or spawning habitats.

During early developmental stages, shape changes are critical to face the pelagic life and posterior recruitment success (Fuiman & Higgs 1997, Frederich *et al.* 2008). Morphological studies may provide useful information about the ecological strategies on early developmental stages in fishes (Frederich *et al.* 2008, Zavala-Muñoz *et al.* 2016, Bernal-Durán & Landaeta 2017, Landaeta *et al.* 2019,

Muñoz-Cordovez *et al.* 2019) and geometric morphometrics has been a useful tool to capture shape changes dealing with the scaling, position, and orientation of the shape through the ontogeny (Toro-Ibacache *et al.* 2010, Klingenberg 2011).

In the present paper, observations of *M. viridis* egg clutches found on *L. trabeculata* sporophytes are presented (Fig. 1). This work aims to describe the spatial location of the spawning site, the timing of embryonic developmental stages, and the main shape changes of recently hatched larvae. Although the kelp forests of central Chile have long been the focus of field studies by fish ecologists, this is the first time spawning of *M. viridis* is described, contributing novel information for the species and the association between the kelpfish and kelp.

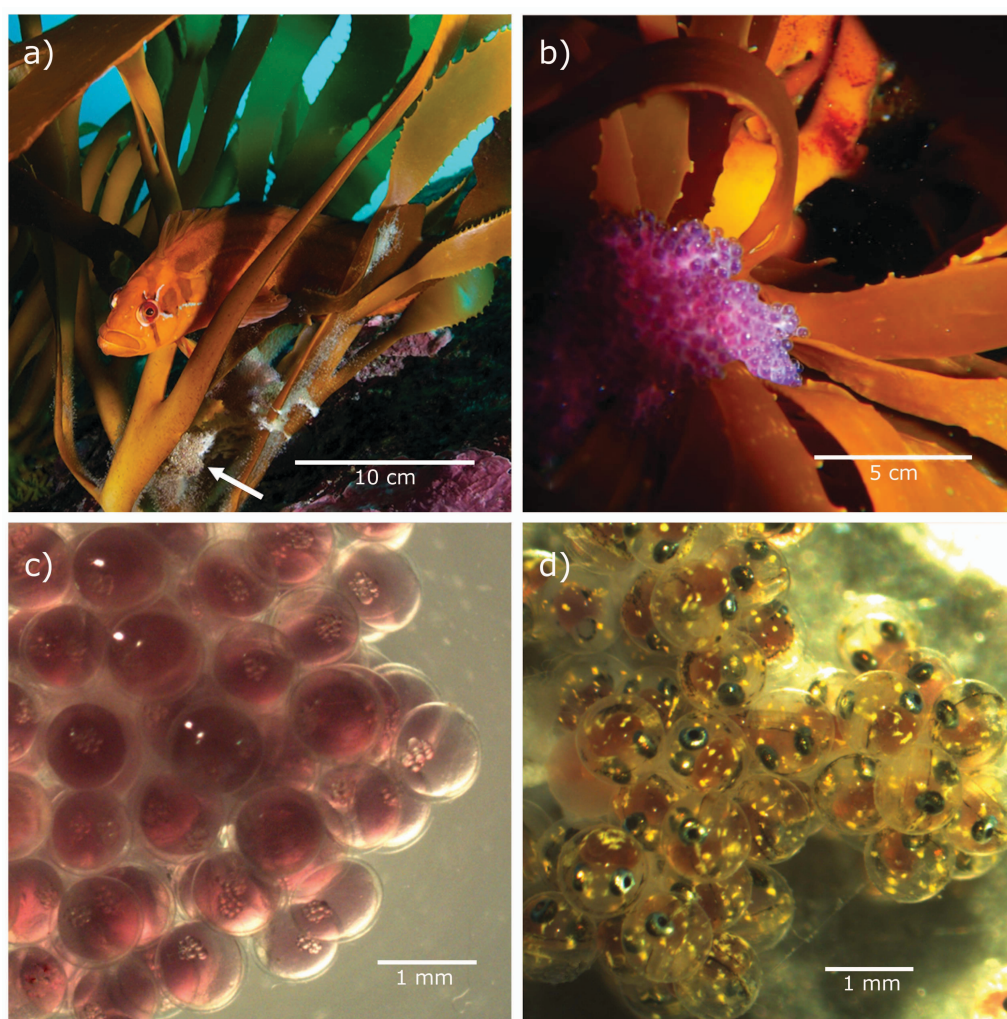


Figure 1. Macroscopic view of: a) *M. viridis* adult caring the egg clutch in the *L. trabeculata* frond, b) recently spawned egg clutch showing its characteristic purple color c) the egg clutch at early development stage and d) at late development stage. Photograph author: a) Eduardo Sorensen, b) Javier Vera / Vista macroscópica de: a) adulto de *M. viridis* cuidando la postura de huevos en la fronda de *L. trabeculata*, b) postura de huevos recién desovados que muestra su característico color púrpura, c) huevos en la etapa de desarrollo temprano y d) en la etapa de desarrollo tardía. Autor de la fotografía: a) Eduardo Sorensen, b) Javier Vera

MATERIALS AND METHODS

Multiple egg masses of *M. viridis*, hereinafter called clutches (Fig. 1), were found during austral autumn-winter months (April to July) of 2016 in shallow subtidal (0-7 m) kelp forests at three sites on the central Chilean coast: Punta de Tralca (33°25'19.93"S, 71°42'14.57"W; three clutches), Las Cruces (33°30'10.16"S, 71°38'1.24"W; two clutches) and Quintay (33°11'31.36"S, 71°42'5.52"W; one clutch). Five egg clutches were found attached to *Lessonia trabeculata*, and one from Las Cruces was attached to a collector (a PVC plastic mesh) fixed to *L. trabeculata* stipes. For each clutch, the presence, size (TL) and behavior of adults (Fig. 1a) were recorded and a sub-sample of eggs (about 10% of egg clutch) was collected. All samples were transported in individual 20 L plastic tanks with aerated seawater to the nearby laboratory of the Estación Costera de Investigaciones Marinas (ECIM) at Las Cruces. Once in the laboratory, the eggs from each clutch were maintained in individual 50 L containers with filtered (0.45 µm) running seawater (14-15 °C) and constant aeration. In addition, an adult fish guarding the nest at Quintay was captured to determine sex via gonad analysis.

In order to describe embryonic development of *M. viridis*, eggs were monitored daily until hatching. Each day, ten randomly-chosen eggs per clutch were carefully removed with a pipette and photographed (stereomicroscope Olympus SZ61, camera Micro Imaging CMOS, resolution 5.0 MPixel). Afterward, sampled eggs were fixed with 4% formaldehyde for 24 h and further preserved in 95% ethanol. The development was categorized into stages according to characteristics such as oil droplet position, eye development, pigmentation patterns, and tail formation, following Kingsford (1985) and Stepien (1986). Embryonic development time was estimated within each egg clutch as the number of days to reach hatching from specific development stages. In two clutches, more than one stage was observed; in those cases, only the most advanced stages were considered. Finally, egg diameter was measured and volume (estimated as sphere volume with egg radius) was determined for five individuals per development stage per clutch through photo analysis using ImageJ software 1.49v.

Once larvae hatched, 30-50 individuals from each clutch were maintained in 50 mL containers, with constant aeration and temperature (14 °C) and seawater replacement twice per day. From these, 10 individuals were randomly selected each day and anesthetized with 0.1 mL of benzocaine (BZ®-20 Veterquímica S.A.), measured (total length, TL) and photographed along the left lateral side. Afterwards, the individuals were allowed to recover and returned to the experimental 50 mL containers.

Geometric morphometric analysis was used to evaluate body shape changes and was based on ten landmarks: 1) snout tip, 2) upper head margin, 3) projection of anus over the dorsal margin, 4) posterior tip of the notochord, 5) anus, 6) lower jaw angle 7) ventral margin of eye, 8) anterior margin of eye, 9) dorsal margin of eye, and 10) posterior margin of eye (Fig. S1, and see Miller & Kendall 2009). A total of 91 pictures of larvae were selected to digitize landmarks with the software TpsDig2 (version 2.22, Rohlf 2006). To assess the relative amount of variation among individuals and potential measurement error, the digitalization of landmarks was repeated for the same 91 individuals and a Procrustes ANOVA was performed. To test for different body shapes, Principal Component Analysis (PCA) was used. Specifically, variation in larval body shape during the 7 days after hatching was evaluated and larvae were grouped into three categories according to post-hatching time A) 0-24 h (n= 28), B) 2-4 days (n= 33), C) 5-7 days (n= 30). Finally, a multivariate regression analysis was performed to determine whether size could affect the body shape through development (allometry) using centroid size as an independent variable and Procrustes coordinates as dependent variables (regression score). All analyses were performed using the program MorphoJ (version 1.06d, Klingenberg 2011).

RESULTS AND DISCUSSION

At all sites, one egg clutch was found on a single individual of *L. trabeculata*. All clutches were attached by elastic filaments to the stipes of the kelp, just below the blade origin (Fig. 1a, b). All egg clutches were actively guarded by one large (>15 cm TL) *M. viridis* (Fig. 1a), except for one clutch at Punta de Tralca, where beside the large specimen, two smaller (10 cm TL) conspecifics were found closer to the egg clutch. Gonad analysis of the large adult collected revealed it to be a male. Although no other adults were analyzed, they showed a similar size (*i.e.*, >15 cm TL) and guarding behavior, so it was assumed that all guarding specimens were male. Guarding adults did not exhibit aggressive behavior towards the divers but remained close to the eggs.

Eggs were spherical with an average diameter of 1.04 mm (\pm 0.003 SD) and volume of 0.6 mm³ (\pm 0.005 SD). The eggs were either purple in color, during early embryonic development (Fig. 1c), turning to bright golden in late stages (Fig. 1d).

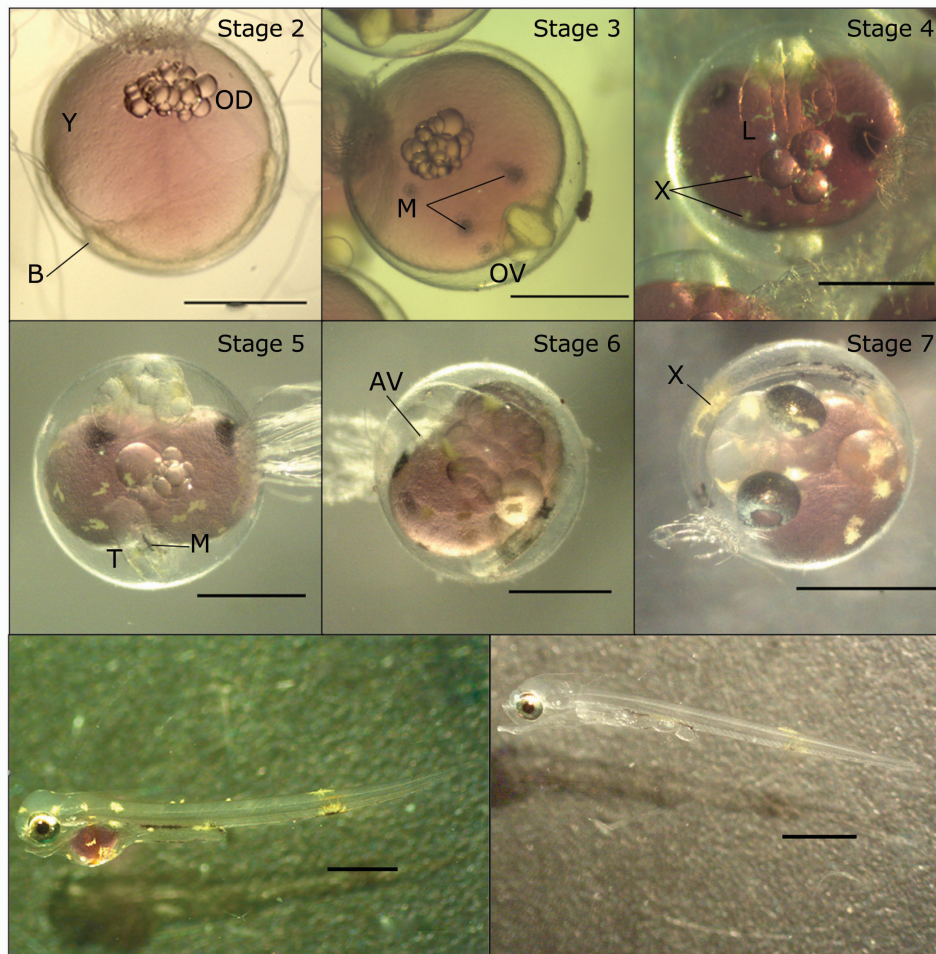


Figura 2. Embryonic developmental stages of *M. viridis* with representative photographs of each one. Pictures of larvae show first day post-hatching and the seventh day of hatching. Letters indicate principal structures of each stage (Y: yolk, B: blastodisc, OD: oil droplet, M: melanophores, OV: optic vesicle, X: xanthophores, L: leucophores, T: tail, AV: auditory vesicle). Scales bars on eggs represent 0.5 mm and in larvae 1 mm / Etapas de desarrollo embrionario de *M. viridis* con fotografías representativas de cada uno. Imágenes de larvas muestran el primer día después de la eclosión y el séptimo día posterior a la eclosión. Escalas en huevos representan 0,5 mm y en larvas 1 mm. Las letras indican las estructuras principales de cada etapa (Y: vitelo, B: blastodisco, OD: gota de aceite, M: melanóforos, OV: vesícula óptica, X: xantóforos, L: leucóforos, T: cola, AV: vesícula auditiva)

Based on structure development and pigmentation, eggs/embryos were grouped into 7 different stages (Fig. 2). As the earliest stage observed from field-collected eggs was neurula, all previous stages, including cleavage and gastrulation, were grouped together as 1st stage. During ~48 h, the stage of neurula (2nd stage) presented an 80% of the egg volume composed of yolk, of which a third corresponded to the blastodisc, and multiples oil droplets were present. The 3rd stage was characterized by cephalization, optic vesicle development, with anterior and posterior regions identifiable by the end of day 4. Four melanophores appeared and multiple oil droplets remained. After 6 days, a 4th stage was indicated by head feature and full eyes, thickened tail, and reduced yolk exhibiting a “kidney” shape. Somites and xanthophores were visible at this stage. The 5th stage (>8 days) was defined by the appearance of a melanophore on the tail, the separation of tail from the yolk, and the presence of the auditory vesicle. The beginning of eye pigmentation, an increase in iridescence and movements characterized the

6th stage which lasted up to 12 days. Lastly, at pre-hatching stage (7th stage), eye pigmentation became bluish in color, xanthophores were dispersed around the body, the yolk was reduced by half and the embryo was in constant movement. This last stage persisted about 4 days until hatching. Although variable, most of the oil droplets coalesced into just one large droplet during the last two stages.

Size of newly hatched larvae, measured within 24 h after hatching, was on average 5.20 (\pm 0.39 SD) mm TL, ranging from 4.04-5.98 mm. Larvae hatched with fully pigmented eyes, a well-developed mouth, and active swimming abilities. The yolk sac was entirely consumed by the 5th day post-hatching. Most of the melanophores were branched and one in the ventral post-anal region overlapped with a xanthophore. Other branched xanthophore and melanophores were present in the gut, but xanthophores gradually disappeared during development and by the 7th day post-hatching only the melanophores in the tail and gut remained (Fig. 2).

Results from Procrustes ANOVA estimate an error of 5.5% (Individual effect: SS= 0.226, MS= 1.57×10^{-4} , df= 1440, F= 18.09, *P*-value < 0.0001; Residual effect: SS= 1.27×10^{-2} , MS= 8.69×10^{-6} , df= 1456). Ontogenetic allometry progressed on average at a rate of 10.6%, where the permutation test against the null hypothesis of independence gave a *P*-value = 0.001. Principal Component Analysis (PCA) described the primary sources of larval body shape variation defined by PC1 and PC2, which explained 52.3 and 16.0% of variation, respectively. Variation in PC1 explained the deformation across the dorso-ventral axis (Fig. S2). For PC2, shape variation was mainly in the head, specifically a displacement of the eye upward relative to the maxilla, which resulted in an elongated and pointed head (Fig. S2).

Regarding the characters of the newly hatched larvae, two-branched xanthophores and a melanophore present in the post-anal region confirm this as a useful taxonomic character (Zavala-Muñoz *et al.* 2016). Also, a well-developed mouth, vigorous swimming movements and yolk consumption suggest that *M. viridis* larvae could be able to feed on plankton early in their larval development. In fact, smaller sizes of *M. viridis* larvae have been reported with gut contents (Ochoa-Muñoz *et al.* 2013) and the main body shape changes the head region are directly related development of anatomical structures involved in prey localization (vision) and capture (as jaws) necessary for the transition from endogenous yolk consumption to exogenous prey feeding (Sanderson & Kupferberg 1999, Yúfera & Darias 2007).

Regarding the reproductive patterns of clinids, our results indicate some common features about the reproductive traits between *M. viridis* and *Heterostichus rostratus* (Myxodini), the North American giant kelpfish. First, incubation time and egg size seem to be similar in these two species, as *H. rostratus* eggs are 1.4 mm of diameter and develop slightly faster (12-17 days at 18 °C; Stepien 1986). Also, structure development timing in the embryos of both species seems similar, ending with a hatching stage characterized by a yolk-sack larva with observable jaws, eye pigmentation and pectoral fins (Stepien 1986); where *H. rostratus* larvae hatch with a 6.2 mm TL. In this study, neither fertilization nor first embryo development stages (*i.e.*, blastula and gastrula) were observed, but it is suggested they are likely similar to *H. rostratus* because of the similarity in environmental temperature, egg sizes and timing of events observed here. Finally, these two species spawn in a vegetal substratum without previous parental care modifications, however, *H. rostratus* select mainly turf algae rather than kelp (Coyer 1982).

Parental care is a common strategy in blennioids, where male size has been reported as a selected trait by females (Neat & Lengkeek 2009). An interesting observation was the presence of smaller conspecifics near a male guarding the same egg clutch, and the occurrence of different stages of eggs within the egg clutch. It has been reported that when males are in charge of parental care, it is common that multiple females spawn in the same egg clutch (see Coleman & Jones 2011). Our observations contrast that described for *H. rostratus* behavior, in which guarding males chase away all conspecifics and egg clutches are composed of eggs of similar developmental stage (Coyer 1982, Stepien 1986).

Finally, the choice of an appropriate spawning site is one of the fundamental processes affecting offspring success (Neat & Lengkeek 2009, Hastings & Petersen 2010). The consistent sighting of *M. viridis* egg postures in kelp fronds across sites separated by >10 kms suggest that this substrate represents a prime place for spawning in central Chile. Considering their ability to mimic kelp fronds, it makes sense that *M. viridis* spawning onto kelp could facilitate avoidance of predators, as in other species that camouflage their nest or themselves (Smith & Wootton 1995). Parental care within the kelp forest may also be enhanced indirectly via food provisioning, as indeed the diet of *Myxodes viridis* is characterized by kelp fronds' epifauna, specifically small crustaceans such as peracarid amphipods and small decapods (Quijada & Cáceres 2000, Thiel & Hinojosa 2009, Winkler *et al.* 2017). Furthermore, *L. trabeculata* might contribute directly to egg development by providing a constant oxygen supply produced by kelp photosynthesis, and also reducing water flow of the shallow subtidal area (Trujillo *et al.* 2019).

The present study provides information of the interaction between *L. trabeculata* kelp and *M. viridis* (for other examples see: Garmendia 2016, Ruz *et al.* 2018), highlighting the importance of kelp for early life history of some reef fishes. Examples of use of this kelp species fronds as reproduction zones are not limited to this clinid fish, as other fish species are known to use kelp stipes to deposit their egg capsules, such as the redspotted catshark (*Schroederichthys chilensis*), skates (*Sympterygia brevicaudata*) (Trujillo *et al.* 2019) and also invertebrates such as squid (*Doryteuthis gahi*) (Carrasco & Pérez-Matus 2016). Other examples extend to small peracarids crustaceans that use the kelps during their entire life history, such as the amphipod *Peramphithoe femorata*, which actively fold and “glue” fronds to build nests in both *Macrocystis pyrifera* and in *L. trabeculata* (Thiel & Hinojosa 2009). As the egg clutches described here were found while surveying only subtidal kelp forest of *L. trabeculata*, it remains to be determined what other type of reproduction sites exist, and the relative importance of kelp as a spawning and rearing habitat.

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Contributions: All the authors actively participated in the research and contributed substantially to the manuscript. VG, CR and APM found the eggs; RMC, VG and CR worked in the laboratory, and all authors actively participated in data processing, analyses, and writing the manuscript.

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SUPPLEMENTARY FIGURES

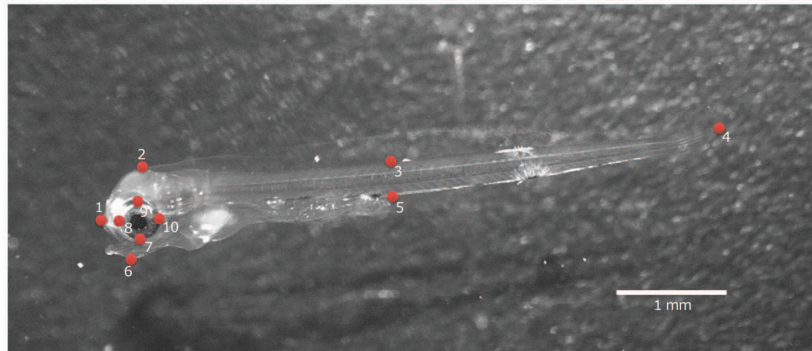


Figure S1. Location of landmarks digitized for the geometric morphometrics analysis of *Myxodes viridis* larvae / Posición de los landmarks digitalizados para el análisis de morfometría geométrica en larvas de *Myxodes viridis*

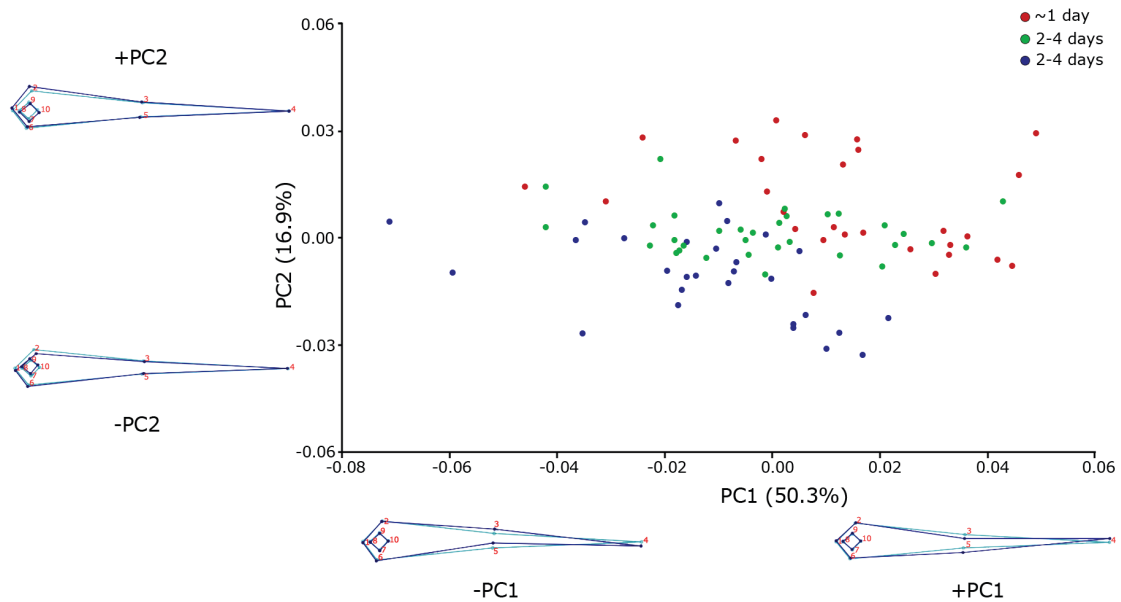


Figure S2. Principal Component Analysis (PCA) plot of variation in larval body shape. Colors represent to larval time of development in lab (red: recently hatched larvae ~1 day, green: larvae between 2 and 4 days, blue: larvae between 5 and 7 days). Wireframes indicate the extreme shape along both PC axes / Gráfico del Análisis de Componentes Principales (PCA) de la variación la forma del cuerpo de la larva. Colores indican el tiempo de desarrollo larval en laboratorio (rojo: larvas recién eclosionadas ~1 día, verde: larvas entre 2 y 4 días, azul: larvas entre 5 a 7 días). Modelo representativo de las formas de la larva. Se muestran las formas extremas a lo largo del eje de los componentes