SYMPOSIUM

Taking the Plunge: California Grunion Embryos Emerge Rapidly with Environmentally Cued Hatching

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Synopsis The process of hatching has been well studied in some model species of teleosts: the medaka Oryzias latipes, the mummichog Fundulus heteroclitus, and the zebrafish Danio rerio. These models are compared to the California Grunion, Leuresthes tenuis that has some unique features of reproduction related to tidal synchrony of spawning and environmentally cued hatching (ECH). During oviposition at spring tides, this marine teleost spawns out of water to bury its clutches on sandy beaches in the high intertidal zone. After embryos of L. tenuis reach hatching competence, hatching can be triggered at any time. Incubation above the water line inhibits hatching until ECH is triggered by rising tides during the following lunar phase, and hatching occurs within a few seconds. We review the embryo’s response to environmental cues at hatching and the effects of the surrounding medium on the chorionase and chorion for this form of ECH. Leuresthes tenuis shares some similarities as well as some important differences with the model species. Comparison of hatching across teleostean taxa indicates great variability in stage at hatching and in duration of incubation that suggest hatching plasticity in response to environmental cues may be more widespread than currently appreciated.

Introduction The process of hatching from an egg-encased embryo to a free-swimming larva is an important life history switch point that may be initiated by a genetic program or by the embryo’s response to some environmental cue (Warkentin 2011). In this article we provide comparisons in the physiology of hatching between model species of teleost fish that hatch according to a developmental timetable, and our study organism, a marine teleost with environmentally cued hatching (ECH).

The spawning behavior of California Grunion, Leuresthes tenuis, was first described (Hubbs 1916) in a slightly skeptical repeat of an old fisherman’s tale of spectacular spawning runs, with thousands of fish emerging from water to spawn on sandy beaches under a full moon. A few years later, the first scientific study of reproduction in L. tenuis (Thompson and Thompson 1919) described some of the unique features of this species. Its early life history is equally spectacular, involving tidal synchrony of ECH and an incubation period of indeterminate duration that can last from one to five weeks. Tides are rhythmic environmental cues that synchronize many aspects of reproduction in marine organisms. In L. tenuis, both the spawning runs and subsequent hatching are cued by the highest tides of the lunar cycle (Walker 1952). Spawning occurs as the tides fall within a couple hours after the highest spring tides of full or new moons. Adult fish emerge from receding waves and crawl on the sand for a few minutes. Females bury themselves tail first into the soft sand and lay clutches of 1000–3000 eggs that are fertilized by milt from males curled around them on the sand surface (Walker 1952).

Eggs incubate terrestrially, buried in coarse sand. Burying the clutch of eggs under sand on shore is a form of parental care (Martin et al. 2004). Above the
water line the buried embryos have increased availability of oxygen and reliable moisture; increased temperatures may also enhance rate of development (Martin and Strathmann 1999). Protective as this microhabitat is for the egg-encased embryos, the damp sand would be deadly to any tiny, transparent hatchling that emerged and tried to swim or use its gills. Thus, responding to an environmental cue to hatch is adaptive.

Occasionally clutches of eggs from two different spawning periods can be seen side by side in the sand (Martin et al. 2011), indicating an extended incubation for the older clutch. The difference in ages can be identified easily by changes in the amount of yolk and in the amount of pigmentation of the embryo’s melanophores (Moravek and Martin 2011). Embryos attain hatching competence after a set period of time, but do not hatch unless and until the cue is presented (Smyder and Martin 2002). Thus, some individuals from one date of fertilization can hatch and continue development as larvae, while others of the same cohort remain as egg-encased embryos.

The duration of the last embryonic stage can be extended as long as hatching is delayed (Moravek and Martin 2011), an example of heterokary (Spicer and Burggren 2003) that reflects plasticity in the extension of the final embryonic stage and in the initiation of the larval phase. This form of extended incubation is very different from fish embryos extending incubation by undergoing diapause (Podrabsky et al. 2010). Embryonic L. tenuis must maintain awareness in preparation for the cue that may appear at any time, and disappear just as suddenly (Darken et al. 1998). An embryo has a limited energy supply and no mobility, yet the cue for hatching is not totally predictable in this environment. These embryos reach hatching competence in ~8–10 days, but can remain unhatched within the chorion, ready and waiting, for up to 36 days post-fertilization (Martin 1999; Smyder and Martin 2002; Moravek and Martin 2011).

**ECH in L. tenuis**

Hatching typically occurs before the highest tides of the new or full moon, a few days in advance of the next spawning run (Walker 1952). As the water rises, the egg-encased embryos wash free of the sand to hatch as they tumble out to sea (Griem and Martin 2000; Speer-Blank and Martin 2004), less than a minute after the cue arrives. In the natural environment of coastal California, one wave follows another every 15 s or so. In this precarious habitat, conditions for hatching are transitory, and if embryos do not respond to the cue quickly, another 10 days could pass until conditions are right again. Eggs are demersal and settle on the substrate if they do not hatch when first washed out. If buried by sand under water, they die from lack of oxygen.

Thus, although the embryos of L. tenuis may wait an indefinite and unpredictable amount of time for the environmental cue, once it presents, they hatch nearly instantaneously. Most other fish take more than 30–60 min to initiate hatching (Yamagami 1988). Even hatchlings of Fundulus heteroclitus, a teleost with ECH, emerge no earlier than half an hour after receiving the cue for hatching (DiMichele and Taylor 1981).

Hatching for egg-laying teleostan fishes requires a two-stage process, first enzymatic and then mechanical (Yamagami 1988, 1996). First, hatching enzymes or chorionases are secreted from hatching glands, softening and swelling the inner membrane. Second, after the inner membrane dissolves, the intact but thin outer membrane can be mechanically ruptured by active movements of the embryo’s tail. If either of these processes is inhibited, hatching cannot occur (Yamagami 1988).

During hatching in L. tenuis the chorion weakens and deforms slightly from its previous spherical shape. A small amount of perivitelline fluid can be seen escaping from a small opening in the egg membrane (Speer-Blank and Martin 2004). The embryonic L. tenuis twists and turns within the membrane, then lashes against the chorion with its tail and emerges tail-first. Occasionally an embryo hatches incompletely and swims about with the head still trapped within the chorion.

**Comparisons with model fish species**

To understand the differences and similarities between ECH in L. tenuis and hatching in other fish, we compare L. tenuis to two model teleost species. The mechanism of hatching has been well studied in teleost fishes in two model species: the freshwater medaka or ricefish Oryzias latipes, and the estuarine killifish, the mummichog Fundulus heteroclitus. L. tenuis is typically marine but individuals or populations may spend some of the life cycle in estuaries, harbors, or bays (Allen et al. 2002; Roberts et al. 2007; Johnson et al. 2009). The speed of hatching and the small opening on the chorionic membrane before
emergence indicate *L. tenuis* may be using a different mechanism to escape from the chorion than do other teleosts.

These three species are in different Orders within the same Series, Atherinomorpha. The embryonic development of *L. tenuis*, *O. latipes*, and *F. heteroclitus* are all similar; all hatch at a late stage of development and are able to swim and feed immediately (Martin et al. 2009). All three species have eggs of similar size and reach hatching competence at almost the same time, with some differences in the later stages of development.

Spawning in *F. heteroclitus* takes place in estuaries and bays of the Atlantic coast of North America at semilunar high tides (Taylor 1999). Early development follows the same sequence of stages (Armstrong and Child 1965) as those of *O. latipes* and *L. tenuis* (Martin et al. 2009). However, at the same temperature *F. heteroclitus* takes substantially longer than *L. tenuis* to complete early stages of cleavage, gastrulation and neurulation (Martin et al. 2009). Although both *L. tenuis* and *F. heteroclitus* synchronize spawning and hatching to the tides, *F. heteroclitus* at 20°C requires 228 h (9.5 days) to reach hatching competence. At the same temperature *L. tenuis* from the outer coast needed only 178 h (7.5 days), but *L. tenuis* resident in San Francisco Bay took 224 h, or 9.3 days, nearly the same as *F. heteroclitus* (Martin et al. 2009). Clutches of *F. heteroclitus* are exposed to air intermittently during daily low tides rather than constantly as is the case in clutches of *L. tenuis*.

The early development of *O. latipes* takes approximately the same amount of time but spawning and development are not synchronized with tides because they live in freshwater (Iwamatsu 2004).

**Teleostean chorion membranes**

Two chorionic membranes surround teleostean eggs, a thin outer layer and a thicker inner layer made of pseudokeratins and polymers of proline, glutamate, and glutamine (Yamagami 1996; Kanamori et al. 2003; Sano et al. 2008). After fertilization, cross-linkages between protein polymers “harden” the chorion and protect the embryo within (Yamagami 1988). This protects the egg from abrasion and prevents entry of many environmental chemicals and microbes. Still, the embryo is vulnerable to physical conditions such as desiccation (Middaugh et al. 1983; Taigen et al. 1984; Podrabsky et al. 2010); changes in salinity (Matsumoto and Martin 2008); UV irradiation (Blaustein et al. 1994; Saito and Taguchi 2002); crushing (Martin et al. 2006); pollutants (von Westerhagen 1988); disease (Wedekind 2002); and predators (Warkentin and Caldwell 2009). Demersal eggs, such as those of *L. tenuis*, tend to have thicker and more resilient envelopes than do those of pelagic fish (Miller and Kendall 2009).

**Chorionase is conserved**

Protective as the chorion is, it prevents the escape of the embryo. Chorionases are enzymes that allow escape from the chorion during hatching and are conserved across species of Euteleosts (Yasumasa et al. 1989, 2010; Kawaguchi et al. 2005, 2010; Sano et al. 2008). Called high choriolytic enzyme (HCE) and low choriolytic enzyme (LCE), for high and low activity respectively, the enzymes from the same protein family complement each other’s actions (Yamagami 1988; Kawaguchi et al. 2010) by cleaving different protein sequences (Yasumasa et al. 2010). In *O. latipes*, HCE swells and softens the inner chorionic membrane, while LCE dissolves it. Together they completely digest the inner chorionic membrane within 1 h. The inner layer thins evenly in most species, but the outer chorion remains intact (Yamagami et al. 1992). Fractions of the solubilized glycoproteins of the inner chorion are large polypeptides, not free amino acids, indicating the enzymes break cross-linkages without hydrolyzing proteins (Yamagami 1996; Yasumasa et al. 2010).

All known fish chorionases are highly conserved metalloproteins in the astacin family (Hiroi et al. 2004; Kawaguchi et al. 2007, 2010). The chorionase in *L. tenuis* appears to fit this pattern. We aligned conserved sequences from *O. latipes* (HCE and LCE), the crayfish *Astacus*, the frog *Xenopus*, and the carp, and developed primers to isolate an ~260 bp cDNA from egg-encased embryos using RT–PCR that appears to code for chorionase in *L. tenuis*.

Purified extract of the fluid in which *L. tenuis* was hatched shows proteolytic activity on HPA *in vitro*, this is blocked by addition of 2 mM EDTA, indicating a metalloprotease. EDTA in seawater also blocks hatching in *L. tenuis* *in vivo*. Working with different EDTA concentrations we found that a concentration of 2 mM in seawater is fatal to embryos, but that effective inhibition is possible at EDTA concentration of 0.2 mM, reversible after 20 min recovery in plain seawater.

**Hatching glands secrete chorionase**

Hatching glands are large single cells that secrete chorionase by exocytosis and disappear after
hatching (Yamagami 1988). In several species these are mesendodermal in origin from the embryonic shield (Inohaya et al. 1999). Hatching glands are found in and around the head and anterior body in many species of fish, and may also occur on the yolk sac of zebrafish (Willemse and Denue 1973; Ishida 1985). In *O. latipes* hatching glands only occur in the inner pharynx (Inohaya et al. 1999), while *F. heteroclitus* has hatching glands on the pharynx, operculum (DiMichele and Taylor 1981), and periphery of the mouth (Kawaguchi et al. 2005). Digestion of the chorion takes time, and hatching typically occurs 30–60 min after chorionase is secreted (Yamagami 1988).

Hatching glands on *L. tenuis* are present along the lateral surface of the body below the lateral melanophores (Fig. 1), as in some other fish (Ishida 1985). These glands are visible on embryos dechorionated artificially before hatching, but absent in hatchlings that hatch naturally (Moravek and Martin 2011). The position of these glands may help spread the chorionase when the embryo actively tries to escape from the chorion (Yamagami 1988). The position of the hatching glands on grunion is appropriate considering the fish hatch tail first. In other marine teleosts, embryos from larger eggs tend to emerge tail first, while embryos from smaller eggs emerge head first (Miller and Kendall 2009).

**Escape behavior**

After the chorion is weakened, muscular effort is necessary for hatching to succeed, as the second of the two-stage hatching process (Yamagami 1988). This activity requires increased energy, resulting in increased metabolic rate in *L. tenuis* during hatching (Fig. 2). Larval *L. tenuis* have a higher rate of oxygen consumption than do embryos, but of course they are now actively swimming. Addition of 0.33 mM of the anaesthetic MS-222 to seawater temporarily paralyzes *L. tenuis* embryos and reversibly inhibits hatching. After 3.5 h of recovery in plain seawater, hatching can be cued successfully by agitation.

**Mechanisms: oxygen levels cue environmental hatching in some species but not *L. tenuis***

One of the most important environmental cues for hatching for teleost fishes is hypoxia, known to trigger hatching in *F. heteroclitus* (DiMichele and Taylor 1981); Atlantic salmon *Salmo salar* (Oppen-Berntsen et al. 1990); *O. latipes* (Yamagami 1988); pike *Esox lucius* (Gulidov 1969); bream *Aramis brama* (Gulidov and Popova 1977); and whitefish, *Coregonus lavaretus* and *C. albula* (Czerkies et al. 2001). Hypoxia upon return to water is the cue for hatching also in some
terrestrially incubating amphibians (Petranka et al. 1982; Seymour and Bradford 1995). Eggs of *F. heteroclitus* hatch in still water as the tides fill the estuary, and pond-dwelling *O. latipes* also hatch in quiet, still water.

When the stimulus to hatch is hypoxia, hatching is preceded by increased respiratory movements that apparently cause hatching glands near the head and operculum to secrete their enzymes (DiMichele and Taylor 1981; Yamagami 1988). For these species, hatching is inhibited by agitating the water to aerate it, or by creating hyperoxic conditions (Gulidov 1969).

In contrast, for *L. tenuis* hatching occurs in waves that are filled with air bubbles. Hatching occurs in agitated, hyperoxic water (Griem and Martin 2000), the same conditions that inhibit hatching in other teleosts (Table 1). For *L. tenuis*, hypoxia inhibits hatching slightly, rather than stimulating it, (Griem and Martin 2000), and severe aquatic hypoxia results in death without hatching.

**Mechanisms: environmental cue of water movement and shear stress**

In some cases movement of water over hatching glands may stimulate them to release their contents. A stream of water directly onto the pharynx of embryonic *O. latipes* initiates hatching, apparently by stimulating secretion by the hatching glands there (Yamagami 1988). Conversely, agitated water on the external surface of the chorion typically inhibits hatching for *F. heteroclitus*, possibly because this aerates the egg-encased embryos. When shaking stops, boundary layers form and hypoxia develops, respiratory movements deepen, chorionase is secreted (DiMichele and Taylor 1981), and eggs hatch within 30–60 min.

In contrast, if embryos of *L. tenuis* are held in still water, they do not hatch (Hubbs 1965; Griem and Martin 2000). Terrestrial incubation is not required; the embryos developed normally if incubated in water rather than their usual terrestrial habitat (David 1939). In water, mature eggs can remain viable without hatching for days if the water is still, hatching only when the water is agitated. We have confirmed this observation many times. However, the slightest movement may trigger hatching in a mature egg under these conditions, and the motion of a swimming hatchling is enough to agitate the remaining eggs into hatching soon thereafter.

One hypothesis for *L. tenuis* is that the cue for hatching involves the sensation of water movement and shear on the surface of the chorion. Shear stress from moving water can cause damage or death to fish eggs over the long term (Morgan et al. 1976).

![Fig. 2](https://academic.oup.com/icb/article-abstract/51/1/26/639598) Metabolic rate was measured as instantaneous oxygen consumption before, during, and after hatching for *Leuresthes tenuis*. Individual eggs (*n = 23*) were measured using Diamond General Clark-type microelectrodes in seawater with a flow-through system.

**Table 1 Environmental cues influence hatching differently in these three species of teleosts**

<table>
<thead>
<tr>
<th>Condition</th>
<th><em>L. tenuis</em></th>
<th><em>F. heteroclitus</em></th>
<th><em>O. latipes</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Air Emergence</td>
<td>Inhibits</td>
<td>Inhibits</td>
<td>Inhibits</td>
</tr>
<tr>
<td>Hyperoxic water</td>
<td>Permissive</td>
<td>Inhibits</td>
<td>Inhibits</td>
</tr>
<tr>
<td>Still H₂O, low oxygen</td>
<td>Permissive</td>
<td>Stimulates</td>
<td>Stimulates</td>
</tr>
<tr>
<td>Still H₂O, Normal oxygen</td>
<td>Permissive</td>
<td>Inhibits</td>
<td>Inhibits</td>
</tr>
<tr>
<td>Agitated H₂O, high oxygen</td>
<td>Stimulates</td>
<td>Inhibits</td>
<td>Inhibits</td>
</tr>
<tr>
<td>Agitated, Hypoxic water</td>
<td>Stimulates</td>
<td>Stimulates</td>
<td>Stimulates</td>
</tr>
<tr>
<td>Hatching glands</td>
<td>Lateral surface of body</td>
<td>Pharyngeal cavity and periphery of mouth</td>
<td>Pharyngeal cavity and periphery of mouth</td>
</tr>
<tr>
<td>Speed of hatch</td>
<td>0.5–5 min</td>
<td>30–60 min</td>
<td>30–60 min</td>
</tr>
<tr>
<td>MS-222</td>
<td>Inhibits</td>
<td>Inhibits</td>
<td>Inhibits</td>
</tr>
</tbody>
</table>

We developed an apparatus that allowed us to agitate water around an egg, without the egg itself moving. Individual hatching-competent egg-encased embryos were positioned by mild suction at the tip of a glass Pasteur pipette, held steady on a ring stand. Each egg was fully immersed in seawater in a glass chamber built for microelectrode respirometry (Diamond General). At the bottom of the chamber a tiny magnetic stir bar spins in response to a motorized electromagnet, ~0.5 cm below the egg’s stationary position. A total of 32 egg-encased embryos were held in place while water swirled around for two to five min. Three embryos hatched during this agitation, perhaps from the effects of initial handling. The motor was then turned off, and the suction released so the eggs dropped from the pipette to settle on the bottom of the chamber. No additional eggs hatched then, nor did any for the following 10 min of immersion in still water. At this point the water was spun again for 30 s, and most of the remaining eggs hatched within the next 2 min (Fig. 3). Control eggs placed individually in the chamber without suction onto a pipette in still water did not hatch for 15 min until after the motor spun them for 30 s, then 90% hatched. This indicates that shear stress on the chorion is not sufficient to cue hatching in L. tenuis.

When hatching-competent L. tenuis embryos are placed in different compositions of artificial seawater, the ionic composition of the seawater influences hatching success after agitation (Fig. 4B). Simple NaCl is not significantly different from seawater as a medium for hatching, nor is saltwater made solely with CaCl₂. Agitation in water containing either solely KCl or solely NaHCO₃ triggers hatching in L. tenuis but in both of these artificial media, the hatchlings immediately die, even if removed the instant they hatch.

Developmental stage at hatching
Hatching glands develop during maturation of fish embryos. Attainment of a specific developmental stage is necessary, but not sufficient, to initiate hatching. For all fish, not just those known to respond to environmental cues, “some triggering stimuli, extrinsic or intrinsic” (Yamagami 1988, p. 480) is necessary to induce secretion of hatching enzyme. In many species of fish, hatching within one clutch may take place over a period of hours or days. For example, the stages in Danio rerio have been well described, and hatching may happen at any time within any of three different developmental stages (Kimmel et al. 1995). Hatching occurs across a broad interval between 48 and 72 h post-fertilization;
thus the duration of incubation for the last hatchlings is half again longer than that for the first hatchlings. During this time the hatchlings and embryos continue to progress through the same developmental stages at the same time. Even if they hatch at 48 h, they do not begin feeding until 72 h.

By contrast, *L. tenuis* begin actively moving their eyes and pectoral fins nearly two days before they are ready to hatch, and are actively opening and closing their jaws well before hatching. Hatchling *L. tenuis* swim constantly and are ready to feed (Martin et al. 2009).

*Gadus morhua* hatch nearly synchronously, but the stage at hatching is affected by temperature (Jordaen et al. 2006) and may not be the same even within one clutch (Hall et al. 2004). For different species, different stage at hatching shows heterochrony. Time of hatching in whitefish is not connected to a particular developmental stage but occurs asynchronously (Luczynski and Kolman 1987).

Not only does hatching occur at different stages within many individual species, but attempts to standardize staging series across species are complicated because characters that define a stage in one species may be scattered across several stages in another species (Haynes 1995; Shardo 1995). Unlike the situation in amphibians, universal schemes for fishes are problematic (Richardson et al. 1997; Martinez and Bolker 2003). Comparisons of morphological landmarks must take into consideration the phylogenetic history of each species and the constraints of the habitats in which they spawn and incubate (Parenti 1981), as well as of temperature and the behavior of the embryos. Phylogenetic and adaptive significance of these developmental differences are unknown, indicating the difficulty of comparing species of fish with different body types, habitats, stages of development at hatching, and life-history strategies (Metscher and Ahlberg 1999).

**Other possible environmental cues: temperature, light**

Hatching of halibut *Hippoglossus hippoglossus* is blocked by light, and can be synchronized by darkness that occurs between 14 and 16 dpf (Helvik and Walther 1993). However, if hatching is blocked too long past this time, it will not occur at all. In *D. rerio* and *O. latipes*, more hatching occurs during the light part of the circadian cycle than in darkness (Yamagami 1988). Light is not a cue for hatching in *L. tenuis* and does not seem to affect incubation success (but see Hubbs 1965).

**Electrical stimulation**

Electrical stimulation applied to eggs can trigger premature hatching in some species (Luczynski and Kolman 1987; Yamagami 1988). In rainbow trout, this takes an hour and repeated stimulation; in *O. latipes*, one dose is sufficient to initiate secretion of chorionase within 5 min and hatching takes place after an hour. This cue has not been tested in *L. tenuis*.

**Inhibition of hatching: emergence into air**

Hatching is inhibited during incubation in air for many species of fish that spawn on beaches (Martin et al. 2004), including *F. heteroclitus* (Taylor 1999); *L. tenuis* (Walker 1952); mudskippers *Periophthalmodon schlosseri* (Ishimatsu et al. 2009; Ishimatsu and Graham, 2011); and *Takifugu nigropunctatus*, the fugu puffer (Yamahira 1996). Emergence of eggs into air inhibits hatching also in *O. latipes* (Yamagami 1988), although eggs of this freshwater species are never naturally exposed to air. All of these species’ eggs must be submerged in water in order to hatch. It is not known whether this inhibition is caused by high oxygen or some other factor.
In *L. tenuis*, emergence into air inhibits hatching, but oxygen levels are not an environmental cue for hatching (Griem and Martin 2000).

What is the mechanism for inhibition of hatching in air? The mechanism whereby air inhibits hatching in air may be similar in all fish, and may be simply insufficient hydration of the chorion for chorionase to work. On the other hand, some anamniotic eggs do hatch out of water. For example, the capelin, *Mallotus villosus*, a smelt, spawns on gravel beaches during high wave events that do not necessarily occur during a high tide (Frank and Leggett 1981). Unlike *L. tenuis*, capelin broadcast their gametes onto the beach, where chorions attach individually onto gravel and pebbles.

Also unlike most species that incubate in air, *M. villosus* hatch on a developmental schedule, whether in water or not (Frank and Leggett 1981). Gravel beaches may trap tiny pockets of water, and the large interstitial spaces there than in sand make it less likely that embryos will be crushed. Still, hatchlings are unable to navigate territorially away from their site of hatching on the beach until washed out by waves high enough to release them (Frank and Leggett 1981). Waiting for this to happen, the stranded larvae lose condition, and few survive long in this inappropriate habitat. It would seem adaptive for this species to develop ECH with this type of beach spawning.

Some salamander eggs during incubation are left emerged in air by drying ponds, and do not hatch until immersed in water (Petranka et al. 1982; Gunzburger 2003). On the other hand, some frog species can hatch in air, including *Pseudophryne bibronii* (Geiser and Seymour 1989), foam-nesters (Shepard and Caldwell 2005), and red-eyed tree frogs (Warkentin 2007, 2011).

For *L. tenuis*, agitation in water appears to be necessary for hatching. To separate the effects of agitation from the presence of water, we placed 8–10 egg-encased embryos in a small amount of sand in a petri dish. The dish was vigorously shaken for 2 min. Other samples were placed in sand in petri dishes for 2 min and not shaken. Afterwards we carefully added seawater without further agitation. This water was left still for 5 min to observe any hatching; then the dishes were agitated to trigger hatching of any remaining embryos. For those that were shaken in air, 49% (*n* = 304) hatched in water before any further agitation, and only 7% (*n* = 308) of the unshaken control eggs hatched in the 5 min of still water, a significant difference (*χ^2^ = 793, df = 1, *P* < 0.001). After agitation in water, a total of 87% of the agitated and 84% of the control eggs hatched within 5 min, not significantly different (*χ^2^ = 1.39, df = 1, *P* > 0.05). Apparently agitation prepared the egg-encased embryos in some way that allowed them to quickly emerge from the chorion once placed into seawater.

### The ecological role of delayed hatching and extended incubation for *L. tenuis*

Extended incubation for clutches of *L. tenuis* occurs rarely in nature. The longer clutches incubate, the longer the exposure to terrestrial predators and infection; successful hatching decreases over time (Darken et al. 1998; Smyder and Martin 2002). Delayed hatching with extended incubation is relatively rare among teleosts (Martin 1999; Martin et al. 2004). The congener *L. sardina*, the Gulf Grunion, has a much smaller yolk and may be unable to delay hatching (Moffatt and Thomson 1978), although this should be re-evaluated. Among others in the Series Atherinomorpha, many Fundulidae can delay hatching to some extent until prompted by an environmental trigger (Greeley and MacGregor 1983; Greeley 1984; Greeley et al. 1986). Spawning on beaches has evolved multiple times independently in the Osmeridae and Atherinidae (Martin and Swiderski 2001), and it is likely that delayed hatching is more common than currently appreciated.

During extended incubation, there are tradeoffs between continued growth and development versus continued survival for an uncertain duration without new energy inputs (Bradford and Seymour 1985). After reaching hatching-competence, embryos of *L. tenuis* arrest development but maintain active metabolism until hatching occurs (Moravek and Martin 2011). The yolk becomes depleted over time (Darken et al. 1998). Embryos respond to light and movement, but there is very little growth within the chorion (Moravek and Martin 2011) and few morphological changes show the passage of time in the embryo. If cues for hatching are never perceived, the embryo dies without hatching (Martin 1999).

As soon as *L. tenuis* hatch, larval development begins (Martin et al. 2011; Moravek and Martin 2011). Thus, *L. tenuis* from a spawning cohort with the same fertilization date may contain individuals that are all the same chronological age, at different stages of larval development (Moravek and Martin 2011). Larvae grow at a similar rate, regardless of the date on which they hatch, and proceed through stages based on the date of hatching, an example of heterokary (Spicer and Burggren 2003). Survival is not affected by duration of incubation until its upper...
limits, when both hatching success and larval survival decline dramatically (Martin et al. 2011).

**Summary and future questions**

In a broad sense, developmental morphology and sequence of stages appear to be relatively stable across many species of fish, but the amount of time between the appearance of different features, and both the stage at hatching and duration of incubation are highly variable. It may be difficult to group embryonic stages of fishes into standardized periods but it seems useful for making comparisons across taxa, particularly to show differences in stage at time of hatching in relation to particular morphological milestones.

A summary of the major differences between hatching in *L. tenuis* and in well-studied model species is shown in Table 1, including speed of hatching and responses to environmental cues. Given the rapidity and form of escape from the chorion, future studies should discover whether these differences may be the result of differences in the chorionase enzyme, differences in the chorion, differences in behavioral or sensory processes, or differences in some other factors.

The chorionase extracts we have examined for *L. tenuis* so far seem to fit the usual teleost pattern of a metalloprotease in the astacin family. An important next step is to sequence the protein and cDNA for comparison with known chorionases.

The structure of the chorion of *L. tenuis* may be such that it responds differently to chorionase. There is not sufficient time for the entire chorion to respond to the enzyme in ECH for *L. tenuis*. We occasionally observe partially hatched larvae of *L. tenuis* with the chorion remaining around the head, indicating that the entire inner chorion does not become thin as it does in other teleosts.

We hypothesize there may be a particular region of the chorion of *L. tenuis* that responds to the enzyme, perhaps with a unique cross-linkage pattern that can be cleaved quickly. Many observations of ECH confirm that only one part of the chorion of *L. tenuis* expands and releases perivitelline fluid before hatching occurs (Speer-Blank and Martin 2004), indicating a weakening and some sort of break in the outer, as well as the inner, chorion membranes.

How mechanical stimulation is transduced into secretion of chorionase and into changes in the chorion are not known. Agitation in air does not cause hatching, and resting in still water also does not trigger hatching; however, agitation in seawater results in rapid hatching. We hypothesize that the embryo is actively sensing the environment and the suitability of initiating larval life, to initiate hatching at just the right moment via some deliberate, volitional behavior. The surrounding medium must also permit the hydration of the chorion so the activity of the chorionase can provide an opening for escape of the embryo.

Looking forward, it is intriguing that so many species of fish, including model species, show heterokairy, either in the timing of, or the stage at, hatching. A next step toward a better understanding of reproductive strategies and their phylogenetic patterns should compare developmental heterochrony among species and heterokairy within species, to explore how these affect reproductive and hatching plasticity in response to an imperfectly predictable, transient habitat for embryos.

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