External Morphology of the Chorion of the Annual Fishes
Cynolebias (Cyprinodontiformes: Rivulidae)

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In summary, this study has verified that the presence of a single medial chin pore can be used to accurately distinguish between individuals of *C. marginatus* and *C. beldingi*. This distinction is important because *C. beldingi* occurs throughout most of the limited distributional range of *C. marginatus*. Unlike *C. beldingi*, which is ubiquitous in the Pacific Northwest, *C. marginatus* is endemic to the Blue Mountains of Washington and Oregon. *Cottus marginatus* is currently listed as a sensitive species that could eventually be upgraded to a threatened status. For this reason, accurate field identification of *C. marginatus* is critical for scientists in their effort to learn more about the ecological requirements and management needs of this species.

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LITERATURE CITED


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External Morphology of the Chorion of the Annual Fishes *Cynolebias* (Cyprinodontiformes: Rivulidae)

MARCELO LOUREIRO AND RAFAEL O. DE SÁ

Members of the family Rivulidae (killifishes) inhabit temporary bodies of freshwater in South and Central America (one species is also found in North America). The most remarkable characteristic of the family Rivulidae is that species have an annual lifecycle with a drought-resistant egg during the dry season. Parenti's (1981) analysis of the order Cyprinodontiformes considered a single genus, *Cynolebias*, whereas Costa's (1990) phylogenetic analysis of the family Rivulidae separates *Cynolebias* from *Cynopoecilus*. One of Costa's synapomorphies to separate *Cynopoecilus* is the unique structure of their egg's chorion, which is shared with *Leptolebias* and *Campellolebias*.

Characteristics of the chorion of teleost fishes have been used to cluster fish eggs in relation to spawning and other ecological characteristics (Ivanov, 1956; Gotting, 1966, 1967). In addition to its ecological significance, the characteristics of the chorion are of systematic value (Ivankov and Kurdyayeva, 1972; Lonning, 1972). For example, the differences in thickness
and density of the filaments found on the egg's surface of *Fundulus heteroclitus* have helped to differentiate close populations (Brummet and Dumont, 1981; Morin and Able, 1983).

*Cynopoecilus* eggs' chorion consist of two concentric layers. Surface patterns result from the structural organization of the outermost layer, the secondary envelope, which is secreted by the follicle cells in the ovary (Wourms and Sheldon, 1976). Light and scanning electron microscopy (SEM) indicate that teleost eggs are ornamented with threads, filaments, pores, puffball-like plugs, and flattened mounds (Brummet and Dumont, 1981; Hart et al., 1984; Johnson and Werner, 1986). In most members of the family Rivulidae, eggs are covered with hairlike fibrils (Costa, 1990). In *Cynopoecilus*, the surface of the egg's chorion consists of adjacent hexagons (Scheel, 1969) with uniformly spaced, macroscopic, and hollow conical projections (Wourms and Sheldon, 1976). The ornamentations and patterns found on the surface of the egg's chorion in cynolebitines may be species-specific (Wourms, 1976). The chorion's surface of eggs of *Cynolebias whitei* is composed of regular projections of different and alternating size and shape between the hairlike projections exhibited as seen by light microscopy (Carvalho, 1957). A SEM study of the egg's chorion of *Cynolebias bellottii* reported the surface possessing hairlike projections (Müller and Sterba, 1963).

Phylogenetic relationships within the genus *Cynolebias* are not yet resolved; however, tentative clustering of species into species groups have been proposed based on morphological characters and patterns of coloration (Amato, 1986; Costa and Brasil, 1990; Costa et al., 1990). Variation on the structure and surface ornamentation of the eggs' chorion could provide additional information to support or refute the reality of the species groups. The goal of this study is to describe and compare the morphology of the egg's surface in several species of *Cynolebias* using SEM analyses.

**Materials and methods.**—The genera used in this study are from South America. One of us (ML) obtained fertilized eggs of the following species in Uruguay: *Cynopoecilus melanotaenia*, *Cynolebias luteoflamulatus*, and *Cynolebias cheradophilus*. In addition, we purchased eggs from captive bred specimens of the following commercially available (i.e., pet trade) species of *Cynolebias*: *gymnoventris*, *cyanaeus*, *adloffi*, *affinis*, *boitonei*, *myers*, *white*, *wolterstorffi*, *flammeus*, *duraznensis*, and *Cynolebias* sp. from Pelotas, Brazil. Limited amount of eggs were available to us for SEM observation; except for *C. duraznensis* and *C. myers*, of which we processed two eggs of each species, we processed one egg from all other species. We prepared eggs using standard techniques as follows. We first ultrasonically cleaned eggs for 15 min; then we fixed them in 3–4% solution of glutaraldehyde for 2 h at room temperature, followed by three 15-min washes with 0.1 M phosphate buffer. Subsequently, we post-fixed them in a 1% solution of osmium tetroxide for 2 h at room temperature and repeated three 15-min washes in 0.1 M phosphate buffer. Eggs dehydrated in 15-min changes of the following graded ethanol series: 35%, 50%, 70%, 80%, 95%, and three 100% changes. Specimens were critical point dried in liquid CO₂, mounted on aluminum stubs and sputter coated with gold/palladium, 22 nanometers thick, using a Hummer VII sputtering system. We examined eggs with a Hitachi S-2500 scanning electron microscope at 15 kV, 20 kV, and 25 kV and photographed them using Polaroid 55 positive/negative film. We scanned most of the egg surface (except the area of the egg that was glued to the supporting stub). We measured thickness of the filaments at the base of the distal segment and measured fibril length by placing them perpendicular to the beam of electrons. We made measurements on one egg per species and on 10 filaments reporting average measurements in the descriptions.

**Results.**—Although eggs were ultrasonically cleaned, some particles remained attached to the chorionic surface in all eggs. Overall, the chorion of most *Cynolebias* eggs exhibited a fine granulated surface with filamentous hairlike fibrils consisting of two parts, identified here as the basal and distal segments. A summary of chorion characteristics is provided in Table 1 for comparative purposes.

*Cynolebias boitonei* (Fig. 1A–B) exhibited a chorion surface that possessed the shortest hairlike fibrils among all species examined. These fibrils had an average length of 5.6 micrometers (μm) and projected from slightly cone-shaped bases that covered the egg's surface at a density of approximately 25 fibrils/50 μm². The egg’s surface between the hairlike projections exhibited a finely rugged appearance. The surface of the chorion of *Cynolebias gymnoventris* was covered (approximately 100 fibrils/50 μm²) by short hairlike fibrils, approximately 5.9 μm in length (Fig. 1C). In this species, the basal segment was round but dorsoventrally depressed; it was unique in possessing a small, blunt lateral projection (Fig. 1D). The egg’s surface among the basal segments was rugged and had pores (approximately 200 nm in diameter). The chorion’s surface of eggs of *Cynolebias affinis* (Fig.
### Table 1. Summary of the Characteristics of the Ornamentation in Species of *Cynolebias*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Length (x/SD)</th>
<th>Thickness (x/SD)</th>
<th>Density/50 μm²</th>
<th>Basal segment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. boitonei</em></td>
<td>5.6/4.6 μm</td>
<td>&lt; 0.5 μm</td>
<td>25</td>
<td>differentiated cones</td>
</tr>
<tr>
<td><em>C. gymnoventris</em></td>
<td>5.9/2.13 μm</td>
<td>&lt; 0.5 μm</td>
<td>100</td>
<td>diff. and flattened</td>
</tr>
<tr>
<td><em>C. affinis</em></td>
<td>20.1/5.09 μm</td>
<td>0.77/0.22 μm</td>
<td>50</td>
<td>diff. and circular</td>
</tr>
<tr>
<td><em>C. cyaenus</em></td>
<td>20.0/9.81 μm</td>
<td>0.72/0.16 μm</td>
<td>30</td>
<td>diff. and circular</td>
</tr>
<tr>
<td><em>C. adloffi</em></td>
<td>35.5/9.26 μm</td>
<td>0.62/0.18 μm</td>
<td>30</td>
<td>differentiated</td>
</tr>
<tr>
<td><em>Cynolebias sp.</em></td>
<td>39.5/15.0 μm</td>
<td>0.64/0.28 μm</td>
<td>50</td>
<td>differentiated</td>
</tr>
<tr>
<td><em>C. luteoflamulatus</em></td>
<td>23.5/3.5 μm</td>
<td>2.33/0.26 μm</td>
<td>20</td>
<td>poorly differentiated</td>
</tr>
<tr>
<td><em>C. cheradophilus</em></td>
<td>22.0/6.75 μm</td>
<td>1.27/0.15 μm</td>
<td>35</td>
<td>diff. and rounded</td>
</tr>
<tr>
<td><em>C. whitei</em></td>
<td>?</td>
<td>&lt; 0.2 μm</td>
<td>20</td>
<td>round protuberances</td>
</tr>
<tr>
<td><em>C. wolterstorfi</em></td>
<td>23.0/11.2 μm</td>
<td>&lt; 0.5 μm</td>
<td>50</td>
<td>diff. and rounded</td>
</tr>
<tr>
<td><em>C. flammeus</em></td>
<td>6.2/1.23 μm</td>
<td>&lt; 0.5 μm</td>
<td>100</td>
<td>diff. and rounded</td>
</tr>
<tr>
<td><em>C. duraznensis</em></td>
<td>16.2/4.34 μm</td>
<td>&lt; 0.5 μm</td>
<td>50</td>
<td>diff. and rounded</td>
</tr>
<tr>
<td><em>C. myersi</em></td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
</tbody>
</table>

1E) and *C. cyaenus* (Fig. 1F) were similar to each other; they both had hairlike projections approximately 20 μm long with distinct basal and distal segments. Both species had circular and slightly dorsoventrally flattened basal segments; the egg’s surface had a granular appearance. However, in comparing *C. affinis* and *C. cyaenus*, the density of fibrils differs, approximately 50/50 μm² and 30/50 μm², respectively.

The overall morphology of the hairlike fibrils of *Cynolebias duraznensis* (Fig. 1G) was similar to that described for *C. cyaenus* and *C. affinis*. Furthermore, the chorion’s surface among the basal segments was also granular, but in this species, the surface granularity was more dispersed than in the two species previously described. *Cynolebias adloffi* (Fig. 1H) and *Cynolebias sp.* (Fig. 1I) had longer hairlike fibrils, between 35 and 40 μm in length, and the basal and distal segments were poorly differentiated from each other.

These two species differed from each other in the density of the hairlike fibrils, approximately 30/50 μm² in *C. adloffi* and approximately 50/50 μm² in the undescribed species. The diameter of the hairlike fibrils of all species described so far was less than 1 μm thick. *Cynolebias luteoflamulatus* was unique in having the thickest hairlike fibrils, approximately 2.3 μm thick, which were of intermediate length, 23 μm. The basal and distal segments were poorly differentiated, overall of similar thickness, with the basal segment slightly expanded.

In this species, the density of the hairlike projections was only 20/50 μm² (Fig. 2A,C). Moreover, they were approximately perpendicular to the surface of the egg, probably because of their greater thickness, whereas in other species the more flexible hairlike fibrils commonly bent over the eggs’ surface. In many cases, the distal segment of the fibrils was not present and only the basal segment remained (Fig. 2A); this was probably an artifact of the technique due to the reduced flexibility of the thicker fibrils.

*Cynolebias cheradophilus* (Fig. 2B,D) also had fibrils of intermediate length, approximately 22 μm, and 1.25 μm thick (density approximately 35/50 μm²). However, in *cheradophilus*, the basal segments were well differentiated, overall rounded, and easily distinguishable from the distal segment. The chorion’s surface appeared granular. Some of the distal segments were also lost in this species.

*Cynolebias wolterstorfi* had similar characteristics to those of *cheradophilus* (Fig. 2E), but the hairlike projections were more dense, approximately 50/50 μm², and the filaments were extremely thin, less than 0.2 μm. The basal segments of *C. whitei* were taller and have a pearl-like shape (Fig. 2F). Their density was approximately 20/50 μm². The distal segments were very thin, less than 0.2 μm thick and highly convoluted, making it impossible to measure the hairlike projections’ length. The surface of the chorion among the basal segments exhibited low, rounded protuberances; each protuberance had a pore (Fig 2F).

In *C. flammeus*, the egg’s surface was covered by hairlike projections at a density approximately 100/50 μm² (Fig. 2G–H). It possessed round basal segments from which relatively short (approximately 6.0 μm) and thin (less than 0.5 μm) distal segments originated. The chorion’s surface among the basal segments appeared strongly rugged in this species. The egg of *C. myersi* had an unique chorion surface; it lacked

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Fig. 1. Egg surface of species of *Cynolebias* examined. (A) *boitonei* (×500), (B) *boitonei* (×2000), (C) *gymnoventris* (×500), (D) *gymnoventris* (×8000) vertical arrow points to “pore” and horizontal arrow points to the lateral projection on the basal segment, (E) *affinis* (×500), (F) *caenus* (×500), (G) *duraznensis* (×3000), (H) *adloffi* (×500), (I) *Cynolebias* sp (×500).

Fibrils, and it was covered with overall rounded and large protuberances of approximately 8.5 μm in diameter at a density approximately 30/50 μm². We also observed ring-shaped structures (approximately 11.9 μm in diameter) that sat on top and among four or five of the rounded protuberances (Fig. 21). These ring structures were continuous with the surface protu-
Fig. 2. Egg surface of species of *Cynolebias* examined. (A) *luteoflamulatus* (×500), (B) *cheradophilus* (×500), (C) *luteoflamulatus* (×2000) upper arrow = distal segment and lower arrow = basal segment, (D) *cheradophilus* (×6000) vertical arrow = distal segment and horizontal arrow = basal segment, (E) *wolterstorffi* (×800), (F) *whitei* (×4000), (G) *flammeus* (×6000), (H) *flammeus* (×1000), (I) *myersi* (×1500).

berances and were present at low density (4/50 µm²).

Discussion.—The ornamentation we observed on the chorion of *Cynopoecilus melanotaenia* corresponded to the description reported by Wourms (1976). Species of *Cynolebias* analyzed here exhibited similar characteristics, except *C. myersi* in which the surface of the egg chorion completely lacked hairlike fibrils and presented only large, round protuberances that supported disperse ring-shaped structures over the sur-
face. All other species exhibited hairlike projections distributed over the external surface of the egg. These fibrils, however, showed variation in length, thickness, and degree of differentiation between the basal and distal segments. There was also variation on the chorion's surface characteristics among the fibrils' basal segment.

In most species, e.g., *affinis*, *cyaneus*, etc., this background surface had a granular appearance; however, it was rugged in *boitonei* and strongly rugged in *flammeus* where it appeared highly folded. Furthermore, the rugged surface of *gymnoventris* possessed pores, whereas the only other species in which pores were found was *C. whitei*. The hairlike fibrils of *C. boitonei* were the smallest and could only be observed with the highest magnification. The hairlike fibrils of *C. affinis*, *C. cyaneus*, *C. duraznensis*, *C. cheradophilus*, and *C. walterstorffi* were similar in length and had a differentiated, overall circular, basal segment. The undescribed species, together with *C. adloffi*, stood out among the other species because they had the longest hairlike projections. Although the basal segment was recognizable, it did not acquire the overall flattened and circular configuration found in the other species. In addition, the species also differed in the density of fibrils and the thickness of the distal segments.

The fibrils were least abundant in *C. luteoflamulatus* and *C. whitei* whereas *C. gymnoventris* and *C. flammeus* exhibited the highest density. *Cynolebias luteoflamulatus* had the thickest distal segments and in addition could be easily identified by the combination of the thicker hairlike fibrils with a poorly differentiated basal segment. The filaments of *C. gymnoventris* and *C. flammeus* were similar in length, thickness, and density; furthermore, the exposed surface of the chorion among the fibrils was rugged in both species. However, *C. flammeus* possessed distinct folding and lacked the pores that were clearly visible in *gymnoventris*. Also, *C. gymnoventris* could be easily identified by the presence of a lateral projection on the basal segment.

Our observations on the eggs of 13 species of *Cynolebias* support Wours’ (1976) suggestion that the characteristics of the egg's chorion are species-specific. The combination of characteristics of the ornamentation presented here can be used for diagnostic purposes for some species. Further fieldwork is needed to obtain additional eggs to determine intra- and interindividual variation and spawn-related variation. However, we suggest that this type of analysis may be valuable for identifying wild-collected eggs from dried ponds when adults are no longer available, particularly from those ponds that are inhabited by more than one species. At the same time, the present data suggest caution in using egg characteristics in phylogenetic analysis until further data are available on the egg's physiological and ecological requirements.

On one hand, species that currently are clustered together in a species group, showed very different egg characteristics, e.g., *C. whitei* and *C. myersi* (whitei group), and *C. affinis*, *C. gymnoventris*, and *C. luteoflamulatus* (luteoflamulatus group). On the other hand, the following five species that exhibited similar egg characteristics currently are clustered in two separate species groups: *C. affinis*, *C. cyaneus*, and *C. duraznensis* are in the *luteoflamulatus* group, whereas *C. walterstorffi* and *C. cheradophilus* are placed in the *porosus* group. These clusters of species based on egg characteristics could be suggesting phylogenetic relationships as well as adaptive convergence among the species. However, it is interesting to note that this data may provide additional support to Costa and Brasil’s (1990) suggestion that the *luteoflamulatus* and the *porosus* groups could be closer to each other and that the *luteoflamulatus*-*porosus* clade is closer to the *adloffi* group.

The pores observed in *C. whitei* and *C. gymnoventris* and the ring-shaped structures found in *C. myersi* may correspond to the external opening of mucus glands. However, histological data are needed to confirm this suggestion.

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Comments on an Intercalar Path for the 
Glossopharyngeal (Cranial IX) Nerve 
as a Synapomorphy of the 
Paracanthopterygii and on the 
Phylogenetic Position of the 
Gobiesocidae (Teleostei: Acanthomorpha) 
ANTHONY C. GILL

In the most recent treatment of the mono-
phly and intrarelationships of the Paracan-
thopterygii, Patterson and Rosen (1989:33) list-
ed four synapomorphies for the taxa they in-
cluded in that group (Percopsidae, Aphredoder-
oiodei, Carapidae, Ophididae, Bythitoidei, 
Batrachoidiformes, Lophiiformes, Gadiformes, 
†Libonius and †Sphenocelphalus): (1) full neural 
spine on PU2 (primitively short in ctenosqua-
mates); (2) two epurals (primitively three in 
ctenosquames); (3) a single supraneural (prim-
itively three in ctenosquames) behind first or 
second neural spine; and (4) “intercalar en-
larged, containing glossopharyngeal [IX] fora-
en, and forming part of cranial wall.” The 
first three of these synapomorphies occur rel-
atively widely in acanthomorph fishes and are, 
therefore, of dubious value in defining the Par-
canthopterygii. For example, a full neural 
spine also occurs in polyromiiforms, zeiforms, 
many perciforms, non-pssettodid pleuronectiforms, 
and most smegmamorphs; a count of two or 
fewer epurals also occurs in zeiforms, many 
smegmamorphs, non-psettodid pleuronectiforms, 
and many perciforms; and a reduced 
number of supraneurals (0 or 1) is also found 
in zeiforms, most smegmamorphs, pleuronect-
iforms, and many perciforms (Johnson and 
Patterson, 1993:559). At least informally, if not in 
the literature, recent acanthomorph workers 
have therefore placed considerable weight on 
the fourth paracanthopterygian synapomor-
phy, the glossopharyngeal nerve foramen in the 
intercalar. Here, I question the value of this 
character as a paracanthopterygian synapo-
morphy by drawing attention to the presence of 
glossopharyngeal foramina in the intercalars 
of non-paracanthopterygian fishes and to vari-