

REGULAR PAPER

The beach-spawning California grunion *Leuresthes tenuis* eats and digests conspecific eggs

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Funding information

Some work by K.A.D. was done while serving at and being supported by the National Science Foundation. Material by A.R.F. is based upon work supported by the National Science Foundation Graduate Research Fellowship under Grant No. DGE-1321846.

Apparent egg cannibalism was investigated in the beach-spawning California grunion *Leuresthes tenuis*. Three hypotheses were tested to determine whether *L. tenuis* regularly consumes and efficiently digests conspecific eggs. First, examination of the gut contents of adults collected at four spawning sites over two seasons showed that the intestines of most fish from all the sites (57–87%, $n \geq 30$, each site) contained *L. tenuis* eggs. The two other hypotheses focused on digestion of the eggs. First, the force required to crush cannibalized eggs was significantly less than that for uncannibalized eggs (fertilized or unfertilized), indicating that ingestion weakens the egg chorions. Second, conspecific eggs fed to fish held in the laboratory visibly degraded as they passed through the gut. The eggs lost c. half of their protein content and about two-thirds of their lipid content as they passed from proximal to distal regions of the gut, indicating that digestion occurred. Digestive enzyme activities of the gut further confirmed that *L. tenuis* can break down the contents of ingested eggs. Trypsin activity decreased and aminopeptidase activity increased posteriorly along the gut, whereas amylase and lipase activities exhibited less clear patterns by gut region. As far as is known, this study is the first to show that *L. tenuis* is an egg cannibal.

KEYWORDS

Atherinopsidae, biochemical composition, California grunion, cannibalism, digestive enzymes

1 | INTRODUCTION

Cannibalism is the act of killing and consuming an individual belonging to the same species, irrespective of its stage of development (Smith & Reay, 1991). Proposed benefits of cannibalism include nutrition, reduced resource competition, parental manipulation and sexual selection (Pereira *et al.*, 2017; Polis, 1981; Smith & Reay, 1991). Cannibalism occurs in diverse taxonomic groups including insects, birds and mammals (Mehlis *et al.*, 2010; Payne *et al.*, 2002) and in 390 fish species belonging to 104 teleost families (Pereira *et al.*, 2017). For teleosts, these numbers represent slightly more than 1% of the known species and nearly 25% of known families based on Nelson (2006). Investigations of fish cannibalism in nature remain uncommon, with nearly 40% of studies based only on captive species (Pereira *et al.*, 2017). Offspring cannibalism in teleosts has been categorized as either filial, non-kin, or a combination of both types (Bose *et al.*, 2016;

Fitzgerald & Whoriskey, 1992; Pereira *et al.* 2017; Smith & Reay, 1991) and eggs are the developmental stage most often cannibalized (Pereira *et al.*, 2017).

A potential egg cannibal of the non-kin type is the beach-spawning California grunion *Leuresthes tenuis* (Ayres 1860). This species is a member of the fish family Atherinopsidae (New World silver-sides) and occurs mainly along the coasts of southern California and northern Baja California (Eschmeyer *et al.*, 1983; Miller & Lea, 1972). It is well known for spawning intertidally on sandy beaches during nights following the highest semilunar tides (Figure 1; Martin, 1999; Taylor, 1999; Walker, 1952); individual fish may spawn multiple times in a season (Walker, 1949). Typically, adult *L. tenuis* are washed onto shore by waves and then remain on the beach to spawn as the waves recede (Fritzsche *et al.*, 1985; Walker, 1952).

Although females lay their eggs about 5 cm under the surface of the sand (Figure 1; Thompson, 1919; Walker, 1949), some eggs may

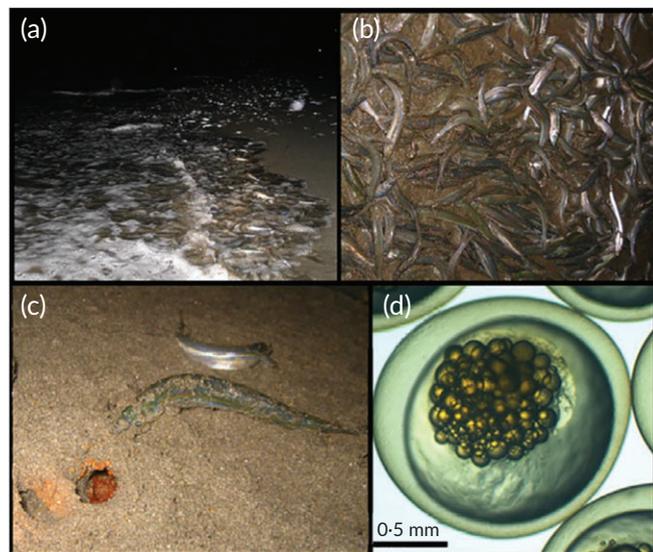


FIGURE 1 Spawning event of *Leuresthes tenuis* at Cabrillo Beach, Los Angeles, California, USA. (a) Fish aggregated on the beach. (b) Adult *L. tenuis* stranded on the sand. (c) A female has dug a hole and deposited eggs in the sand. (d) Unfertilized *L. tenuis* egg, with orange oil droplets and chorion visible. Egg diameter 1.7 mm

become available for consumption by spawning males and females. Fish may cannibalize eggs while in the water just before or after a spawning event, or while on the sand during or immediately after the spawning act. Eggs consumed from the water may come from gravid females that have released eggs just before they come ashore or from a spawning event if the eggs were washed out of the sand by wave action (Demartini & Sikkell, 2006; Thompson, 1919; Walker, 1949). These eggs could be ingested from the water or from the surface of the sand. During a heavy run, many eggs are strewn across the shore (Figure 1), presumably making them available for consumption by the adults.

The impetus for this study of egg cannibalism in *L. tenuis* was based on four observations and experiments variously made by the authors. First, we noticed that a few fish that were dissected for another purpose had conspecific eggs in their guts. Second, following this observation, a laboratory experience for school eighth grade students found that some *L. tenuis* guts they dissected contained conspecific eggs (Cavanagh *et al.*, 2014). Third, we found that externally applied proteases do not digest the *L. tenuis* chorion and release embryos, as they do in zebrafish (Westerfield, 2000) and other fishes. Moreover, Carter (2015) found that the chorion is virtually impermeable to several compounds. Fourth, we attempted to digest eggs in vitro using a commercial digestive-enzyme bath and an *L. tenuis* gut homogenate, yet we found no evidence of digestion after 5 h of exposure in either preparation (Appendix S1 in Supporting information). The present investigation was designed to examine the guts of fish from multiple sites extending over 2 years with the objective of determining whether the apparent egg cannibalism by *L. tenuis* is a common or isolated event. Logically, another objective was to establish whether the fish can digest cannibalized eggs. Three hypotheses were tested: *L. tenuis* ingests conspecific eggs during spawning episodes at multiple sites involving more than 1 year along its breeding range in southern California; the amount of force required to crush eggs that

have been ingested is less than that required for uncannibalized eggs, whether fertilized or unfertilized; conspecific eggs fed to *L. tenuis* are digested as they move down the gut based on numerical reduction, physical degradation and digestive enzyme concentration. Overall, the study tested whether *L. tenuis* cannibalizes eggs and whether it can digest the eggs to their component monomeric units that are potentially useable in metabolism.

2 | MATERIALS AND METHODS

2.1 | Fish collection

For all parts of this study, adult *L. tenuis* were collected by hand at spawning beaches in southern California. Fish examined for *L. tenuis* eggs in their guts were obtained under California State University, Fullerton (CSUF) IACUC protocol 09-R-07 in 2009 from the following three beaches: Belmont Shore (33° 45' N; 118° 08' W), Long Beach Pier (33° 45' N; 118° 08' W) and Malibu Surfrider (34° 02' N; 118° 41' W) (Figure 2). These fish were euthanized with an overdose (1 g l⁻¹) of MS-222, transported to the lab on dry ice and kept in a -80°C freezer until examined for *L. tenuis* eggs in the gut. The fish designated for egg counts from the fourth spawning locality, Cabrillo Beach (33° 43' N; 118° 17' W) (Figure 2) in 2008 and 2009, had been previously collected as mortalities from a spawning run under CSUF IACUC protocol 08-R-06, dissected for another purpose (Higgins & Horn, 2014) and their guts stored in 10% formalin. To collect cannibalized eggs for the crushing force measurements, adults were obtained under CSUF IACUC protocols 11-R-07 and 14-R-07 at Cabrillo Beach in May 2013 and July 2014 and transported on ice to CSUF, where eggs were removed from the anterior region of the digestive tracts. Uncannibalized eggs for the crushing force measurements were obtained under CSUF IACUC protocols 11-R-07 and 14-R-07 by stripping females at Cabrillo Beach in 2013 and 2014. To obtain adults for the in vivo digestibility experiment, fish were collected under University of California, Irvine (UCI) IACUC protocol 2011-2989 at Cabrillo Beach in April 2014 and housed at Cabrillo Marine Aquarium, San Pedro, California.

2.2 | Egg counts in *L. tenuis* guts

The entire stomachless (Horn *et al.*, 2006) gut (proximal, mid and distal intestines) of *L. tenuis* was examined for the presence of eggs based on one collection per site ($n = 30$ – 31 for the first three sites and $n = 53$ for Cabrillo Beach) for a total of 143 fish from the four sites. The conspicuous appearance of the eggs, including the size (1.5–1.9 mm; Carter, 2015) and presence of multiple orange oil droplets (Figure 1(d)), made them readily identifiable with $\times 10$ and without magnification (Walker, 1949).

2.3 | Egg-crushing experiment

2.3.1 | Acquisition of fertilized and unfertilized uncannibalized eggs

Eggs were stripped from *L. tenuis* directly into filtered seawater in plastic containers. To fertilize a portion of the eggs, sperm were also

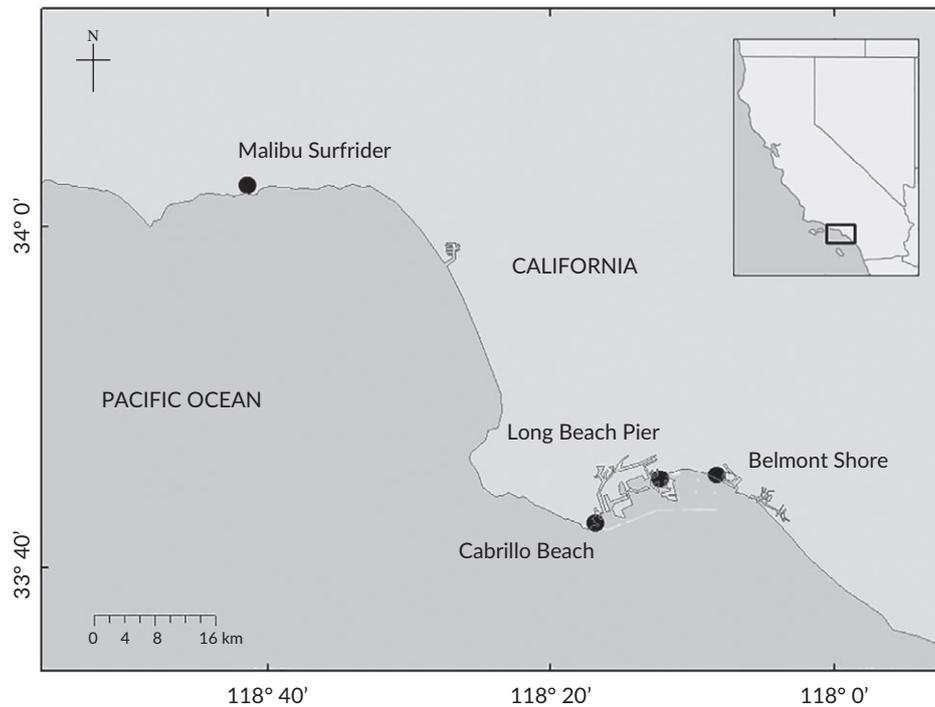


FIGURE 2 The four spawning/collection sites (●) in southern California, USA, for adult *Leuresthes tenuis*. Map from Google Earth

stripped from fish and added to eggs in one of the containers. After approximately 20 min, the fertilized and unfertilized eggs were rinsed with filtered sea water three times, placed onto damp paper towels in separate containers and taken to CSUF, within 2 h of fertilization. Eggs were kept overnight in containers with damp sand in an incubator set at 20°C.

2.3.2 | Acquisition of cannibalized eggs

Intact cannibalized eggs were gently pushed out of the anterior region of the intestine, rinsed with filtered seawater and placed between two pieces of filter paper moistened with seawater in Petri dishes and kept in a 20°C incubator overnight. Some eggs found in the intestines were developing embryos; these eggs were not used in the crushing force measurements. The cannibalized eggs were not tested to determine whether they were fertilized.

2.3.3 | Measurement of force required to crush eggs

The force required to crush cannibalized eggs ($n = 38$) was measured and compared with values for unfertilized ($n = 20$) and fertilized ($n = 50$) eggs collected during the same *L. tenuis* runs (Carter, 2015). Eggs were individually transferred to a glass slide and the force (N) required to crush or rupture the chorion was measured using a Type #9203 piezoelectric high-sensitivity force sensor and #5995A charge amplifier (Kistler; www.kistler.com). The voltage output value at the rupture point was recorded and converted to force (N) with a calibration curve established using objects of known mass multiplied by acceleration from gravity (9.81 m s^{-2}). The system was calibrated twice daily, before and after conducting tests with the eggs.

2.4 | In vivo digestibility experiment

2.4.1 | Fish maintenance and feeding protocol

Adult *L. tenuis* ($n = 150$) were housed in two 422 l cylindrical tanks (75 fish per tank) with recirculating, filtered seawater maintained at salinity 32.0 ± 0.5 and $20.00 \pm 0.25^\circ\text{C}$ at Cabrillo Marine Aquarium. The fish were allowed to acclimate to the tank environment for 2 weeks during which time they were fed ad libitum frozen mysid crustaceans, which are major food items of *L. tenuis* (Higgins & Horn, 2014; Horn *et al.*, 2006). The fish were deprived of food for 5 days before the start of the experiment to ensure that their guts were empty and that they would feed on the eggs provided. Eggs were stripped from females at the next spawning event and these uncannibalized and unfertilized eggs were fed to the captive fish within 24 h. Once the fish began feeding, four fish were collected from each tank every 15 min for the first hour post-feeding and then every hour thereafter for a total of 10 h. The fish were euthanized in 1 g l^{-1} MS-222 and their intestines removed and divided into proximal, mid and distal segments of equal length (German *et al.*, 2015; Horn *et al.*, 2006). Gut contents (eggs) were squeezed from each of the three segments, placed in individual 1.5 ml vials, frozen in liquid nitrogen and transported to UCI where they were stored at -80°C until counted (within 1 month). The eggs were thawed and then counted under a dissection microscope at $\times 10$ magnification. An additional eight fish from each of the two tanks were collected 90 min post-feeding for use in assays of egg nutrient concentrations and digestive enzyme activities. Their guts were similarly divided into three sections and the eggs and gut tissues were frozen separately (German *et al.*, 2015).

2.4.2 | Sample preparation for biochemical assays

The eggs and gut tissue samples designated for analyses of egg nutrient concentrations and digestive enzyme activities were stored at

–80°C until homogenized (within 1 month). When homogenized, the eggs or tissues from each gut region of each fish were individually homogenized in ice-cold 25 mM Tris-HCl pH 7.5 using a Polytron homogenizer (Brinkman Instruments; www.brinkmann.com). Samples were then centrifuged at 9400g for 2 min at 4°C. The supernatant was recovered and stored in 100–200 µl aliquots at –80°C until used in assays (German *et al.*, 2015). Egg samples destined for lipid concentration assays were homogenized with bile salts (5.8 mM sodium cholate) used as an emulsifier.

2.4.3 | Nutrient content and digestive enzyme assays

Homogenates of the eggs were used to determine egg nutrient concentrations (proteins, lipids and carbohydrates). Protein content of the eggs was measured using the bicinchoninic acid assay (Smith *et al.*, 1985). Lipid content was measured using the charring method outlined by Marsh and Weinstein (1966), following the solvent extraction method of Bligh and Dyer (1959). The total soluble carbohydrate content of the eggs was measured using the method developed by DuBois *et al.* (1956) as described by Pafilis *et al.* (2007). The activity of four digestive enzymes (amylase, lipase, trypsin and aminopeptidase) was measured at 17°C using the gut-tissue homogenates from the 90 min post-feeding samples, following the methods of German *et al.* (2004), Horn *et al.* (2006) and German *et al.* (2015).

2.5 | Statistical analysis

Prior to all parametric tests, a Bartlett's or Levene's test for homogeneity of variance was performed on the data and a Shapiro-Wilk test of normality was performed on the residuals. Where necessary, data were log transformed before analysis. All tests were run using R Studio 3.2.1 (www.r-project.org). Mean crushing force measurements among uncannibalized eggs, both unfertilized and fertilized, and cannibalized eggs were compared with ANCOVA (with collection date as a covariate), followed by Tukey's honest significant difference (HSD) test. Egg protein and carbohydrate contents were compared among the intestinal regions using repeated measures ANOVA and Tukey's HSD test. The lipid content data were not normally distributed and had unequal variances and so a Kruskal-Wallis non-parametric test was conducted to compare means. Using data from Horn *et al.* (2006), a sample size of $n = 8$ was deemed appropriate for comparisons of digestive enzyme activity among gut regions using a power calculator (HyLown Consulting LLC, 2013). Using luminal nutrient concentration data from German (2009), egg nutrient concentration sample sizes of $n = 4$ were sufficient according to the power analysis. For egg crushing force, data from Davenport *et al.* (1986) suggested that sample sizes of six were appropriate to achieve sufficient power ($1 - \beta = 0.9$, $\alpha = 0.05$).

3 | RESULTS

3.1 | Egg counts in *L. tenuis* guts

The numbers of eggs found in the guts of *L. tenuis* at the four spawning sites are summarized in Table 1. More than 80% of the fish collected at three of the sites (Belmont Shore, Cabrillo Beach and Malibu Surfrider) had eggs in their guts and > 50% at the fourth site (Long Beach Pier). Mean egg number per fish (zeroes included) ranged from almost five at Long Beach Pier to nearly 18 at Malibu Surfrider. Long Beach Pier fish also had the lowest median number of eggs and Malibu Surfrider the highest. Also, more than 80% of the fish collected at the spawning sites were male. The proportions of females that cannibalized eggs (33–67%) were lower than those of male cannibals (63–92%), but the small sample sizes of females ($n = 3$ –5) did not warrant statistical comparison. Numbers of fish with cannibalized eggs were not compared statistically among the sites because only one sample was collected at each site.

3.2 | Egg-crushing experiment

Mean forces required to crush eggs differed significantly by egg status and with collection date (egg status: $F_{2,107} = 60.04$, $p < 0.001$; collection date: $F_{1,102} = 4.10$, $p < 0.05$), but the interaction term of egg status \times collection date was not significant ($p > 0.05$). For both collection dates, less force was required to crush eggs recovered from *L. tenuis* guts compared with fertilized and unfertilized eggs that had not been consumed ($p < 0.001$; Figure 3), indicating that the chorions were weaker after ingestion. Crushing force did not differ significantly between fertilized and unfertilized eggs (Figure 3).

3.3 | In vivo digestibility experiment

Post-feeding, cannibalized eggs moved through the *L. tenuis* intestines, decreased in number (Figure 4) and became visibly degraded (Figure 5). Protein content of ingested eggs decreased ($F_{2,14} = 12.20$, $p < 0.001$) as the eggs moved from the proximal to distal region of the intestines (Figure 6(a)). Trypsin, the digestive enzyme that breaks down large proteins and polypeptides into smaller dipeptides and oligopeptides, followed a similar, decreasing trend moving down the gut ($F_{2,30} = 7.83$, $p < 0.01$; Figure 6(b)). Aminopeptidase, the digestive enzyme that breaks down dipeptides and oligopeptides to produce absorbable amino acids, followed the opposite activity pattern, increasing ($F_{2,25} = 49.89$, $p < 0.001$) distally along the gut (Figure 6(b)). Although eggs in the mid region contained significantly lower carbohydrate content than those in the proximal

TABLE 1 Data summary of conspecific eggs found in the guts of *Leuresthes tenuis* collected at the four sites in southern California, USA

Spawning site sample size (n)	Fish with eggs in gut (%)	Number eggs per fish (mean \pm s.d.)	Number eggs per fish (median)	Number of males	Males with eggs in gut (%)	Number of females	Females with eggs in gut (%)
Belmont Shore 31	87.1	7.9 \pm 7.2	7	28	89.3	3	66.7
Cabrillo Beach 53 ^a	86.8	10.0 \pm 12.9	6	–	–	–	–
Long Beach Pier 30	56.7	4.8 \pm 7.8	1	27	63.3	3	33.3
Malibu Surfrider 31	80.6	17.9 \pm 20.0	10	26	92.3	5	40.0

^a Sex of fish collected from Cabrillo Beach was not determined.

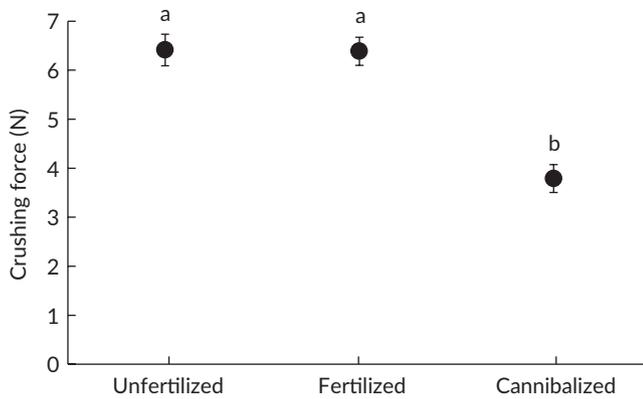


FIGURE 3 Force (mean \pm s.e.) required to crush unfertilized ($n = 20$) and fertilized ($n = 50$) uncannibalized eggs, and cannibalized ($n = 38$) eggs recovered from intestines of *Leuresthes tenuis*. Different lower-case letters indicate significant differences ($p < 0.05$)

region ($F_{2,12} = 6.94$, $p < 0.01$; Figure 6(c)), carbohydrate content in the eggs of the distal region was not lower than that in the proximal region (Figure 6(c)). Amylase activity was significantly lower in the

distal region compared to the mid region, but not the proximal region ($F_{2,20} = 6.02$, $p < 0.01$; Figure 6(d)). Lipid content of the eggs was lower in the distal region than in the proximal and mid regions (Figure 6(e)) and, similarly, lipase activity decreased distally along the gut; the decline, however, was not statistically significant ($F_{2,28} = 1.36$, $p > 0.05$; Figure 6(f)).

4 | DISCUSSION

This study showed that *L. tenuis* eats and digests conspecific eggs. The first hypothesis was supported in that the fish regularly but variably had conspecific eggs in their digestive tracts at multiple spawning sites in southern California over a two-year period. In support of the second hypothesis, eggs taken from the digestive tract were more easily crushed (i.e. they had weaker chorions) than uncannibalized eggs, whether the latter were fertilized or unfertilized. The third hypothesis was also supported in that eggs fed to *L. tenuis* in the laboratory showed clear signs of being digested.

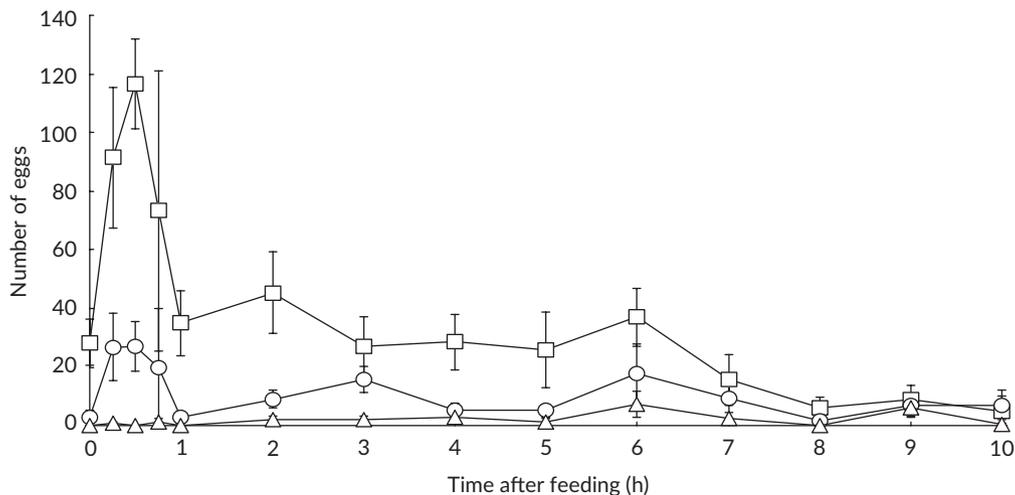


FIGURE 4 Number (mean \pm s.e.) of ingested *Leuresthes tenuis* eggs recovered from proximal (□), mid (○), and distal (△) regions of *L. tenuis* intestines from immediately post-feeding up to 10 h post-feeding

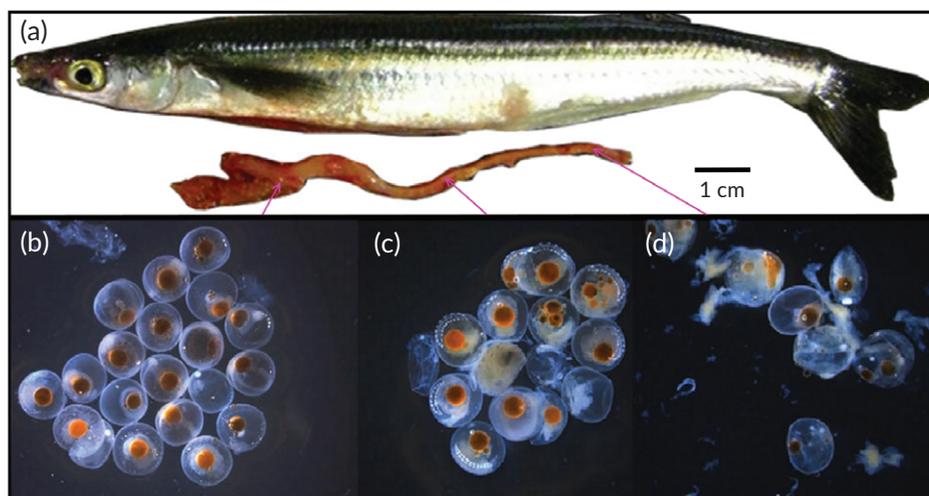


FIGURE 5 (a) *Leuresthes tenuis* with a dissected gut full of conspecific eggs and the qualitative degradation and presumed digestion of the eggs extracted from (b) the proximal, (c) mid and (d) distal regions of the intestine

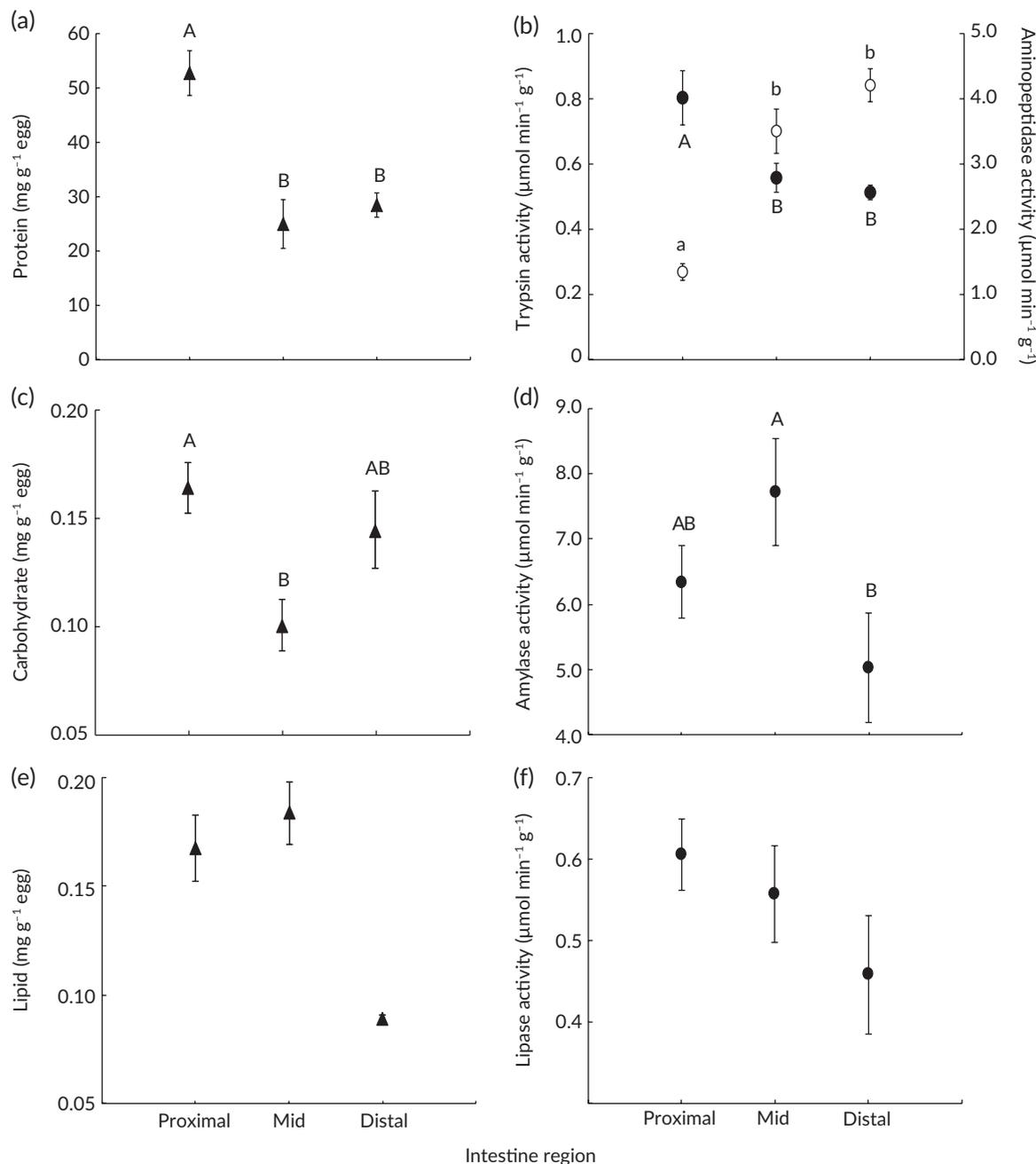


FIGURE 6 Nutrient concentrations of ingested eggs (▲) and corresponding enzyme activity (mean \pm s.e.) of: (a) protein and (b) trypsin (●) and aminopeptidase (○); (c) carbohydrate and (d) amylase; (e) lipid and (f) lipase recovered from the proximal, mid, and distal intestinal regions. Different letters indicate significant differences ($p < 0.05$) [in (b) capital letters apply to trypsin and lower-case letters to aminopeptidase]

The wide variation in proportion of *L. tenuis* with eggs in the gut may signal the opportunistic nature of egg ingestion based on several factors. Differences in the number of cannibalized eggs among the sites probably depend on the time of collection at each site and the size of the spawning runs. Larger runs should result in more eggs laid, which ought to allow more eggs to be consumed by each fish. Although data on run sizes were not available, Malibu Surf rider may have experienced a larger run and Long Beach Pier a smaller run, on the dates fish were collected. The sex ratio of spawning *L. tenuis* is known to be biased towards males (Thompson, 1919; Walker, 1949; the present study) and, in filial cannibals at least, males can recover more of the energy expended in reproduction than females (Manica,

2002). This bias contributes to the variation in number of cannibalized eggs because males had higher proportions of eggs in the gut.

Leuresthes tenuis is probably a non-kin cannibal, because eggs in the gut are probably unrelated to the cannibal (Dominey & Blumer, 1984; Smith & Reay, 1991). In support of this claim, *L. tenuis* has a promiscuous mating system with multiple paternity (Byrne & Avise, 2009) and is a non-guarder in terms of parental care (Balon, 1975). A few other beach-spawning fish species consume conspecific eggs (Martin, 2015), including the Atlantic silverside *Menidia menidia* (L. 1766) (Middaugh, 1981) and the Pacific herring *Clupea pallasii* Valenciennes 1847 (Hourston & Haegle, 1980). However, a search of the literature (Web of Science, 2018) revealed that *L. tenuis* is the only

beach-spawning fish that has been shown to cannibalize eggs and digest them.

Cannibalism by fishes in the wild remains poorly understood, seldom reported and is perhaps rare in nature (Eigaard *et al.*, 2014; Pereira *et al.*, 2017). Even though eggs in the developmental stage are most often cannibalized, ingestion of conspecific eggs may be frequently overlooked because they can be difficult to identify and are digested faster than larval fish (Pereira *et al.*, 2017). Expanded focus on fish species in the wild that show cannibalism in captivity might increase its perceived occurrence and importance in nature.

Both the egg crushing and feeding experiments indicate that *L. tenuis* can digest cannibalized eggs. During ingestion, the villiform pharyngeal teeth of *L. tenuis* (pers. obs.) may have punctured or otherwise physically weakened the egg chorions to initiate breakdown by the digestive enzymes, but no data were available to test this possibility. Changes in the biochemical composition of the ingested eggs and the digestive enzyme profile of the gut showed that the fish breaks down egg proteins to absorbable units (*i.e.* dipeptides and amino acids; Clements & Raubenheimer, 2006). Cannibalized eggs, therefore, almost certainly provide a nutritional boost to *L. tenuis*. In this species, the spawning act is arduous and even repeated by individual fish during a season (Walker, 1949). The fish eats mainly mysids offshore (Higgins & Horn, 2014), but spawning fish are often found with empty guts (Horn *et al.*, 2006) or with eggs in their digestive tracts (this study). Future studies could show how much this opportunistic source of nutrition contributes to the metabolism of *L. tenuis*, including a comparison of energy replenishment in spawning males and females.

ACKNOWLEDGEMENTS

The authors thank A. Fredell for allowing the dissection of digestive tracts from the *L. tenuis* he had collected from spawning sites in southern California for an otolith study; the GEAR UP program at CSUF for providing supplies that facilitated dissecting and counting conspecific eggs in *L. tenuis* digestive tracts; K. Darrow and her staff for allowing the egg digestibility experiments to be conducted at Cabrillo Marine Aquarium; the Division of Academic Affairs at CSUF for supporting the crushing force measurements; A. Lappin, California State Polytechnic University, Pomona, for advice on techniques to measure crushing force; S. Huynh, CSUF, for aiding in the measurement of nutrient concentrations of cannibalized eggs and digestive enzyme activities of *L. tenuis* guts; H. Aryafar, CSUF, for photographs (Figure 1 (a)–(c)). Any opinion, findings and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

Authors' contributions

A. J. Santos and A. R. Frederick made an equal contribution to this work.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Santos AJ, Frederick AR, Higgins BA, et al. The beach-spawning California grunion *Leuresthes tenuis* eats and digests conspecific eggs. *J Fish Biol.* 2018;93: 282–289. <https://doi.org/10.1111/jfb.13734>