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Reproduction, early development, and larval rearing strategies for two sponge-dwelling neon gobies, *Elacatinus lori* and *E. colini*

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ABSTRACT

A major goal of the aquaculture industry is to reduce collection pressure on wild populations by developing captive breeding techniques for marine ornamental species, particularly coral reef fishes. The objective of this study was to develop a rearing protocol for two recently described species of neon gobies that are endemic to the Mesoamerican Barrier Reef: 1) *Elacatinus lori*; and 2) *Elacatinus colini*. First, the current study describes the reproductive behavior and larval development of both species. Second, it evaluates the effects of different rotifer and *Artemia* densities on the survival and growth of *E. lori* and *E. colini* larvae. Third, it compares the survival and growth of *E. colini* larvae fed wild plankton to those fed a combination of rotifers and *Artemia*. Once acclimated, pairs of *E. lori* began spawning in 53.2 ± 12.4 *d* (mean ± sd), while pairs of *E. colini* took only 12.2 ± 10.3 *d*. *E. lori* produced more embryos per clutch (1009 ± 477) than *E. colini* (168 ± 83). *E. lori* larvae hatched 8.18 ± 0.4 days after initial observation with a notochord length of 3.67 ± 0.2 mm. In comparison, *E. colini* larvae hatched 6.8 ± 0.4 days after initial observation with a notochord length of 3.51 ± 2.3 mm. Both species settled as early as 28 days post hatch at 9–9.5 mm standard length, following the fusion of the pelvic fins to form a pelvic disc. During rotifer density trials, from 0 to 6 days post hatch, there was no significant difference in survival or standard length between treatments fed 10, 15 or 20 rotifers ml^{-1} for either species. During *Artemia* density trials, from 6 to 14 days post hatch, control treatments fed solely on 15 rotifers ml^{-1} had significantly higher survival than treatments that were fed rotifers in combination with 3, 6 or 9 *Artemia* ml^{-1}. Finally, *E. colini* larvae that were fed wild plankton had significantly higher survival and growth than those fed with a combination of 15 rotifers ml^{-1} and 3 *Artemia* ml^{-1}. The results of this study suggest that *Artemia* nauplii are not a suitable prey for *E. lori* or *E. colini* larvae. Our results demonstrate the feasibility of rearing *E. lori* and *E. colini* to settlement, and suggest that 10–20 rotifers ml^{-1} and wild plankton provide a viable starting point for optimizing the survival and growth of *Elacatinus* spp. larvae.

1. Introduction

Coral reef ecosystems are declining rapidly in response to global climate change and anthropogenic activities that threaten reef resilience (Bruno and Valdivia, 2016). Among these activities, the marine aquarium trade has been cited as a potential threat to the biodiversity of coral reefs (Dee et al., 2014; Domínguez and Botella, 2014; Moorhead and Zeng, 2010; Rhyne et al., 2012, 2014). Indeed, recent estimates suggest that > 11 million marine ornamental fishes, representing 1802 species, are imported into the U.S. annually for distribution in the marine aquarium trade (Green, 2003; Rhyne et al., 2012; Wabnitz, 2003). Of these, < 1% of specimens are cultured in captivity, while the vast majority are wild caught from reefs in South-East Asia and the Caribbean (Domínguez and Botella, 2014; Rhyne et al., 2012). In some areas, overexploitation and destructive fishing practices have led to the localized decline of reef fish populations and have compromised the ability of reef ecosystems to recover (Domínguez and Botella, 2014). Despite these issues, the demand for marine ornamentals is expected to expand as new technologies simplify the care and maintenance of home aquaria (Moorhead and Zeng, 2010). Therefore, a major goal of the aquaculture industry is to reduce collection pressure on wild populations by developing captive culture techniques for marine ornamental species, particularly coral reef fishes.

Neon gobies of the genus *Elacatinus* were among the first reef fishes to be cultured for distribution in the aquarium trade (Feddern, 1967; Moe, 1975; Valenti, 1972). The genus is composed of 26 species that are primarily distributed on coral reefs throughout the Western Atlantic (Colin, 1975; Froese and Pauly, 2016). Their vibrant coloration and

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Table 1
Characteristics of reproduction from different *Elacatinus* species that have bred in captivity.

Species	Time to 1st spawning (days)	Spawning interval (days)	Clutch size (eggs clutch^{-1})	Chorion width (mm)	Chorion length (mm)	Incubation time (days)	Hatching success (%)	Reference
Elacatinus evelynae	21	14 9–20	200–250	–	–	7	94 ± 3	Colin (1975), Olivotto et al. (2005)
Elacatinus figaro	20.3 ± 5.9 24–31	11.2 ± 2.1 8–10	648 ± 183 140–1020	0.58–0.7	1.8–2.1	6.8 ± 8 5–8	69.7 ± 24.1 34–100	Côrtes and Tsuzuki (2012), da Silva-Souza et al. (2015), Meirelles et al. (2009), Shei et al. (2010, 2012)
Elacatinus genie	–	9 8–10	–	–	1.8–1.9	–	–	Colin (1975)
Elacatinus horsti	–	–	–	–	2.5	–	–	Colin (1975)
Elacatinus louisae	–	–	–	–	2.8–2.9	–	–	Colin (1975)
Elacatinus multifasciatus	< 1 month	–	250	–	–	5–8	–	Wittenrich (2007)
Elacatinus oceanops	1–3.5 months	10–28	50–600	–	2–3.3	6–10	–	Colin (1975), Feddern (1967), Moe (1975), Valenti (1972), Wittenrich (2007)
Elacatinus puncticulatus	–	7–10	153 ± 28	0.55 ± 0.11	–	6–7	98.5 ± 0.6	Pedrazzani et al. (2014), Wittenrich et al. (2007)
Elacatinus xanthiprora	–	12 7–14	–	–	1.7–2.0	–	–	Colin (1975)
Elacatinus colini	12.2 ± 10.3 3–44	7.8 ± 1.7 2–16	168 ± 19 19–388	–	–	6.8 ± 0.4 6–7	86 ± 16 48–100	Majoris et al. (this study)
Elacatinus lori	53.2 ± 12.4 30–69	19.0 ± 7.2 13–35	1009 ± 477 564–1763	0.66 ± 0.22	2.54 ± 0.05	8.18 ± 0.4 8–9	98.2 ± 2 96–100	Majoris et al. (this study)

Time until 1st spawn was measured in days after pairs were acclimation to aquaria. Spawning interval is reported as days between spawning events. Incubation time is reported in days after a clutch was first observed.

Table 2
Feeding protocols under which attempts have been made to culture *Elacatinus* larvae in captivity.

Species	Greenwater	Diet	Prey ml^{-1}	*Artemia* introduction	Reference
Elacatinus evelynae	*N. oculata*	EU + BR, BR + BP, BP, WP + A	10 R 5 A	15 dph	Colin (1975), Olivotto et al. (2005)
Elacatinus figaro	*N. oculata*	BR + A, BP, En-BP, BP + EU, BP + WP, BP + A	3–9 R 0.1–3 A	12–18 dph	Côrtes and Tsuzuki (2012), da Silva-Souza et al. (2015), Meirelles et al. (2009), Shei et al. (2010, 2012)
Elacatinus genie	–	WP + A	–	14 dph	Colin (1975)
Elacatinus horsti	–	WP + A	–	14 dph	Colin (1975)
Elacatinus louisae	–	WP + A	–	14 dph	Colin (1975)
Elacatinus multifasciatus	*N. oculata*	BP + A	10–15 R	12–14 dph	Wittenrich (2007)
Elacatinus oceanops	–	R + A, WP + A	–	10 dph	Colin (1975), Feddern (1967), Moe (1975), Valenti (1972), Wittenrich (2007)
Elacatinus puncticulatus	*N. oculata* Paste	EU, BR + BP + P, WP, R + A	10 R –	15 dph	Pedrazzani et al. (2014), Wittenrich et al. (2007)
Elacatinus xanthiprora	–	WP + A	–	14 dph	Colin (1975)
Elacatinus colini	*N. oculata* Paste	BR, BR + A, BR + WP	10–20 R 3–9 A	6 dph	Majoris et al. (this study)
Elacatinus lori	*N. oculata* Paste	BR, BR + A, BR + WP	10–20 R 3–9 A	6 dph	Majoris et al. (this study)

Diet: *N. oculata*, *Nannochloropsis oculata*; BR, *Brachionus rotundiformis;* BP, *Brachionus plicatilis*; R, rotifer species not specified; A, *Artemia* spp. nauplii; EU, *Euplotes* sp. ciliates; P, *Paramecium* sp.; WP, wild zooplankton; En-, indicates that prey have been enriched; +, indicates a combination of prey.

peaceful disposition have made them a favorite among saltwater aquarium hobbyists. Within the genus, many *Elacatinus* spp. remove parasites from other reef fishes (Côté and Soares, 2011). This unique behavior makes them an important member of coral reef communities, and highly valued in the aquarium trade. However, their propensity for cryptic speciation and relatively limited geographic distributions of *Elacatinus* spp. make them potentially vulnerable to exploitation through wild collection (Colin, 1975, 2010; D'Aloia et al., 2017; Gasparini et al., 2005; Meirelles et al., 2009; Shei et al., 2010; Taylor and Hellberg, 2005). The aquaculture industry has the potential to reduce collection pressure on *Elacatinus* spp. by providing a source of captive cultured livestock for the aquarium trade.

Captive spawning and successful rearing has been reported for 12 species of *Elacatinus*, many of which are commercially available in the aquarium trade (Sweet, 2016). In captivity, breeding pairs typically lay clutches of several hundred to a thousand demersal eggs on the inner surface of PVC tubes (Table 1). The male cares for the eggs until hatching occurs 5–10 days post fertilization (Table 1). *Elacatinus* larvae are often cultured using green water technique and a standard diet of 10–20 rotifers ml^{-1} (*Brachionus* sp.), transitioning to 0.5–9 *Artemia* ml^{-1} at 15–18 days post hatch (dph) (Table 2). However, recent studies have demonstrated that the culture of reef fish larvae is dramatically influenced by the density and types of live prey provided, especially early in development (Anto et al., 2009; Côrtes and Tsuzuki, 2012; da Silva-Souza et al., 2015; Leu et al., 2015; Moorhead and Zeng, 2011; Olivotto et al., 2005; Pedrazzani et al., 2014; Wittenrich et al., 2007). As a result, larval nutrition has been a primary focus of efforts to improve aquaculture protocols (for review see: Holt, 2003; Moorhead and Zeng, 2010; Olivotto et al., 2011, 2017).

To understand the effect of nutrition on larval development, a

Table 3
Characteristics of larval development from different *Elacatinus* species that have been cultured in captivity.

Species	Hatching	Yolk-sac absorption	Flexion	Settlement	Survival to settlement	Reference
Elacatinus evelynae	– –	–	–	– 30–40 dph	10–50%	Colin (1975), Olivotto et al. (2005)
Elacatinus figaro	3.0–3.12 TL 0 dph	–	–	8.5 TL 24–44 dph	2–30.6%	Côrtes and Tsuzuki (2012), da Silva-Souza et al. (2015), Meirelles et al. (2009), Shei et al. (2010, 2012)
Elacatinus multifasciatus	3.0[a] 0 dph	–	–	– 29–35 dph	–	Wittenrich (2007)
Elacatinus oceanops	3–4[a] 0 dph	–	–	10[a] 18–45 dph	–	Colin (1975), Feddern (1967), Moe (1975), Valenti (1972), Wittenrich (2007)
Elacatinus puncticulatus	2.95 ± 0.35[a] 0 dph	–	–	– 36–57 dph	–	Pedrazzani et al. (2014), Wittenrich et al. (2007)
Elacatinus colini	3.51 ± 0.23 NL 0 dph	4 NL 1 dph	4.76 ± 0.36 SL 10 dph	9–9.5 SL 28–58 dph	36.6 ± 18.6% 13.6–65.2%	Majoris et al. (this study)
Elacatinus lori	3.69 ± 0.20 NL 0 dph	4 NL 1 dph	5.00 ± 0.24 SL 10 dph	9–9.5 SL 28–58 dph	15.9 ± 14.4% 3.5–34.1%	Majoris et al. (this study)

Measurements of size over age in mean ± sd or range: NL, Notochord length in mm; SL, standard length in mm; dph, age in days post hatch; %, percent survival to settlement; –, value not reported in reference. [a]Measurement type unspecified in reference.

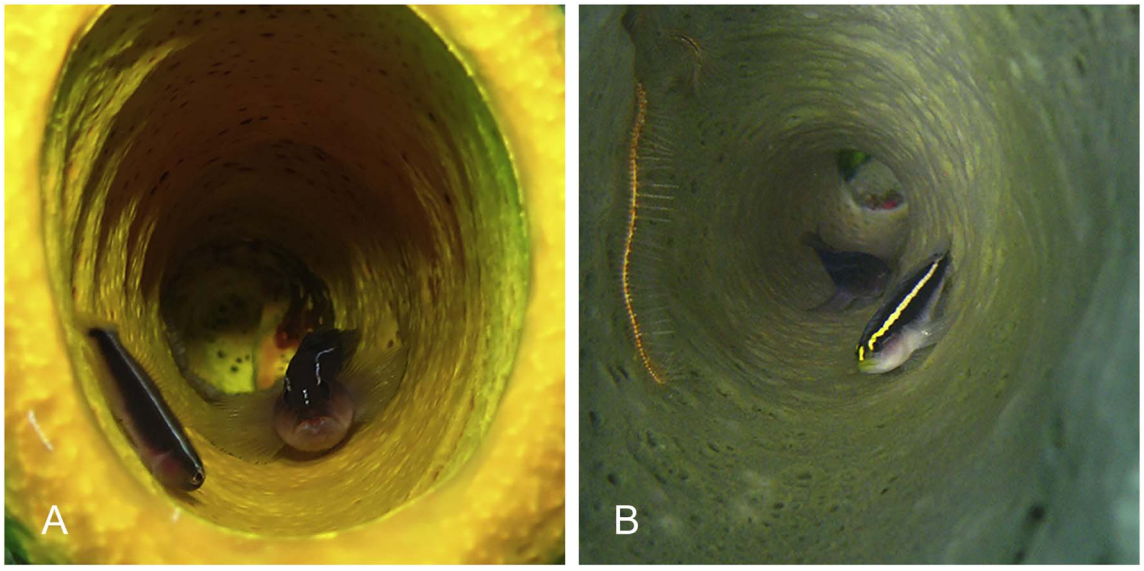


Fig. 1. Photographs of *E. lori* and *E. colini* on reefs near Carrie Bow Caye, Belize. A) Breeding pair of *E. lori* in an *A. fistularis* tube sponge, the smaller female is in the foreground and larger male in the mid-ground. Note the clutch of eggs adhered to the inner sponge lumen in the background to the left of the male. B) Breeding pair of *E. colini* in a tube sponge, with the female in the foreground and male in the background. Note the darker courtship colors of the male.

detailed description of measurable morphological and behavioral traits is necessary as a benchmark for evaluating rearing protocols (Table 3). *Elacatinus* larvae hatch with a limited yolk reserve and begin feeding exogenously within 1–3 days (Table 3). Cultured larvae settle between 18 and 58 dph after the pelvic fins have fused to form a pelvic disc, one of the defining characteristics of the family Gobiidae (Table 3). Despite the successful rearing of several *Elacatinus* spp. to settlement, few studies provide a detailed description of larval development (Table 3).

The objective of this study was to develop a rearing protocol for two recently described species of neon gobies, that are endemic to the Mesoamerican Barrier Reef: 1) *Elacatinus lori* (Colin, 2002); and 2) *Elacatinus colini* (Randall and Lobel, 2009). We describe the reproductive behavior and larval development for both species and evaluate the suitability of rotifers, *Artemia* and wild-caught plankton as prey for culturing *E. lori* and *E. colini* larvae. Specifically, we evaluate the effect of different rotifer and *Artemia* densities on the survival and growth of *E. lori* and *E. colini* larvae. Finally, the survival and growth of *E. colini* larvae fed wild plankton is compared with those fed a combination of rotifers and *Artemia*.

2. Materials and methods

2.1. Description of reproductive behavior and larval development

2.1.1. Broodstock maintenance

Broodstock pairs of *E. lori* and *E. colini* were collected by divers from reef habitats near Carrie Bow Caye, Belize (Fig. 1). Following shipment to the U.S., breeding pairs were established in 75-l aquaria connected to a recirculating seawater system at Boston University, USA. The room was maintained on a 14 L:10 D light cycle. The broodstock system water quality was maintained at a salinity of 33–35 ppt, a temperature of 27–28 °C, a pH of 8.0–8.3, NH_3 levels of 0–0.25 ppm, and undetectable levels of NO_2 and NO_3. These values were similar to water quality conditions under which other species of *Elacatinus* have been reported to spawn in captivity (SI Table 1). Pairs were fed with a varied diet of frozen mysid shrimp, frozen brine shrimp, and pellet food. Each pair was provided with a grey PVC pipe (diameter: 2.5-cm, length: 15-cm) that served as a spawning shelter. Once the first clutch was observed in the shelter, pairs were monitored daily to record reproductive behavior, spawning frequency, and clutch size.

2.1.2. Live feed culture

E. lori and *E. colini* larvae were cultured using small rotifers (*Brachionus rotundiformis*; S-Type, Reed Mariculture, USA) and newly hatched brine shrimp nauplii (*Artemia salina*, INVE Technologies, Thailand). Rotifers were cultured using commercial algae paste (Rotigrow Plus®; Reed Mariculture, USA) fed twice daily, once in the morning (0600–0800 h) and once in the afternoon (1600–1800 h). Condensed rotifers were enriched using a commercial enrichment product (N-Rich™ PL plus; Reed Mariculture, USA) for 2 h, then thoroughly rinsed to remove excess lipids before being fed to larvae. *Artemia* nauplii were fed to larvae within 8 h of hatching without enrichment.

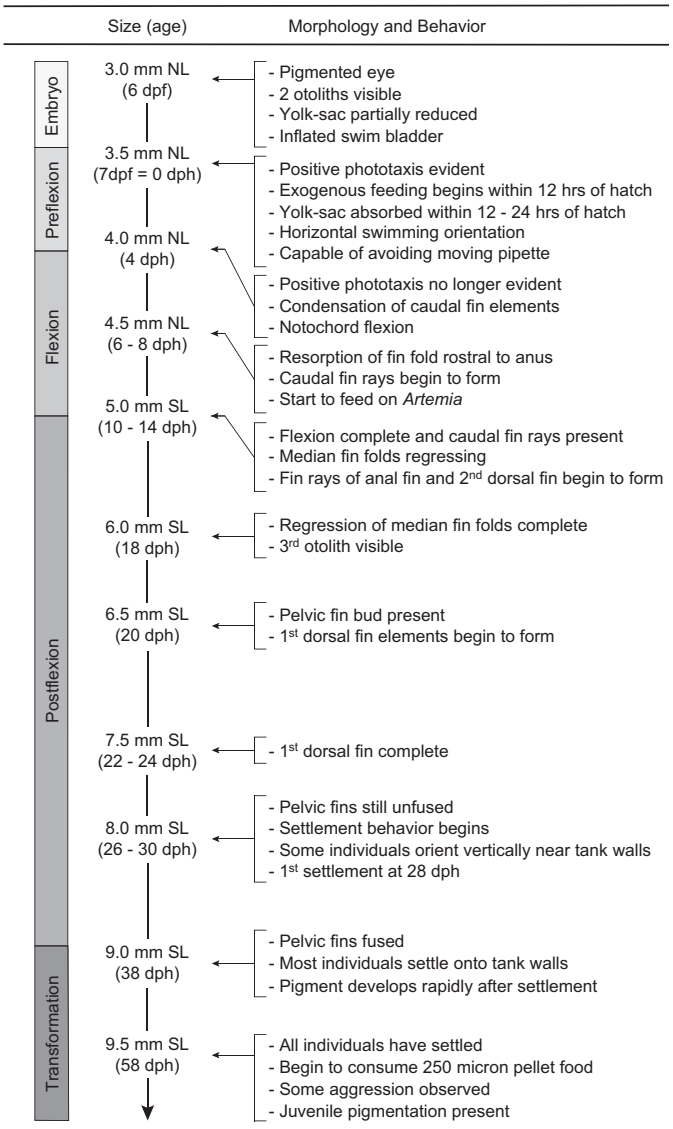
2.1.3. Larval rearing methods to describe early development

To describe larval development and growth rate, 3 clutches of *E. lori* and *E. colini* were reared to settlement. Clutches typically hatched 7 days after being observed in the lab. Therefore, the night before they were expected to hatch, individual PVC pipes with attached eggs were transferred to a cylindrical black 76-l rearing bin. To oxygenate the eggs and stimulate hatching, the PVC pipe was positioned vertically and a gentle stream of air was directed over the eggs using an airstone positioned underneath the pipe. Hatching began immediately following transfer to the rearing bin and was complete by the following morning. Larvae were fed once daily with rotifers (15 ml^{-1}) from hatch through settlement. To maintain the nutritional quality of rotifers and enhance the feeding ability of larvae, 2 ml of Rotigreen *Nanno*™ (Reed Mariculture, USA) was added to the rearing bin during each feeding (Faulk and Holt, 2005; Setu et al., 2010). The room was maintained on a 14 L:10 D light cycle. The water quality of rearing bins were maintained at a salinity of 33–35 ppt, a temperature of 27–28 °C, a pH of 8.0–8.3, NH_3 levels of 0–0.25 ppm, and undetectable levels of NO_2 and NO_3. These values were similar to water quality conditions under which other species of *Elacatinus* have been cultured in captivity (SI Table 2).

2.1.4. Larval development and growth

To describe larval development and assess growth, 3 larvae were sampled from each clutch every other day. Larvae were anesthetized using sodium bicarbonate buffered MS-222 and transferred to a petri dish containing 3% methylcellulose. *E. lori* larvae were photographed using a Canon 60D digital SLR camera equipped with a Canon MP-E

Table 4
General ontogeny of the neon gobies *Elacatinus lori* and *Elacatinus colini*. Labels indicate the size at which > 50% of the individuals possess the identified morphological or behavioral characteristics. NL = notochord length; SL = standard length; dpf = days post fertilization; dph = days post hatch.



65 mm 5 × macro lens (Canon Inc., Japan). The camera was mounted on a 'Stackshot' focus-stacking rail (Cognysis Inc., USA). The camera and rail system were automated using 'Helicon Remote' and the photo stacks were compiled into a single image using 'Helicon Focus' (HeliconSoft, Ukraine). *E. colini* larvae were photographed using a Canon 60D digital SLR camera mounted on a Zeiss Stemi 2000-C stereo dissection microscope (Carl Zeiss, Germany). The standard length (SL) and body depth (BD) of larvae were measured from photos using ImageJ (NIH, USA).

2.2. Prey density and composition experiments

2.2.1. Obtaining larvae in the field

To determine the effects of prey density on larval survival and growth, we reared *E. lori* and *E. colini* larvae in a wet lab at the International Zoological Expeditions (IZE) field station on Southwater Caye, Belize. *E. colini* larvae were obtained from broodstock that were maintained in the lab following the same protocol as described above.

Table 5
The size of *E. lori* and *E. colini* larvae fed a standard diet of 15 rotifers ml^{-1}, 0–30 days post hatch (dph). NL, notochord length; SL, standard length; BD, body depth; *n*, sample size.

	E. lori	*E. colini*
0 dph	NL = 3.69 ± 0.20	NL = 3.51 ± 0.23
	BD = 0.51 ± 0.02	BD = 0.47 ± 0.03
	n = 19	*n* = 18
10 dph	SL = 5.00 ± 0.24	SL = 4.76 ± 0.36
	BD = 0.75 ± 0.06	BD = 0.65 ± 0.09
	n = 7	*n* = 22
20 dph	SL = 6.55 ± 0.71	SL = 6.36 ± 0.59
	BD = 1.04 ± 0.16	BD = 0.93 ± 0.13
	n = 12	*n* = 18
30 dph	SL = 8.08 ± 8.10	SL = 8.23 ± 0.37
	BD = 1.30 ± 0.01	BD = 1.41 ± 0.12
	n = 2	*n* = 13

Intriguingly, *E. lori* took more time to begin spawning than *E. colini*. Therefore, to obtain enough *E. lori* larvae to complete prey density and diet experiments during the summer field season, SCUBA divers visited a transect of 60 yellow tubes sponges *Aplysina fistularis* each day to observe the spawning activity of resident males and track the age of clutches. Clutches were collected using a slurpgun on the day prior to their anticipated hatch (i.e., 7 days after their first observation on the reef).

2.2.2. Larval rearing methods during experimental replicates

Following acquisition of larvae, either from broodstock maintained in the lab or from the reef, individuals from a single clutch were acclimated communally in a 38-l bin. Larvae displaying normal swimming behavior were then distributed to 24 cylindrical black 6.5-l rearing bins. Each rearing bin was connected to a flow through seawater system with 5 μm pre-filtration. To maintain water quality and remove residual prey items between feedings, water exchange was provided to each rearing bin for 1.5 h each morning at a flow rate of 115 ml min^{-1}. Following water exchange, residual prey densities were counted within each rearing bin and additional flow was provided if prey exceeded 1 ml^{-1}. New prey items and green water (0.5 ml of Rotigreen *Nanno*™) were dosed to the rearing bins between 0800 and 1000 h. The water quality parameters of larval rearing bins including temperature (27.6–29.2 °C), salinity (33–36 ppt), and pH (8.0–8.3) were monitored daily; while NH_3 (< 0.17 ppm), NO_2 (0 ppm; i.e., undetectable), and NO_3 (< 0.08 ppm) were monitored every third day.

2.2.3. Plankton collection

In addition to culturing rotifers and *Artemia*, wild plankton were collected daily from the dock at IZE using a plankton pump. A 500 gal $hour^{-1}$ bilge pump and an LED light were mounted to a piling on the dock at IZE. The pump delivered water to a collection bucket that retained plankton > 55 μm in size. The pump was operated nightly from 18:30 to 22:00 h. Plankton retained within the collection bucket were size sorted, and those between 55 and 150 μm were maintained overnight in clean seawater with gentle aeration. Condensed plankton were enriched using a commercial enrichment product (N-Rich™ *PL plus*) for 2 h, then thoroughly rinsed to remove excess lipids before being fed to larvae.

2.2.4. Rotifer density optimization

To determine optimal rotifer density for newly hatched *E. lori* and *E. colini*, survival and growth of larvae were evaluated under 4 different rotifer density treatments: 0 (unfed control), 10, 15, and 20 rotifers ml^{-1}. Twelve, 6.5-l rearing bins were set up for each species, allowing for 3 replicates per density treatment. On the day of hatch (0 dph), 25 larvae were transferred to each rearing bin. Rotifer density treatments were assigned to bins at the start of trials using a complete

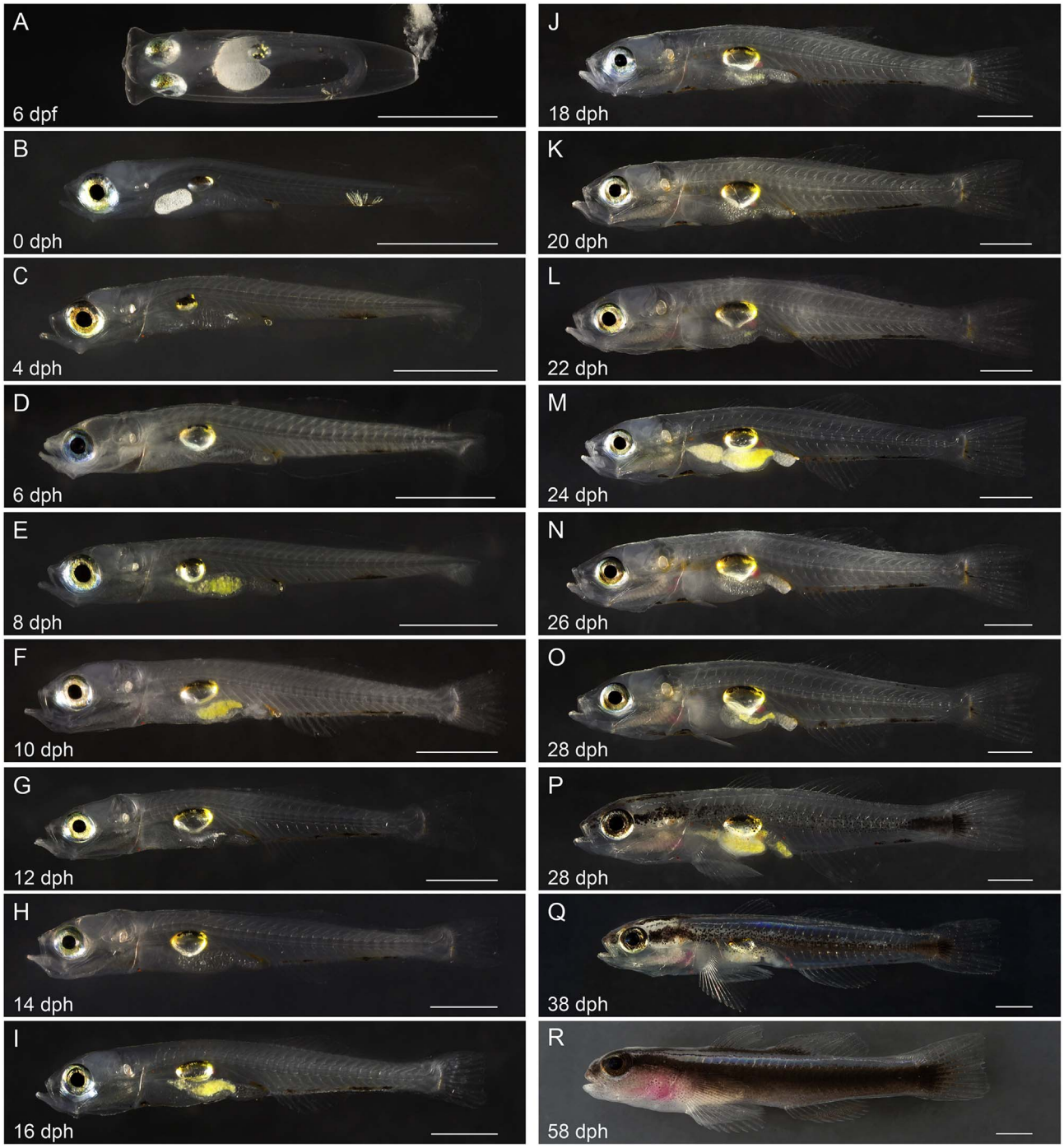


Fig. 2. The early life history of *Elacatinus lori*. Shown are multiple individuals. White scale bars = 1 mm. (A): A 6-dpf (days post-fertilization) embryo with pigmented eyes, two visible otoliths, an inflated swim bladder, and a yolk-sac. The chorion has protuberances at the anterior end and adhesive filaments at the posterior end. (B) A newly-hatched preflexion larva with a functional mouth, reduced yolk reserve, median fin fold, and ventral pigment. (C) 4-dph (days post-hatch) preflexion larva with condensation of the caudal fin elements. (D) 6-dph larva with initial flexion of the notochord. Note that eye color is influenced by position of camera lighting. (E) 8-dph flexion larva with initial formation of caudal fin rays and resorption of the minor fin fold rostral to anus. Note the presence of rotifers in the gut. (F) 10-dph postflexion larva with hypural plate and caudal fin rays. Resorption of the median fin folds begins along the trunk, and development of the anal fin rays is more advanced than fin ray development in the second dorsal fin. (G–I) 12–16-dph postflexion larvae, dorsal and anal fin formation, and fin fold resorption continue. (J) 18-dph postflexion larva with complete resorption of the fin folds and emergence of the 3rd otolith. (K–M) 20–24-dph postflexion larvae, pelvic fin bud and first dorsal fin begin to form. (N) 26-dph postflexion larva, the pelvic fins remain unfused and snout begins to elongate. (O) 28-dph presettlement larva, beginning of pelvic fin fusion. (P) 28-dph earliest settler with fused pelvic fins forming a complete pelvic disc and first dorsal fin. At settlement, a dark spot develops on the caudal peduncle and a strip begins spreading from the head onto the trunk. (Q) 38-dph settler (3 days after settlement) dark pigment and blue stripe, characteristic of adults, have progressed onto the trunk. (R) 55-dph settler, fully pigmented. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

randomized block design. Following daily water exchange, each rearing bin was dosed with the assigned rotifer density. There was no significant difference in water quality parameters among rotifer density treatments (all Kruskal-Wallis tests, $p > 0.05$). On day 6, all surviving larvae were collected from the rearing bins, counted and photographed using a dissection microscope. The photographs of larvae were used to compare larval size (SL) among rotifer density treatment.

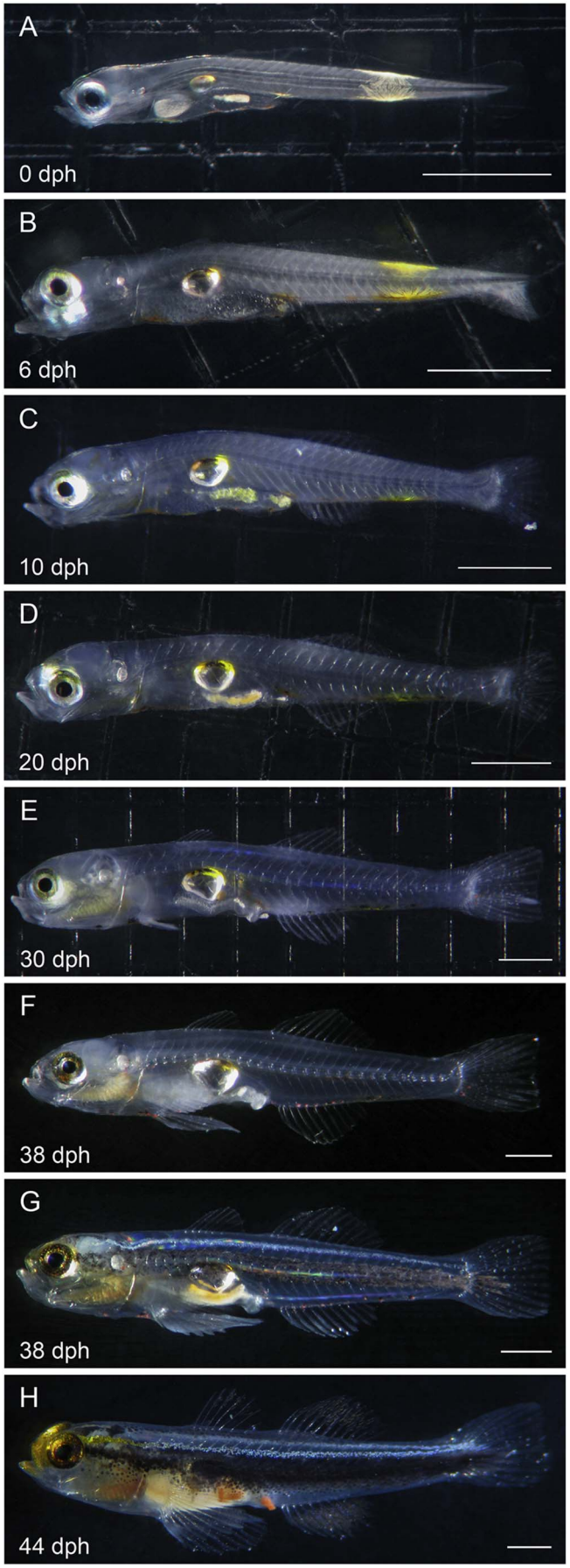


Fig. 3. The early life history of *Elacatinus colini*. Shown are multiple individuals (age in days post hatch at lower left). White scale bars = 1 mm. (A) A newly-hatched preflexion larva with a functional mouth, 2 visible otoliths, reduced yolk reserve, inflated swim bladder, median fin folds, and dorsal and ventral trunk pigments. (B) 6-dph preflexion larva with initial condensation of the caudal elements. (C) 10-dph larva nearing completion of flexion with fin fold resorption beginning along the trunk. (D) 20-dph postflexion larva, dorsal and anal fin rays have developed, and fin fold resorption is complete. (E) 30-dph postflexion larva, the first dorsal fin has developed and the pelvic fins have begun to fuse. (F) 38-dph pre-settlement larva, beginning of pelvic fin fusion. (G) 38-dph settler with fused pelvic fins forming a complete pelvic disc. At settlement, a band of dark pigment develops from the head through the trunk, followed by golden coloration on the snout and a blue strip that spreads rapidly from the head through the caudal peduncle. (H) 44-dph settler, fully pigmented. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

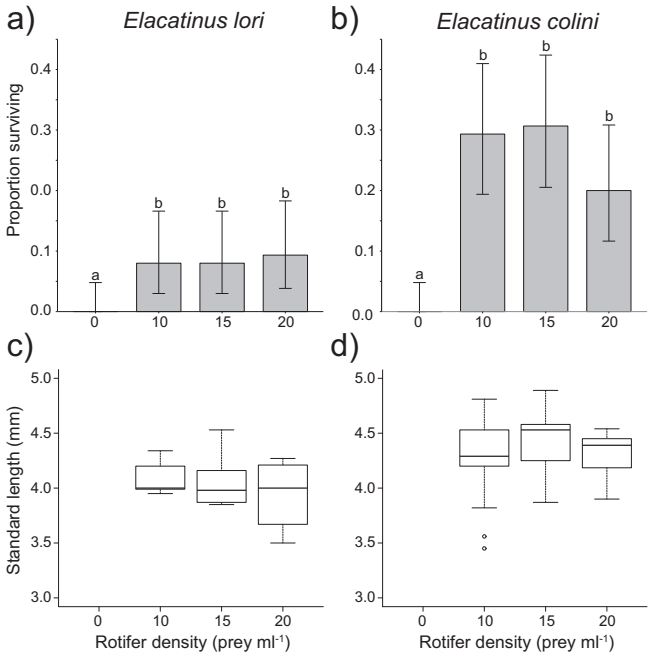


Fig. 4. The effect of rotifer density on the survival and standard length of *E. lori* and *E. colini* larvae. a–b) Bar plots displaying the proportion of larvae that survived to 6 dph when fed 0, 10, 15 or 20 rotifers ml^{-1}. 95% confidence intervals (*whiskers*) and significant difference between rotifer density treatments (*letters*) are illustrated. c–d) Box plots showing the standard length (SL) of larvae at 6 dph when fed 0, 10, 15 or 20 rotifers ml^{-1}. There was no significant difference in SL between rotifer density treatments ($p > 0.05$). The median (*centerline*), interquartile range (*box*), minimum and maximum values (*whiskers*), and outliers (*circles*) are illustrated.

2.2.5. Artemia density optimization

To determine the optimal density of *Artemia* for culturing *E. lori* and *E. colini* larvae, the survival and growth of larvae were evaluated under 4 density treatments: 0 (unfed control), 3, 6, and 9 *Artemia* ml^{-1}. A pilot experiment indicated that > 40% of *E. colini* larvae began consuming *Artemia* nauplii at 6 dph. Therefore, for each species, larvae from a single clutch were reared communally in a 38-l rearing bin and fed 15 rotifers ml^{-1} from 0 to 6 dph. On day 6, surviving larvae were distributed evenly among twelve, 6.5-l rearing bins (3 bins per *Artemia* density treatment). Due to differential survival to day 6, the number of larvae distributed among the rearing bins varied by species (*E. lori*: $n = 20$ larvae bin^{-1}; *E. colini*: $n = 14$ larvae bin^{-1}). *Artemia* density treatments were assigned to bins at the start of trials using a complete randomized block design. Following daily water exchange, each bin was dosed with rotifers (15 ml^{-1}) and the assigned *Artemia* density. The water quality parameters in larval rearing bins did not differ significantly between treatments (all Kruskal-Wallis tests, $p > 0.05$). On day 14, all surviving larvae were counted and photographed. The photographs of larvae were used to compare larval size (SL) among *Artemia* density treatments.

2.2.6. Optimized rotifer and Artemia densities vs. wild caught plankton

To determine the suitability of wild caught plankton for rearing larvae in the lab in Belize, the growth and survival of *E. colini* larvae fed a combination of rotifers and *Artemia* (RA) was compared with larvae fed solely on wild caught plankton (P). Prey combination treatments were assigned to bins at the start of trials using a complete randomized block design. On the day of hatch (0 dph), 25 larvae were transferred to each of six, 6.5-l rearing bins (3 bins per prey combination). Rotifers (15 ml^{-1}) or plankton (≤ 10 ml^{-1}) were fed to larvae beginning at 0 dph. However, *Artemia* (3 ml^{-1}) were not included in the RA diet until 6 dph. Due to natural variation in the quantity of plankton collected in the field each evening, the average density of plankton fed to larvae was 5.3 ± 3.8 prey ml^{-1} (mean ± SD). Following daily water exchange, each rearing bin was dosed with the assigned prey combination. Water quality parameters were not significantly different between prey treatments (all Wilcoxon Rank-sum tests, $p > 0.05$). On day 14, all remaining larvae were counted and photographed. The photographs of larvae were used to compare larval size (SL) among prey treatments.

2.3. Data analysis

Fisher's Exact test was employed to determine whether a significant difference ($\alpha < 0.05$) in larval survival occurred among prey density treatments. When a difference was observed, pairwise comparisons with Bonferroni's correction were used to determine which treatments differed with respect to survival. Kruskal-Wallis tests were used to compare the size (SL) of larvae among prey density treatments. An Exact Wilcoxon Rank-Sum test was used to compare the size (SL) of larvae fed wild caught plankton with those fed the optimized diet of rotifers and *Artemia* (see above). All statistical analyses were carried out using R version 3.3.1 (Team, 2014).

3. Results

3.1. General observations of broodstock

E. lori and *E. colini* males were characterized by their large size, canine teeth on their lower jaw, and pointed genital papillae, while females were identified by their smaller size, rounded snout, and square genital papillae. Males spent the majority of their time resting inside the PVC spawning shelter, while females were more active and moved around the tank. Gravid females developed a full rectangular abdomen with a yellow mass visible toward the posterior end of the abdomen. Before spawning, both males and females turned a dark grey to black color throughout the body. *E. lori* pairs began spawning within 30–69 days, and *E. colini* pairs within 3–44 days of being introduced into aquaria (Table 1). Female *E. lori* deposited a dense monolayer of eggs on the inside surface of the spawning shelter every 19 ± 7.2 days, while *E. colini* spawned smaller clutches every 7.8 ± 1.7 days (Table 1). Males incubated the eggs by fanning and mouthing the clutch until hatching occurred (Table 1). On multiple occasions, females were observed consuming recently hatched larvae in the water column. *E. colini* pairs spawned regularly for 12 months, while *E. lori* ceased spawning after 6 months.

3.2. Larval development

The developmental timing of morphological and behavioral characteristics was similar between *E. lori* and *E. colini* (Tables 4–5). For both species, clutches hatched immediately after transfer to a rearing tank. Embryos hatched with a functional mouth, inflated swim bladder, pigmented eyes, and two visible otoliths (Figs. 2A–B; 3A). Larvae are positively phototactic and swim to the surface of the water after hatching. Early in development, larvae were attracted toward light reflecting off the walls of the tank. However, the use of black rearing tanks and addition of green water helped to distribute larvae more evenly throughout the water column. Exogenous feeding on rotifers began within 12 hours post hatch (hph) and yolk reserves were depleted within 24 hph. Notochord flexion is complete by 10 dph (Figs. 2F; 3C). Following flexion, resorption of the fin folds begins along the trunk, and the development of fin rays begin in the anal fin and second dorsal fin (Figs. 2F–J; 3C–D). By 18–20 dph, the fin folds have been completely resorbed, and the pelvic fin bud and first dorsal fin begin to form (Figs. 2J–K; 3D). Between 20 and 28 dph, the pelvic fin continues to elongate, but remains unfused (Fig. 2K–O). Pre-settlement larvae have an elongated snout, partially fused pelvic fins, and complete first dorsal fin (Figs. 2O; 3E–F). Larvae begin settling when the pelvic fins have fully fused to form a pelvic disc, which can be used to suction onto surfaces. In both species, the earliest settlement event occurred at 28 dph. *E. lori* develop a dark spot on the caudal peduncle at settlement and pigment radiates rapidly from the head onto the trunk (Fig. 2P). In a 3 day post-settlement *E. lori*, the dark body pigmentation and radiant whitish blue stripe, characteristic of adult *E. lori*, have developed along the trunk (Fig. 2Q). Similarly, *E. colini* immediately develop a dark strip along the body at settlement. Within a few days, yellow pigment develops on the snout and a blue stripe radiates from the head through the caudal peduncle (Fig. 3G–H). *E. lori* and *E. colini* settle between 28 and 58 dph.

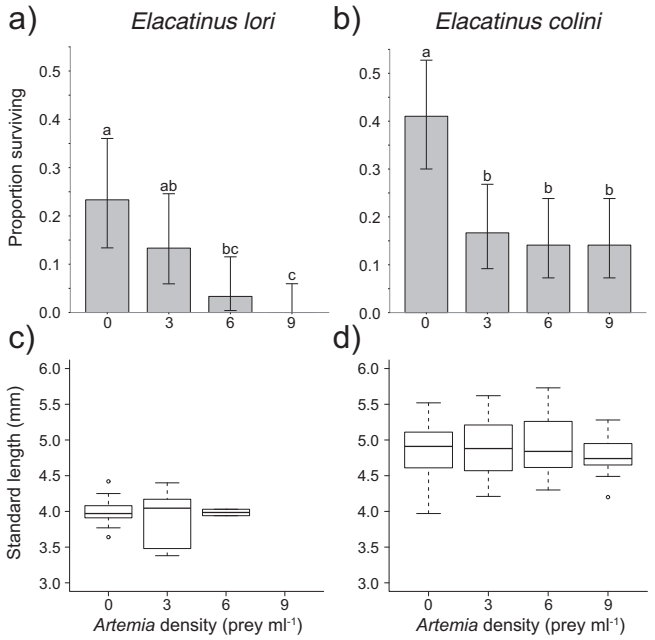


Fig. 5. The effect of *Artemia* density on survival and size (standard length) of *E. lori* and *E. colini* larvae. a–b) Bar plots displaying the proportion of larvae that survived to 14 dph when fed 0, 3, 6 or 9 *Artemia* ml^{-1}. The 95% confidence intervals (*whiskers*) and significant difference between rotifer density treatments (*letters*) are illustrated. c–d) Box plots showing the standard length (SL) of larvae at 14 dph when fed 0, 3, 6 or 9 *Artemia* ml^{-1}. There was no significant difference in SL between *Artemia* density treatments ($p > 0.05$). The median (*centerline*), interquartile range (*box*), minimum and maximum values (*whiskers*), and outliers (*circles*) are illustrated.

3.3. Optimization of rotifer density

For both *E. lori* and *E. colini*, the larval survival in treatments fed rotifers were significantly higher than unfed controls (Fisher's Exact tests: $p_{E.\ lori} = 0.028$; $p_{E.\ colini} < 0.0001$; Fig. 4a–b). However, there was no significant difference in the survival ($p > 0.05$) or standard

length (Kruskal-Wallis tests: $H_{E.\ lori} = 0.83$, $p_{E.\ lori} = 0.66$; $H_{E.\ colini} = 3.57$, $p_{E.\ colini} = 0.17$) of larvae among those fed 10, 15 or 20 rotifers ml^{-1} (Fig. 4a–d). At each rotifer density, the mean survival and standard length of *E. lori* larvae (Fig. 4a, c) were lower than *E. colini* larvae (Fig. 4b, d).

3.4. Optimization of Artemia density

There was a significant difference among *Artemia* density treatments in larval survival to day 14 (Fisher's Exact tests: $p_{E.\ lori} < 0.0001$; $p_{E.\ colini} < 0.0001$; Fig. 5a–b). For both *E. lori* and *E. colini*, the highest survival occurred in control treatments, which were provided with 15 rotifers ml^{-1} without the addition of *Artemia* (Fig. 5a–b). The survival of *E. lori* larvae declined incrementally in treatments fed 3, 6, and 9 *Artemia* ml^{-1}, with no larvae surviving to 14 dph when fed 9 *Artemia* ml^{-1}(Fig. 5a). Despite the adverse effect of *Artemia* density on survival, there was no significant difference in the standard length of surviving larvae among density treatments (Kruskal-Wallis tests: $H_{E.\ lori} = 0.04$, $p_{E.\ lori} = 0.98$; $H_{E.\ colini} = 0.73$, $p_{E.\ colini} = 0.87$; Fig. 5c–d).

3.5. Wild caught plankton feeding experiment

The survival of *E. colini* larvae to day 14 was significantly higher in treatments fed wild caught plankton than those fed a combination of 15 rotifer ml^{-1} and 3 *Artemia* ml^{-1} (Fisher's Exact test: $p_{E.\ colini} = 0.006$; Fig. 6a). The standard length of larvae fed wild caught plankton was also significantly larger than those fed rotifers and *Artemia* (Exact Wilcoxon Rank-Sum test: $W_{E.\ colini} = 603$, $p_{E.\ colini} < 0.0001$; Fig. 6b).

4. Discussion

In this study, we established captive broodstock and reported a rearing protocol for two species of neon gobies, *Elacatinus lori* and *E. colini*. In general, *Elacatinus* spp. adapt well to small aquaria and begin reproducing within a few weeks to months of acclimation (Table 1). *E. lori* spawned less frequently than *E. colini*, but typically produced larger clutches and had higher hatching success (Table 1). The number of embryos in each clutch and incubation times were similar to those reported for other species of *Elacatinus* that have bred in captivity (Table 1). Upon hatching, yolk-sac absorption and first feeding typically occur within 24–48 h (Table 4). For *E. lori* and *E. colini*, notochord flexion was complete by 10 dph and settlement occurred between 28 and 58 dph. Pigmentation developed rapidly on the head and trunk within a few days of settlement (Table 4). The description of larval morphology and behavior in relation to size and age is a valuable tool for evaluating the effect of different rearing protocols on development and is necessary for the comparison of development across species (Tables 3–4).

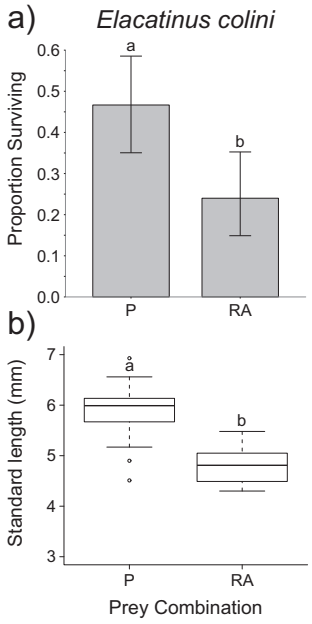


Fig. 6. The effect of wild-caught plankton as a food source on survival and size (standard length) of *E. colini* larvae. a) Bar plot displaying the proportion of larvae that survived to 14 dph when fed wild-caught plankton (P) or a combination of rotifers and *Artemia* (RA). The 95% confidence intervals (*whiskers*) and significant difference between rotifer density treatments (*letters*) are illustrated. b) Box plots showing the standard length of larvae at 14 dph fed wild-caught plankton (P) or a combination of rotifers and *Artemia* (RA). The median (*centerline*), interquartile range (*box*), minimum and maximum values (*whiskers*), outliers (*circles*), and significant difference between density treatments (*letters*) are illustrated.

During rotifer density experiments, there were no significant differences in the survival or growth of *E. lori* or *E. colini* larvae among density treatments that were fed 10, 15 or 20 rotifers ml^{-1}. However, the highest mean survival was achieved in *E. lori* that were fed 20 rotifers ml^{-1}, and *E. colini* that were fed 15 rotifers ml^{-1}. The larvae of other *Elacatinus* species have been successfully reared to settlement when fed densities of 10–20 rotifers ml^{-1} (Table 2; Meirelles et al., 2009; Olivotto et al., 2005; Shei et al., 2010). Taken together, the results of these studies suggest that a density of 10–20 rotifers ml^{-1} provide a reasonable starting point for rearing *Elacatinus* spp.

During *Artemia* experiments, the survival of *E. lori* larvae declined incrementally with increasing *Artemia* nauplii density. Intriguingly, for both species, the highest survival occurred in control treatments in which larvae were only fed rotifers, suggesting that *Artemia* were inappropriate prey for *E. lori* and *E. colini* larvae at this stage of development. In contrast to our findings, a recent study demonstrated that providing *Artemia* nauplii at day 12 accelerated the timing of metamorphosis in *E. figaro*, when compared with *Artemia* addition on day 18 (da Silva-Souza et al., 2015). It is possible that introducing *Artemia* to *E. lori* and *E. colini* later in development may have positive rather than negative effects on their development.

There are several possible explanations for the negative effect of *Artemia* on the survival of *E. lori* and *E. colini*. The majority of larvae were capable of consuming *Artemia* nauplii by 6 dph. However, *Artemia* may have had a direct effect on survival if larvae had not developed the ability to digest this larger, more complex prey, or if larvae derived less nutritional value from *Artemia* than enriched-rotifers. Alternatively, *Artemia* may have had an indirect effect on survival by influencing water quality in the rearing bins. Increasing *Artemia* densities may have depleted oxygen in the rearing bins, resulting in higher mortality due to asphyxiation. While low oxygen concentrations cannot be ruled out, the rearing bins were well aerated to provide oxygen during *Artemia* density trials, and larval densities were low. Therefore, it is unlikely that low oxygen concentrations were the cause of mortality. Since all other water quality parameters were the same among treatments, this supports our conclusion that *Artemia* likely had a direct effect on larval survival and were an inappropriate prey item for *E. lori* and *E. colini* early in development.

E. colini larvae fed with wild plankton experienced significantly better survival and growth than those fed with a combination of rotifers and *Artemia*. Côrtes and Tsuzuki (2012) demonstrated similar benefits to survival and growth in *E. figaro* larvae that were fed a diet of wild copepod nauplii and rotifers. In this study, wild plankton samples appeared to be dominated by copepod nauplii, but also included a variety of other organisms and prey sizes. Previous studies have shown that larvae select larger, more complex prey as their feeding abilities improve throughout development (Anto et al., 2009). Thus, wild plankton samples may have enhanced survival and growth by allowing larvae to select prey from an assortment of prey types and sizes. The benefits to survival and growth make the use of wild caught plankton an ideal strategy for rearing reef fish species (Pedrazzani et al., 2014; Wittenrich

et al., 2007, 2010). Our results demonstrate the feasibility of rearing *E. lori* and *E. colini* to settlement, and suggest that 10–20 rotifers ml^{-1} and wild plankton provide a viable starting point for optimizing the survival and growth of *Elacatinus* spp. larvae. The captive rearing protocols presented here could obviate the development of a wild caught fishery for these Belizean endemics.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2017.10.024.

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