Reproduction, larviculture and early development of the Bluebanded goby, *Lythrypnus dalli*, an emerging model organism for studies in evolutionary developmental biology and sexual plasticity

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Abstract

The Bluebanded goby, *Lythrypnus dalli*, is a popular ornamental aquarium species and a key organism for the study of several fundamental biological questions, most notably reversible sex change in adults. To maximize the tractability of this species as an emerging model system, it is essential to have an optimized propagation system and a detailed developmental staging scheme. One limitation to the larviculture of *L. dalli* is the relatively small size of the larvae, which makes the transition from yolk to feeding challenging. We developed a protocol and successfully reared three generations of *L. dalli* in the laboratory. The protocol contains several key innovations for the rearing of diminutive fish larvae, including tank design and co-culturing of microalgae (*Isochrysis galbana*) with copepods (*Parvocalanus* sp.) in the larval rearing tanks. In addition, we describe the embryonic and larval development of *L. dalli* under controlled conditions and in comparison with the model organism *Danio rerio*. We found that at 21°C *L. dalli* larvae hatch in 4 days, reach flexion in 18–25 days and are sexually mature by 3 months. Overall, the embryonic development of *L. dalli* is remarkably similar to *D. rerio* with several striking differences, including the position and shape of the blastomere, size of the neuromasts and corresponding cupula, and relative timing of pigmentation and brain subdivision. The ability to rear this species in captivity is a valuable tool that could be utilized for a variety of similarly diminutive species and to address a greater breadth and depth of biological questions.

Keywords: larviculture, co-culture, *Lythrypnus dalli*, acanthomorph development, fused pelvic fins

Introduction

The rearing and development of marine fishes is of interest to hobbyists, commercial aquarists and biologists for a variety of reasons including the following: to increase our understanding of larval development and comparative embryology, to strengthen inferences of homology of ontogenetic characters and to increase the number of species that can be bred in captivity for ornamental propagation and research (Kendall, Ahlstrom & Moser 1984; Collazo & Bolker 1994). Rearing fish in captivity provides access to embryos, which is a limiting factor in evolutionary developmental biology research. The ability to rear larvae of various species in captivity is essential for comparative and manipulative investigations of the genetic and developmental basis of diversity and novelty. In addition, observations of development may help aquaculturists identify and address significant hurdles in rearing these and similar species. Finally, the number of ornamental marine species
commercially produced in captivity is currently limited, especially compared to freshwater species (Andrews 1990; Meirelles, Tsuzuki, Ribeiro, Medeiros & Silva 2009; Olivotto. Planas. Simoes, Holt, Avella & Calado 2011; Sweet 2014), and this rearing protocol may aid in the development of captive breeding programmes that could potentially alleviate collection pressures on a variety of wild marine fishes targeted by aquarium trade collections.

The developmental processes of teleost fishes, with the exception of a few taxa, have not been well characterized, representing an important gap in our understanding of animal biology. This deficit is particularly striking as teleosts represent the majority of vertebrate diversity, with approximately 27 000 species (Nelson 2006). The most commonly studied teleost is the zebrafish, Danio rerio, a freshwater species widely used in laboratory research. Accordingly, D. rerio development has been well characterized (Kimmel, Ballard, Kimmel, Ullmann & Schilling 1995; Metscher & Ahlberg 1999), which provides a valuable basis for comparative developmental studies. However, D. rerio is not a member of the most speciose and derived group of teleost fishes, the Acanthomorpha sensu Near, Eytan, Dornburg. Kuhn, Moore, Davis, Wainwright, Friedman and Smith (2012), or spiny-rayed fishes. This group includes many economically important species such as tuna, halibut, rockfish, tilapia and mullet (Stiassny, Wiley, Johnson & Carvalho 2004; Nelson 2006; Eschmeyer & Fricke 2012), or spiny-rayed fishes. This group includes many economically important species such as tuna, halibut, rockfish, tilapia and mullet (Stiassny, Wiley, Johnson & Carvalho 2004; Nelson 2006; Eschmeyer & Fricke 2012), or spiny-rayed fishes.

The Gobiidae are the most diverse family of Acanthomorpha and the second-most speciose family of vertebrates (Moyle & Cech 2004; Nelson 2006). Many are of interest to the aquarium trade, including the Bluebanded goby, Lythrypnus dalli, because they are small, brightly coloured and exhibit interesting social and reproductive behaviours (Sunobe & Nakazono 1993; Scaggiane, Grober, Lorenzi & Rasotto 2004; Drilling & Grober 2005; Meirelles et al. 2009). Adult L. dalli are readily collected within their range on shallow reefs using SCUBA. In addition, adults are readily maintained in captivity, breed readily and females lay hundreds of eggs per clutch. Their size at maturity is relatively small (adult TL less than 6 cm. Miller 1972), making them tractable with respect to space limitations in aquaculture and laboratory settings. The eggs of L. dalli are attached to the substrate, cared for by the guarding male (Rodgers, Earley & Grober 2006), and develop into transparent embryos that are hardy and easily monitored. They are abundant throughout most of their range, in coastal marine habitats in the Eastern Pacific from the Gulf of California to Morro Bay, CA (Miller 1972), and are not listed as a species of concern in any category (IUCN Red List, van Tassell, Lea & Bearez 2010). Finally, this species displays several derived traits that make them ideal for studies of evolution and development, such as high contrast pigmentation patterns, pelvic fins that are fused into a sucking disc (a common feature of this family), and bidirectional sex change. Bidirectional sex change represents the extreme in sexual plasticity, and L. dalli are commonly used in studies of sex determination and sex change (St. Mary 1993, 1994, 1996, 1998, 2000; Black, Reavis & Grober 2004; Black, Balthazart, Baillien & Grober 2005, 2011; Rodgers, Drane & Grober 2005; Lorenzi, Earley & Grober 2006, 2012; Rodgers et al. 2006; Rodgers, Earley & Grober 2007; Lorenzi, Earley, Rodgers, Pepper & Grober 2008; Lorenzi, Carpenter, Summers, Earley & Grober 2009; Lorenzi & Grober 2012; Maxfield, Van Tassell, St Mary, Joyeux & Crow 2012).

Despite their popularity and tractability as a model for biological enquiry, the early development of gobies is relatively poorly studied (Nakatsuji, Kitano, Akiyama & Nakatsuji 1997; Patzner, Van Tassell, Kovacic & Kapoor 2011), and L. dalli have previously been considered difficult to rear in captivity (Moe 2008). This difficulty is largely due to the small size of L. dalli at yolk depletion and transition to exogenous feeding (L. dalli larvae measure 2.1 mm at hatching, Patzner et al. 2011), and the difficulty of providing appropriately small live food at first feeding. We developed a protocol to address this hurdle that can be adapted to rear a variety of ornamental or research species with small larvae.

The objectives of this study were to develop a larval rearing system and protocol for L. dalli, and to describe their development in an evolutionary context. We have successfully reared three generations of L. dalli in captivity and characterized their embryonic and larval development in comparison with D. rerio. This comparison highlights the developmental variation among teleosts and the need for greater representation of the Acanthomorphs for studies that address the bases of novel traits in a phylogenetic context.
Materials and methods

Animals (breeding colonies)

All animal procedures were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committees of San Francisco State University (SFSU), Georgia State University, and Roger Williams University. Adult gobies were collected using SCUBA from Catalina Island, California and transferred to SFSU or the Georgia State fish facility. Embryos were then shipped from Georgia State University to Roger Williams University. Embryonic and early larval development descriptions were based on fish housed at SFSU, while the rearing protocol was developed at Roger Williams University.

Breeding colonies were established with five adult females housed in 38-litre (10-gallon) rectangular tanks with darkened sides. The largest female in each group usually changed sex within 2 weeks, resulting in operational sex ratios of 1:4 within each colony. Sex change was confirmed by the presence of fertilized eggs.

Adult tanks were maintained at ambient temperature (~20°C, range = 14.4–22.2°C), with salinity between 30–35, NH₄⁻/NH₄⁺ and NO₂ <1 ppm, and NO₃ <20 ppm. Each tank had isolated, recirculating water filtered with an Emperor 280 BIO-Wheel filter (Marineland Aquarium Products, Moorpark, CA, USA) and received 25% weekly water changes with filtered, sterilized natural seawater (Mt. Hope Bay, Rhode Island). Temperature, salinity, flow, fish health and behaviour were monitored daily and water quality was monitored weekly. A photoperiod of 14:10 hours light:dark was provided by ambient fluorescent lighting, mimicking natural conditions during the breeding season. Tank bottoms were syphoned every day, or as needed. Adults were fed a variety of food including Otohime Fish Diet (Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan), a gelatin based diet with added seafood products, frozen Mysis shrimp and frozen bloodworms 3 to 4 times daily until satiated.

Egg production, incubation and hatching

Each tank contained two 2-inch long, ¾-inch diameter PVC tubes lined with a plastic transparency for egg deposition (Fig. 1a). PVC tubes were visually checked daily for new egg clutches (Fig. 1b). When eggs were found, the clutches were inspected, staged and returned to their designated tubes and tanks for paternal care until hatching, between 4–6 days later at ~20°C.

Eye pigmentation, yolk colour and age of clutches were monitored to predict hatching. Just before hatching, clutches were moved to individual rearing tanks and placed in submerged hatching chambers (Fig. 1c). Hatching was generally induced within a few hours after covering larval tanks to eliminate light penetration. If a partial or no hatch occurred by the next morning, transparencies were removed from hatching chambers, left in the air for up to 5 min, shaken mildly, and replaced.

Larval rearing system

Hatched larvae were reared in 100-L circular tanks connected to a 380-L recirculating system (Fig. 1d). The system contained a biofilter and protein skimmer, and was maintained under the same temperature and water quality conditions as the adult broodstock. Rearing tank water levels were controlled by external T-barbs to maintain the volume at 75 L. A 40-watt aquarium strip light illuminated approximately ⅓ of the tank surfaces, 24 h a day. An 8-cm-diameter cylindrical standpipe screen of 40-µm mesh was attached to the centre drain of each larval tank (Fig. 1e). A bubble ring around the bottom of the screen provided constant gentle aeration and kept larvae and food away from the standpipe drain. Water inlets were placed against the sides of the rearing tanks to minimize turbulence and were fed with constant flow of 0.1–0.15 L min⁻¹. Daily maintenance included system water changes of 10–25%, cleaning of microalgae drip lines and standpipe screens, and syphoning of debris and deceased larvae from rearing tanks.

Plankton Cultures

Larval *L. dalli* require extremely small foods at the onset of exogenous feeding. We found that the copepod nauplii of the genus *Parvocalanus* sp. were well suited for the relatively small larvae of *L. dalli*, similarly to what has been reported for the lemon-peel angelfish (Olivotto, Holt, Carnevali & Holt 2006). *Parvocalanus crassirostris* (Reed Mariculture Inc., Campbell, CA, USA) were cultured in 150-litre tanks at densities of ~10–30 copepods per mL. To maintain these cultures, the microalgae
Isochrysis galbana (CCMP 1324) (Florida Aqua Farms Inc., Dade City, FL, USA) was added twice daily to densities of 1 – 2 \times 10^9 cells mL\(^{-1}\), and water changes of 50\% were conducted three times per week. Copepods were harvested using 40-lm mesh screen, while size-sorted nauplii for larval feeding were harvested by filtering the culture through a 75-lm screen filter, then through a 40-lm screen filter.

Culturing of *I. galbana* was accomplished following the protocols outlined by Creswell (2010). All pieces of microalgae culture equipment were autoclaved or sterilized with bleach. *Isochrysis galbana* were cultured at ambient temperature (20°C) with sterilized salt water (salinity of 35) in sealed culture vessels. Each culture was aerated through a 0.2-\mu m in-line filter. Optimal densities of 8 – 12 \times 10^6 cells mL\(^{-1}\) were reached in 5 – 7 days in algae media. Cultures were started in 1 L Roux flasks with autoclaved media, transferred to 20 L glass carboys and finished in 100 L polycarbonate tanks (20 and 100 L media was pasteurized to 85°C). Algae media was formulated following the manufacturer’s instructions (F/2 Algae Food, Fritz Aquatics, Inc., Texas, USA).

**Larval feeding**

Copepods (*Parvocalanus* sp.) were cultured in *L. dalli* rearing tanks as described above prior to the first feeding of larvae. *Isochrysis* densities were maintained daily (8 – 12 \times 10^6 cells mL\(^{-1}\)) through microalgae drip lines as well as direct addition to the tank. Early larval gobies were able
to consume only the smaller copepod nauplii, and were therefore reliant on reproducing copepods.

Late stage larvae and early-settled juveniles were fed Artemia nauplii (Brine Shrimp Direct, Odgen, UT, USA) in addition to copepod culture maintenance. Culturing of the copepods through the addition of microalgae was halted once all the larvae settled out of the water column. Juveniles were weaned onto a diet of Otohime pellets (100–400 μm diameter) at 2 weeks post settlement.

Developmental staging of Lythrypnus dalli

Developmental observations of L. dalli embryos and larvae were conducted at San Francisco State University. The individuals used for this study were housed according to the protocol above, except that adults were kept in a closed, recirculating sea water system maintained at 15.5°C (14.4–18.3°C). Developmental observations were made at two temperatures, 14.4 and 17.0°C.

Photos of embryos and larvae were taken at 43 different stages of development from 33 different clutches of eggs. Embryonic and early larval observations and photos were taken of live individuals using a Zeiss DiscoveryV.8 stereomicroscope and an AxioCam ICC3 camera with AxioVision software (Carl Zeiss Microscopy GmbH, Jena, Germany). If necessary, larvae were anaesthetized in a 1:25 dilution in seawater of a 0.4% MS-222 solution, pH 7 (Tricaine methanesulfonate; Sigma-Aldrich, Co., St. Louis, MO, USA). Stages of embryonic development are described for L. dalli based on staging criteria for D. rerio (Kimmel et al. 1995) for the following periods: zygote, cleavage, blastula, gastrula, segmentation, pharyngula, hatching and early larval. Observations of late larval development were made on individuals preserved overnight in 4% paraformaldehyde at 4°C, then transferred to 100% methanol and stored at −20°C. The proposed staging of late larval L. dalli was made comparable to D. rerio following Parichy, Elizondo, Mills, Gordon and Engeszer (2009).

In situ hybridization

Whole mount in situ hybridization was done to test the feasibility of the procedure and to observe expression patterns of the posterior Hox gene, hoxal3a, during multiple stages of L. dalli embryonic development. Embryos were dechorionated either before or during overnight preservation in 4% paraformaldehyde at 4°C. Embryos were stored in 100% methanol at −20°C. Whole mount in situ hybridizations were carried out as described by Archambeault, Taylor and Crow (2014).

Results

Fertilization, hatching rates, developmental timing and survivorship

Fertilization and egg deposition of L. dalli occurred most frequently in the 4 hours following first light, 8:00–12:00 hours. (Fig. 2). Average nest size at 21.6°C was 835 eggs (n = 9, range 396–1055), and often represented multiple spawning events. Males guarded the nests until hatching, and filial cannibalism was occasionally observed on clutches that were unfertilized or experienced fungal growth (personal observations). Removal of clutches from broodstock tanks significantly increased the frequency of egg laying: when clutches were removed, new clutches were fertilized every 9.4 days (SE = 0.86, n = 86) compared with every 11.0 days (SE = 0.85, n = 63; Mann–Whitney test: U = 3378, p = 0.0102) when clutches remained with adults for paternal care. However, hatching rates for L. dalli were more consistent with parental care, ~95% with male care at temperatures of 18 – 22°C. Survivorship to sub-adulthood was variable, between 5–40%.

The yolks of L. dalli embryos are orange, making new clutches easy to detect against the white PVC tubes. Like other goby eggs, L. dalli eggs are oblong in shape and are attached to the substrate by adhesive threads, in contrast to the negatively buoyant but free moving spherical eggs of D. rerio (Fig. 3). Tavolga (1950) showed in Bathygobious that eggs stripped from the ovary are equipped with the adhesive threads, which are then activated by contact with water and attach to the substrate. However, in L. dalli adhesive threads are absent from the eggs while still in the ovary, and thus must be added at the time of female egg deposition (Grober, unpublished data). The female external genitalia in L. dalli have two internal chambers, the most ventral of which is most likely used to produce the adhesive threads during egg laying (Schuppe et al., in preparation).

Unlike some gobies, L. dalli eggs lack oil globules (Yokoi & Hosoya 2006; Wittenrich, Turingan & Creswell 2007). Chorion dimensions are uniform
at 1.88 mm by 0.46 mm (length $\times$ SE = 1.878, width $\times$ SE = 0.002; n = 15). Embryonic *L. dalli* grew in size from 0.57 mm diameter at the zygotic stage to 2.72 mm in length at hatching.

*Lythrypnus dalli* embryos hatched in 10.3 days at 14°C, 9.1 days at 17°C and 4 days at 21.6°C, which is slightly faster than other gobiids reared at similar temperatures (Fig. 4), but slower than *D. rerio* at its common rearing temperature, 28.5°C. To directly compare the development rate of *L. dalli* to *D. rerio*, relative hours of development was plotted vs. developmental stage (Fig. 4). Both *L. dalli* and *D. rerio* display a linear relationship between time and developmental stage, thereby making it possible to estimate developmental stage based on time and temperature for *L. dalli* ($y = 0.0189*\text{temp}\times\text{hpf}$, $R^2 = 0.914$, $R^2 = 0.932$ for 17°C and 14°C respectively). Interestingly, *L. dalli* takes 2.3–3 times longer than *D. rerio* to reach the protruding mouth stage, depending on the temperature. This relationship between developmental stage and time is robust to temperature, therefore, the following staging scheme for *L. dalli* is consistent at a range of developmental temperatures.

**Cleavage, Blastula and Gastrula**

The early periods of development are very similar between *D. rerio* and *L. dalli* (Fig. 5a–i), however, there is an observable difference in blastodisc position during early cleavage. The cells formed by early cleavages in *D. rerio* are positioned on top of the yolk whereas the cells of the early blastodisc of *L. dalli* wrap around the yolk, and reach towards the vegetal pole (Figs 3b–c, 5a–c). A third cleavage plane, perpendicular to the original two planes, is established by the 64-cell stage in both *L. dalli* and *D. rerio* embryos, resulting in multiple layers of cells (Fig. 5f). By the seventh cleavage in *L. dalli* the blastodisc is positioned on top of the yolk (Fig. 5f). During early epiboly, the yolk of *L. dalli* becomes relatively oblong, mimicking the shape of the chorion (Fig. 5i). By 90%, epiboly the embryo is spherical again (Fig. 5).

**Segmentation**

By the 6-somite stage, both *D. rerio* and *L. dalli* have a distinct head bulge and tail bud, both of which are more pronounced and relatively bigger in *L. dalli* (Fig. 5k). This difference contributes to an overall oblong shape of *L. dalli*, mirroring the shape of the chorion. This difference in shape appears to be associated with earlier detachment of the tail and head buds from the yolk in *L. dalli* (Table 1). Thus, egg shape may play a role in shaping developmental progress in some features.

During the early segmentation period, the optic placode and somites of *L. dalli* are often obscured by a grainy texture, likely due to cell movement.
(6-somite stage, Fig. 5k). Nonetheless, the optic placode is visible by the 6-somite stage, the otic placode is visible by the 9-somite stage, and the otic vesicle hollows and contains two otoliths by the 12-somite stage (Fig. 5k–m). Also by the 12-somite stage, the lens is present and most somites exhibit the expected chevron-shape.

Differentiation of the brain also occurs earlier in *L. dalli* than *D. rerio*. By the 19-somite stage, the telencephalon, diencephalon and mesencephalon are evident in the fore- and midbrain of *L. dalli*, and are separated from the hindbrain by a distinct cerebellum (Fig. 5n). In contrast, the cerebellar primordium does not become prominent in *D. rerio* until the 26-somite stage (Table 1).

**Pharyngula**

The pharyngula period begins with the posterior advancement of the lateral line primordium over the trunk somites (e.g. prim-5, prim-15). The primordium is a difficult trait to monitor, and requires differential interference contrast optics to visualize. We took advantage of the large and visible neuromasts of *L. dalli* to characterize their developmental stage categorically. For example, when a neuromast was visible on the ninth somite, but not yet on the 14th somite, we categorized the embryo into the prim 9–14 stage. By the end of the pharyngula period, neuromasts were visible on the 5, 9, 14, 23 and 29 somites of *L. dalli*, therefore, we suggest subdividing the pharyngula period into six stages (prim 1–5, prim 5–9, prim 9–14, prim 14–23, prim 23–29 and high pec).

Pigmentation begins in the early prim stages for *L. dalli*. By prim-9-14, *L. dalli* has scattered black and red pigment cells along the ventral line of the body (Fig. 5o). Additional pigmentation appears on a dorsal region of somites 14–23 and 29 somites of *L. dalli*, therefore, we suggest subdividing the pharyngula period into six stages (prim 1–5, prim 5–9, prim 9–14, prim 14–23, prim 23–29 and high pec).

Muscle movement, including a visible heart-beat, begins in *L. dalli* at the prim 9–14 stage. A small pectoral fin bud emerges by this stage as well. The pectoral fin bud develops the apical ectodermal ridge (AER) and reaches a 1:1 height to width ratio at the “high-pec” stage (Fig. 5q). By this stage, many visible changes have occurred in *L. dalli* including heavily pigmented

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**Figure 3** The eggs of *L. dalli* are oblong and attached to the substrate via adhesive threads. (a) The chorions of 4-day post fertilization *L. dalli* embryos remain oblong and attached perpendicularly to the substrate via adhesive threads (arrows in a and b). (b) The *L. dalli* embryo is smaller than the *D. rerio* embryo (c). The *L. dalli* blastomere wraps around the yolk towards the vegetal pole (marked by arrowheads in b) through the 8-cell stage. (c) The *D. rerio* embryo is free-floating, has a spherical chorion, and the blastomere is perched on top of the yolk (arrowheads). (d) Ventral aspect of a cross-section through the body of a female *Lythrypnus dalli*, noting the position of the ovaries (Ov), hypaxial body musculature (M) and genital papilla (GP). The female GP has two lumens. The dorsal lumen is the putative oviduct by which the egg is communicated from the ovary to the genital papilla for adhesion to the substrate. The ventral lumen is the proposed adhesive thread producing structure within the GP and is noted with an arrow. Tissue was stained with Haematoxylin and Eosin. Scale bars in b and c equal 0.50 mm, and in d equals 200 μm. Vegetal poles (in b and c) and ventral surface of the fish (in d) are at the bottom of the images.
eyes, the appearance of the swim bladder just posterior to the yolk, and a constriction in the posterior region of the gut.

**Hatching**

The beginning of the hatching period is characterized by the elongation of the pectoral fin buds reaching a height to width ratio of 2:1. The fin buds arch posteriorly, defining the “long-pec” stage. At this stage, blood circulation is noticeably strong, and the eyes contain iridescent cells (Fig. 6a).

The ‘pec-fin’ stage is characterized by the expansion of the apical ectodermal ridge in the pectoral fins into the paddle shaped apical fold. The blood...
Table 1 Heterochronic developmental characteristics of Danio rerio and Lythrypnus dalli

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Stage in L. dalli</th>
<th>Stage in D. rerio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tail detaches from yolk</td>
<td>9-somite</td>
<td>15-somite</td>
</tr>
<tr>
<td>Head detaches from yolk</td>
<td>19-somite</td>
<td>Prim-20</td>
</tr>
<tr>
<td>Optic placode</td>
<td>6-somite</td>
<td>5-somite</td>
</tr>
<tr>
<td>Otic placode</td>
<td>9-somite</td>
<td>14-somite</td>
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<tr>
<td>Otic vesicle with otoliths</td>
<td>12-somite</td>
<td>20-somite</td>
</tr>
<tr>
<td>Lens</td>
<td>12-somite</td>
<td>20-somite</td>
</tr>
<tr>
<td>Chevron-shaped somites</td>
<td>12-somite</td>
<td>14-somite</td>
</tr>
<tr>
<td>Distinct cerebellum</td>
<td>19-somite</td>
<td>26-somite</td>
</tr>
<tr>
<td>Eye pigmentation</td>
<td>Prim23-29</td>
<td>Prim-15</td>
</tr>
<tr>
<td>Trunk melanocytes</td>
<td>Prim23-29</td>
<td>Prim-15</td>
</tr>
<tr>
<td>Heartbeat</td>
<td>Prim9-14</td>
<td>26-somite</td>
</tr>
<tr>
<td>Muscle movement</td>
<td>Prim9-14</td>
<td>26-somite</td>
</tr>
<tr>
<td>Swim bladder</td>
<td>High-pec</td>
<td>High-pec</td>
</tr>
<tr>
<td>Pectoral fin bud</td>
<td>Prim9-14</td>
<td>Prim-25</td>
</tr>
<tr>
<td>Indescent eye pigment</td>
<td>Long-pec</td>
<td>Long-pec</td>
</tr>
<tr>
<td>Visible cupula</td>
<td>1 dph</td>
<td>4 dph</td>
</tr>
</tbody>
</table>

Grey boxes indicate the species in which the trait appears at an earlier stage.

One intriguing difference between L. dalli and D. rerio at this stage of lateral line development is the relative size of the cupula. The cupula contain kinocilia that project distally from hair cells at the base of each neuromast (Metcalfe, Kimmel & Schabtach 1985). While the neuromasts emerge during the pharyngula period, the cupula become visible around 4 days after hatching in D. rerio. In contrast, within one day of hatching in most L. dalli larvae, four pairs of cupula visibly protrude from the trunk lateral line. Up to eight pairs of cupula are visible along the head and trunk lateral lines of L. dalli by two days post hatching (Fig. 8). Interestingly, the cupula of L. dalli are 2–7 times longer than those of D. rerio, although the larvae themselves are smaller. Finally, the cupula of L. dalli are visible on a stereoscope with the correct lighting, making them significantly easier to observe.

By one-day post hatching, the lower and upper jaws protrude equally anterior (Fig. 6c, e). The development of the jaws suggests readiness for feeding. The gape size of L. dalli at hatching is roughly 110 μm tall by 90 μm wide. The yolk is largely depleted by 4-5 days post hatching at 21°C, at which time food encounters are crucial for survival.

Larval morphogenesis and settling

Flexion occurs between 18 and 25 days post hatching, after which caudal fin rays develop, similar to D. rerio (Parichy et al. 2009). Mesenchymal condensations and formation of the fin rays within the median fin fold quickly follow to form the anal and second dorsal fins (SI = 3.96 mm, SE = 0.09) (Fig. 9a).

The paired pelvic buds emerge after the fin rays have formed in the medial fins including the second dorsal, anal and caudal fins (SI = 5.68 mm, SE = 0.48) (Fig. 9b, e–f). At this stage, several rows of neuromasts are clearly visible on the caudal fin, which does not occur in D. rerio.

Lythrypnus dalli has two dorsal fins, and the first dorsal fin emerges from the dorsal midline anterior to the midline fin soon after the emergence of the pelvic fin buds (SI = 7.35 mm, SE = 0.11) (Fig. 9c), similar to what has been described in Rhinogobius sp. Bl (Yokoi & Hosoya 2006). Most acanthomorphs have two dorsal fins, but the molecular basis of the differentiated first dorsal fin has not been studied.
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Fin rays are evident in the pelvic fins by SL = 7.67 mm (SE = 0.15) (Fig. 9c, g), but the pelvic fins do not fuse to form the adult sucking disc until approximately 11.06 mm SL. The fusion of the pelvic fins occurs in two stages. First, the posterior portions of the two fins fuse together (Fig. 9g), likely as a result of the outgrowth of the fins as a single structure at later stages, although this has not been observed directly. Next, the anterior edges of the pelvic fins fuse together to complete the sucking disc structure. A similar process is pictured in *Rhinogobius* sp. BI (Yokoi & Hosoya 2006).

Juvenile *L. dalli* began to settle out of the water column starting around 40 dph and concurrent with the onset of adult colouration, after the development of the pelvic fin sucking disc (Fig. 10), as has been described for other Gobiidae with pelagic larvae.
There was considerable divergence in settlement time with some larvae taking 90–100 days to settle. The juvenile period lasted roughly 1–2 months, at which time most individuals were sexually mature. This timing is similar to *D. rerio*, which reach sexual maturity in 10–12 weeks (Westerfield 2000).

In situ hybridization

We successfully performed whole mount in situ hybridizations on embryonic *L. dalli* and *D. rerio* for *hoxa13a*. The two species display strikingly similar patterns of expression of *hoxa13a* in the trunk, tail bud, gut and pectoral fin buds during the stages of embryonic development examined (Fig. 11).

Discussion

*Lythrypnus dalli* is interesting to aquarists and evolutionary developmental biologists for its bright colouration, bidirectional sex change, modified pelvic fins, size and patterning of neuromasts and alternative male breeding morphs. In order to promote research and rearing of this derived teleost for comparative studies of vertebrate development and to facilitate large-scale production to supply the aquarium trade, an optimized rearing protocol and developmental staging scheme were developed. Using this rearing protocol, 300–500 juvenile *L. dalli* can be reared in each of several tanks at once, allowing for the production of large numbers of individuals for research or aquarium use.

Some of the difficulties breeders have faced in rearing diminutive species such as *L. dalli* and *Priolepis nocturna* include determining optimal breeding and rearing temperatures, and culturing appropriately small foods for onset of first feeding (Wittenrich et al. 2007). We successfully reared

![Figure 7](image1.png)

**Figure 7** Two different angle measurements proved helpful in staging *L. dalli*. (a) The head-trunk angle (HTA), shown in red, and the forehead slope, shown in blue. (b) The HTA of *L. dalli* increases in a linear fashion similar to *D. rerio*, except at an earlier stage. At the end of the pharyngula period, the jaws of *L. dalli* protrude anteriorly causing an increase in the slope of the forehead.

![Figure 8](image2.png)

**Figure 8** The neuromasts and cupula of the head and trunk lateral lines of *L. dalli* are prominent in recently-hatched larvae. (a) Eight pairs of neuromasts are marked with dots on a 2 day post-hatching larvae. Three pairs are located anterior to the pectoral fins on the head lateral line (blue dots), and five pairs are on the posterior lateral line (red dots). (b) The neuromasts are clearly visible bumps along the posterior lateral line from a dorsal view (arrowheads). (c) The cupula containing the kinocilia extend at least a body width perpendicular to either side of the trunk (arrows).
three generations of *L. dalli* at 18–21°C, near the top of their reported natural temperature range (18–22°C, FishBase 2013). We also observed successful reproduction and development through early larval stages at a range of temperatures, from 14°C–22°C, suggesting that this species can be bred and reared at a range of temperatures. The relatively small gape size of *L. dalli* at hatching and at exogenous feeding have posed a challenge, as common larval marine fish foods such as artemia and rotifers (>120 μm) are too large to consume at first feeding. We therefore developed and optimized the protocol for the co-culturing of copepod nauplii and *L. dalli* larvae in tanks designed to keep the larvae and copepod nauplii in, while allowing for water and waste exchange. This protocol is readily adaptable for rearing other gobies or small marine ornamental species.

A clear understanding of the evolutionary history of ontogenetic characters is crucial to the interpretation of results from developmental research (Metscher & Ahlberg 1999). To build a phylogenetic context for multiple ontogenetic characters, developmental studies that include a variety of vertebrate taxa such as derived teleosts are essential. Studying development within a phylogenetic context identifies interesting developmental and evolutionary novelties and the appropriate organisms in which to study them. Our work is...
the first description of development of a member of the genus *Lythrypnus*, and strengthens the phylogenetic context for ontogenetic characters within gobid species (Sunobe & Nakazono 1987; Gil, Gonçalves, Faria, Almada, Baptista & Carreiro 1997; Privileggi, Ota & Ferrero 1997; Arakawa, Kanno, Akiyama, Kitano, Nakatsuji & Nakatsuji 1999; Borges, Faria, Gil, Gonçalves Emanuel & Almada 2003; Olivotto, Zenobi, Rollo, Migliarini, Avella & Carnevali 2005; Yokoi & Hosoya 2006; Wittenrich et al. 2007; Meirelles et al. 2009; Kondo et al. 2012). In addition, our ability to show comparable expression of *hoxa13a* during the formation of the gut and trunk in *D. rerio* and *L. dalli* using in situ hybridization suggests that *L. dalli* is also tractable for studies of gene expression.

Our comparative analysis has highlighted novelties in *L. dalli*, such as the blastodisc shape and large larval cupula. The blastodisc shape of *L. dalli* may be due to the oblong shape of the chorion. However, this unique blastodisc shape has not been mentioned in other gobid species, and importantly not in *Priolepis nocturna*, a member of a closely related clade to *Lythrypnus* (Wittenrich et al. 2007), despite the conserved fusiform shape of the chorion. Interestingly, the third cleavage in another gobid species, *Leucopsarion petersii*, occurs in the horizontal plane, which results in two layers of cells at the 8-cell stage (Arakawa et al. 1999). In comparison, the third cleavage plane is used during the sixth cleavage in *D. rerio* and *L. dalli* (Kimmel et al. 1995). These cells are therefore

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**Figure 10** Pigment pattern development in *L. dalli*. (a) Pigmentation prior to settling is minimal ensuring that larva are relatively transparent. (b) Once the pelvic fins are well developed and larvae begin to settle, pigmentation begins to fill. (c) The adult pigmentation pattern becomes evident soon after settling occurs.

**Figure 11** Expression of *hoxa13a* in the hindgut and tail of *Lythrypnus dalli* and *Danio rerio*. Whole mount in situ hybridization reveals *hoxa13a* expression in the posterior gut (arrowheads) and tail bud of (a) prim 9–14 stage *L. dalli* embryos and (b) prim-10 stage *D. rerio* embryos. Scale bar is 0.50 mm and all photos are to scale.
perched on top of the yolk, which may be an alternative adaptation to the fusiform chorions of the Gobiidae. Other gobids may share or have additional strategies to deal with the size and shape limitations of their chorions.

Temperature plays a critical role in developmental timing, hatching success and survival of fish embryos (Schirone & Gross 1968; Kimmel et al. 1995; Kucharczyk, Luczynski, Kujawa & Czerkies 1997; Bermudes & Ritar 1999; Arenzon, Lemos & Bohrer 2002; Yang & Chen 2005; Ahn, Yamada, Okamura, Horie, Mikawa. Tanaka & Tsukamoto 2012). Higher temperatures accelerate development and reduces time to hatching in many species, including D. rerio (Kimmel et al. 1995). At temperatures outside this range, higher rates of malformation and lower rates of survival are common (Kimmel et al. 1995). We observed shorter times to hatching at higher temperatures for Lythrypnus dalli in the range tested (14.4–21.6°C), and did not observe higher rates of malformation or death, suggesting that L. dalli development progresses normally within this temperature range. Danio rerio occur in warmer waters than L. dalli, and therefore are kept in warmer water, develop faster and hatch sooner than L. dalli in the lab.

Interestingly, despite the temperature and overall time to hatching differences in L. dalli and D. rerio, we found that both species progress through larval and juvenile stages in a linear fashion. While neither species allocates more time to any particular developmental stage based on the staging criteria proposed here, and following Kimmel et al. (1995), there were several variations in the timing of specific characters, which appeared at different developmental stages between the taxa (Table 1). For example, the otic placode, otoliths and lens develop at earlier developmental stages in L. dalli, and eye pigmentation occurs earlier in D. rerio, indicating variation in timing of individual organs with respect to developmental stage. Interestingly, some characteristics of the segmentation period develop at similar stages in the ice goby, but at later stages in D. rerio (Kimmel et al. 1995; Arakawa et al. 1999), which suggests an evolutionary component to these differences in developmental timing. In addition, L. dalli are smaller than D. rerio at all embryonic stages and times, with the exception of the 14-somite stage, likely due to the lifting of the head and tail bud off the yolk at an earlier stage, which appears to be a shared feature of gobid embryos, again perhaps associated with the shape of the chorion.

The pelvic fins of L. dalli are of interest for several reasons. First, they articulate in the thoracic region as is common in many acanthomorphs, relative to the abdominal pelvic fins of D. rerio. Second, the pelvic fins of L. dalli are fused on both the posterior and anterior edges, as are the basipterygia, the internal bony pelvic girdle supporting the pelvic fins. These modifications to the pelvic fins form a sucking disc – a unique morphological character of many gobies – in a similar manner as described in Rhinogobius sp. BI (Yokoi & Hosoya 2006). These pelvic fin modifications make members of the Gobiidae, including L. dalli, interesting taxa in which to study the evolution of fin modifications and body plan diversity.

An interesting developmental trait of L. dalli is the pronounced neuromasts and cupula of the hatching-stage embryos and early larvae. We found eight pairs of neuromasts and corresponding cupula on newly hatched larvae, which extended up to a body width out of the neuromast. Comparatively, the neuromasts of D. rerio are much smaller and difficult to find. Similarly pronounced, and comparable numbers of cupula have been reported in two Eviota species, E. abax and E. storthynx (Sunobe & Nakazono 1987), but were specifically not found in a more closely related species to Lythrypnus. Priolepis nocturna (Wittenrich et al. 2007). We found no other reports of these cupula in larval gobids, however, preservation in 5% formalin may destroy them (Sunobe & Nakazono 1987). It would be interesting to further examine the prevalence of these large cupula and neuromasts in gobid species as well as in a variety of pelagic and demersal larval fishes.

Several distinct lineages within the Gobiidae family are bidirectional sex changers (St. Mary 1993; Sunobe & Nakazono 1993). This is a rare characteristic in teleosts and represents an extreme in sexual plasticity. Little is known about the development of the gonads of these species, but the limited data suggest similar molecular pathways are involved in initial gonad development and the reallocation process of gonadal tissue during sex change (Miyake, Sakai & Kuniyoshi 2012). In L. dalli, preliminary data indicate that juveniles develop initially into a bipotential phase where gonads contain both eggs and sperm and genitalia are ambiguous (Lorenzi and Grober, unpublished data), followed by social regulation of initial sexual
development that is responsive to social status (Solomon-Lane, et al., in preparation) in the same way that has been shown for adults (Rodgers et al. 2007).

*Lythrypnus dalli* is a small ornamental fish that is easily maintained in a laboratory setting. Furthermore, adults readily lay eggs in captivity, and are of interest for studies of sexual plasticity, sex change and alternative reproductive strategies. This taxon is tractable and evolutionarily interesting for studies of developmental traits and novelties, such as the early neuromasts of the lateral line and modified pelvic fins. This work provides a useful staging scheme that can be used to study the development of several novel features in a representative derived teleost.

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