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Development of European bitterling in the gills of freshwater mussels

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The development of bitterling embryos within the unique environment of a freshwater mussel's gills requires a departure from typical cyprinid embryological development. Eggs are large (2·6 × 1·7 mm), illustrating the low risk of predation, and elliptical; a response to unionid gill morphology and a way of increasing the transfer rates of respiratory and excretory products to and from the tissues. The yolk sac develops elongated lateral processes during early ontogeny; these secure the embryo into the host's interlamellar space. Once the larva is capable of movement (8·2 mm) the lateral processes are lost and the larva becomes less dependent of the host's gills for the provision of oxygen. Hatching (3·3 mm) and pigmentation of the blood (6·4 mm) occur relatively early; this may increase the rate of oxygen supply to the tissues. Pigmentation of the eyes and appearance of the melanophores occurs relatively late (7·4 mm and 7·9 mm, respectively); embryos are not required to detect or avoid predators. Bitterling larvae generally emerge from the host once the yolk sac has been consumed (10·5 mm); this may mark a change in respiratory and nutritional requirements.

Key words: *Rhodeus sericeus*; host choice; unionid mussel; development; egg size; egg shape.

INTRODUCTION

The European bitterling *Rhodeus sericeus,* Pallas 1776 is a small cyprinid fish with a remarkable life history. In early spring, males defend territories around freshwater mussels (Bivalvia: Unionidae). Responsive females are led to the mussel, where they deposit eggs from an extended ovipositor which is inserted into the mussel's exhalant siphon (Wiepkema, 1961; Heschl, 1989). The eggs are lodged, either singly or a few at a time, into the gill cavity of the mussel, after which the male bitterling ejects his sperm into the mussel's inhalant water current (Duyvene de Wit, 1955), such that fertilization takes place within the gills of the host. The bitterling's life history constitutes a complete role reversal of recognized symbiotic relationships: a vertebrate uses an invertebrate as its host, rather than vice versa.

Approximately 60 species of bitterling (sub-family Acheilognathinae) have been described, and all display a reproductive dependency on unionid mussels (Nelson, 1994). While laboratory studies have provided extensive knowledge of the behaviour and hormonal control of spawning in bitterling (Bretschneider & Duyvene de Wit, 1947; Bresse, 1950; Duyvene de Wit, 1955; Pickford & Atz, 1957; Wiepkema, 1961; Asahina *et al.*, 1983; Shimizu *et al.*, 1985; Shimizu &

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Hanyu, 1993), studies of natural populations are surprisingly few. Descriptions of the development of *R. sericeus* within the mussel are often contradictory, and are based mostly on anecdotal references, such as Hardy (1954). For example, Maitland (1977) states that eggs are deposited in the mantle cavity of the host, while Wheeler (1969) cites the gill chamber; Maitland & Campbell (1992) suggest that larvae leave mussels at a length of 9–10 mm, while Wheeler (1969) gives 20 mm; the time period that larvae remain in the host, which is likely to be related to developmental rates and therefore to be temperature dependent, has been quoted frequently as 3–4 weeks (e.g. Wheeler, 1969), but Maitland & Campbell (1992) cite 2–3 weeks, and Bresse (1950) 46 days.

This study attempts to clarify the development of bitterling within the host. Such information is important for a number of reasons. First, the bitterling–mussel relationship provides an excellent model for understanding the evolution of host specialization. The suitability of mussels as hosts to bitterling varies with regard to species (Reynolds *et al.*, 1997) as well as intraspecific differences such as mussel size and sex (Aldridge, 1997). An understanding of the microenvironment in which bitterling develop may reveal the factors important in affecting host quality. Second, the bitterling is an introduced species to Britain and much of the New World (Mayden, 1991) and therefore its impact on the natural ecosystem should be assessed. Third, the world's unionid fauna is declining at a dramatic rate as a result of pollution and human manipulation of natural watercourses (Bogan, 1993; Watters, 1996; White *et al.*, 1996). Similarly, bitterling are declining throughout much of Europe as a consequence of reduced host numbers. A number of Japanese bitterling species are on the IUCN Red List of threatened species (IUCN, 1996). Informed conservation measures can be undertaken only when the ecology of an animal is properly understood.

Developing within such an unusual environment is likely to require a departure from typical cyprinid embryological development. To understand how bitterling are able to exploit mussels, this study addressed the question: how have bitterling adapted their life history to suit this unique environment?

MATERIALS AND METHODS

STUDY SITES

Studies were focused at Wicken Lode, Cambridgeshire, U.K., (National Grid Reference TL56007019 to TL56317050) between April and June 1995. Bitterling are thought to have been introduced here in the late 1970s (Aldridge, 1997). The site has little or no flow, especially during the summer months when macrophyte density is at its height. Dissolved oxygen concentrations are high as a consequence of photosynthetic activity: Painter (1995) recorded summer oxygen concentrations from 6·5 to 8·0 mg l^{-1} and found no summer stratification. Summer pH ranges from 7·8 to 8·2 (Rowell, 1986), and conductivity from 500 to 1100 μs cm^{-1} (D. Aldridge, pers. obs.).

A second site, Little Mere, north Cheshire, U.K., (National Grid Reference SJ688768) was studied in late May 1996, to assess how widely applicable to other bitterling populations were the Cambridgeshire observations of early bitterling ontogeny. Bitterling have been recorded from Cheshire since the 1930s (Hardy, 1954) and Little Mere was chosen because it has held bitterling for more years than Wicken Lode (C. Goldspink, pers. comm.). The Mere is *c.* 300 m long and 200 m wide. It has shallow-shelving banks, so that water depth reaches <1 m at 20 m from any bank. Exposed regions of bed along the fringes indicate that water levels drop during the

summer. When the site was visited in summer 1996, macrophyte density was very low, with only a narrow fringe of *Phragmites australis* Trin. ex Stendl on the northern edge. No submerged vegetation was observed.

MUSSEL GILL MORPHOLOGY

Mussels representing the median size classes of the four commonest species in Wicken Lode (*Anodonta anatina* L., *A. cygnea* L., *Unio pictorum* Philipsson and *U. tumidus* Philipsson; Aldridge, in press) were transported to the laboratory in aerated river water. Mussels were opened by cutting through the anterior and posterior adductor muscles and the internal morphology was examined.

SITES OF BITTERLING DEVELOPMENT

Up to 50 individuals of each of the four mussel species were collected from the dead end of Wicken Lode during May 1995, transported to the laboratory in aerated river water, and opened to reveal their bitterling load. A further 17 *A. anatina* and 19 *U. tumidus* were collected and studied from Little Mere in May 1996. The lengths of bitterling embryos were measured along their longest axis with an eyepiece graticule under a binocular microscope. For well-developed embryos, the total length (from the anteriormost extremity to the end of the tail fin) was measured. Notable changes in the gross anatomy of bitterling were identified which represented distinct developmental stages. The precise location within the mussel in which distinct developmental stages of bitterling are found were identified.

DEVELOPMENTAL REQUIREMENTS

Developing bitterling embryos were removed from the gills of *U. pictorum* and *U. tumidus* during May 1995; the lamellae of each demibranch were separated gently under water with forceps, and the embryos freed with a jet of water from a pipette. After total length measurement, each embryo was transferred with a 3-mm diameter pipette into an individual glass jar, which contained 10 cm^3 of dechlorinated, oxygen-saturated tap water.

The developing embryos were held under two experimental regimes. In the first, 23 early stage embryos (<7 mm) were provided with continuous, gentle aeration (6·5 mgl^{-1} dissolved oxygen) *via* plastic tubing with a 0·6-mm internal diameter which was positioned on the bottom of each jar. In the second, 115 embryos, representative of all sizes removed from the mussels, were held without aeration.

The developing bitterling were held at 20° C, and checked every 1 or 2 days over 3 weeks. At each check, the water in the jar was replaced with dechlorinated, oxygen-saturated tap water, and the total lengths of the developing bitterling remeasured. A number of factors indicated whether the bitterling were alive: there was an increase in length relative to the previous check; the yolk sac remained a bright yellow-orange, rather than a pale yellow colour; older larvae elicited a 'tail-flick' in response to agitation; in older larvae the heart could be observed beating.

RESULTS

MUSSEL GILL MORPHOLOGY

The internal morphology was similar in each mussel species (Fig. 1). On entering the mussel's exhalant siphon, the female bitterling's ovipositor passes into the exhalant cavity at the base of, and dorsal to, the mussel's gills. The inner and outer demibranchs of each gill are divided from each other by a septum close to the exhalant siphon, while the bases of the two inner demibranchs retain a common opening within the exhalant cavity until they meet the foot anteriorly, at which point the demibranchs segregate. Therefore, eggs may enter either the

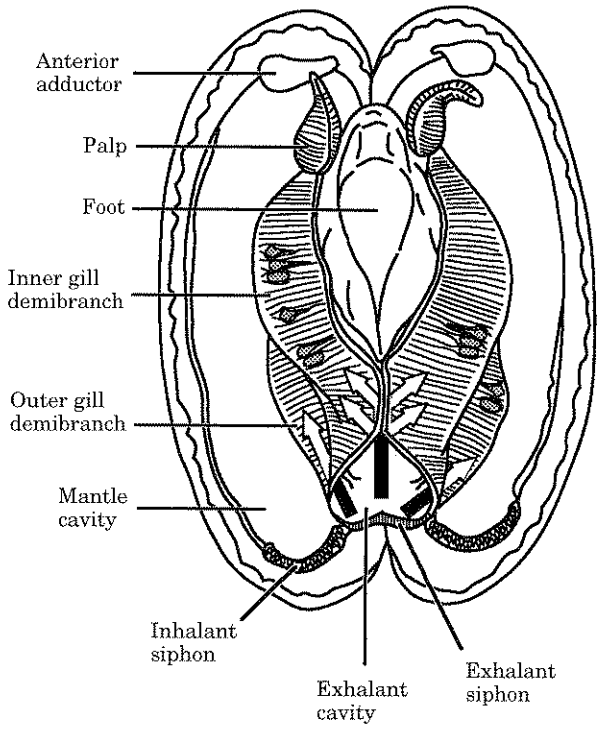

FIG. 1. Diagrammatic representation of an opened unionid mussel to show the access routes to the inner and outer demibranchs which are available to a female bitterling's ovipositor. The anterior of the mussel is at the top of the diagram, and the ventral side is drawn uppermost. The single arrows indicate the independent routes to the outer demibranchs, the double arrow indicates the common opening to the two inner demibranchs. When the valves are closed in the natural position, the two sides of the mantle tissue meet such that the two halves of the inhalant siphon and the two halves of the exhalant siphon form coherent tubes which are then extended slightly from the shell.

left or right inner demibranchs via one common route, or one of the two outer demibranchs by their own discrete routes.

STAGES AND SITES OF BITTERLING DEVELOPMENT

Bitterling eggs are elliptical ($2{\cdot}59 \pm 0{\cdot}04 \times 1{\cdot}74 \pm 0{\cdot}07$ mm, $n=63$), unlike most fish eggs which are spherical. Initially, eggs and larvae appear to be held in place by wedging into the interlamellar space. Indeed, they can be dislodged into the exhalant cavity at the gill base by a jet of pipetted water. However, dislodgement of the embryo from the host's gill is made less likely by the expansion of the anterior portion of the yolk sac into a widened band, which forms two elongated lateral processes either side of a less pronounced ventral ridge (Fig. 2). During development, the yolk sac is resorbed and the polarity is such that, in over 99% of cases, the tail develops towards the open base of the demibranch (Fig. 1).

Bitterling were capable of developing along almost the entire length of both demibranchs. They lay side by side between the gill lamellae, and rarely were found stacked upon one another, either in the dorso-ventral or lateral plane of the demibranch. On rare occasions, one egg followed another into the same interlamellar space. However, no cases were found in which development to

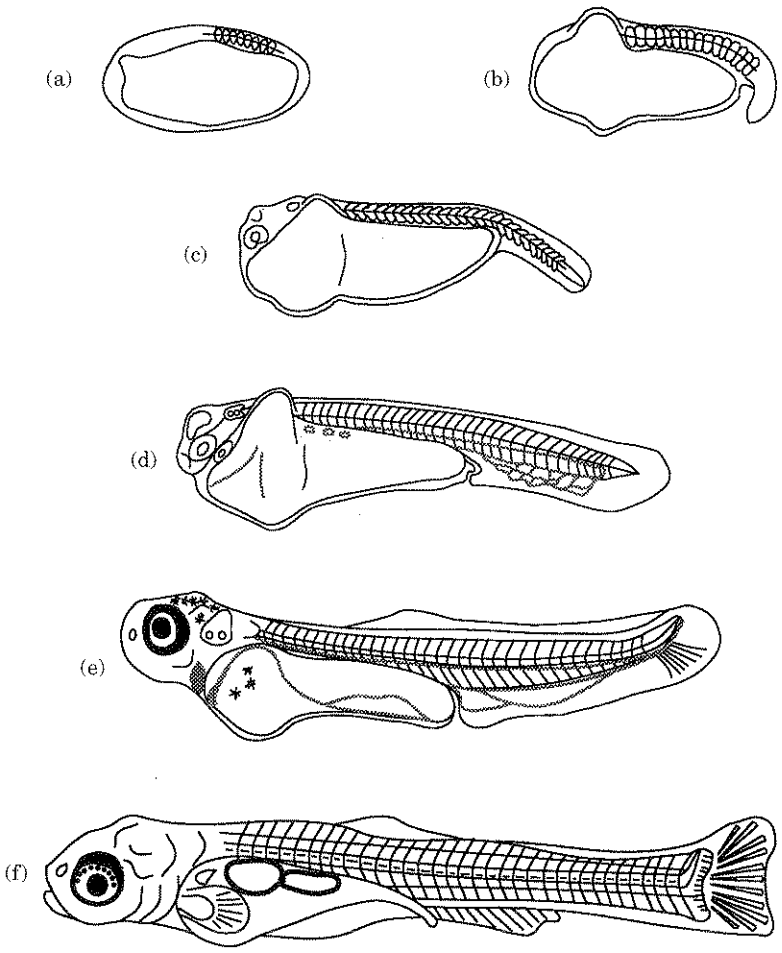

FIG. 2. Distinct stages in development of bitterling embryos (all drawings × 10): (a) egg with embryonic first myotome segmentation; (b) hatched embryo, with initiation of yolk-sac lobe formation; (c) appearance of unpigmented eye; (d) appearance of pigmented blood (shown stippled), lobes of yolk sac become prominent; (e) eyes become pigmented, blood in heart is pigmented and circulatory system extends to tip of tail (shown stippled), first melanophores appear, appearance of rays in caudal fin, fish migrates into host's exhalant cavity and (f) both chambers of swimbladder filled with gas, distinct lateral line, caudal and anal fins ossified, yolk sac fully absorbed, fish leaves mussel.

advanced stages (i.e. >7 mm) had occurred in such conditions. The maximum number of bitterling found in an individual mussel was 63 (a 81 mm male *U. tumidus* from Little Mere).

During development, the embryos increase in length *c.* five-fold and undergo a number of distinct changes which can be divided into three stages:

Development between the host's gill lamellae

The myotomes appear first at 2·7 mm [Fig. 2(a)] and by 2·9 mm the caudal region has developed around the yolk sac. The characteristic lobes of the yolk sac are pronounced by 3·3 mm and the embryo hatches from the chorion such

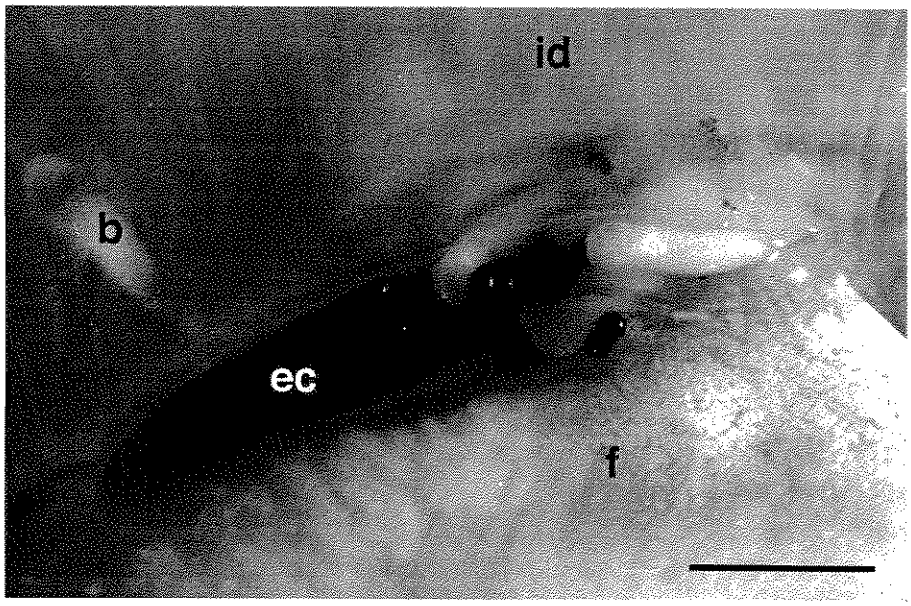

FIG. 3. Bitterling that have migrated into the exhalant cavity at the base of the inner demibranch; b, embryo between the gill lamellae; f, foot of mussel; ec, exhalant cavity of mussel; id, inner demibranch of host's gill. Scale bar=4 mm.

that the tail extends from the body [Fig. 2(b)]. The unpigmented eyes become apparent first at 4·7 mm, and segmentation of the caudal region is complete [Fig. 2(c)]. Once the embryo has reached 6·4 mm the blood becomes pigmented within the ducti Cuvieri; at 6·7 mm pigmented blood is evident dorsal to the yolk sac; and at 6·9 mm pigmented blood can be seen in the heart. At 7·40 ± 0·19 mm (n=30) embryos are capable of limited movement, the eyes become pigmented and the aorta dorsalis extends to nearly the tip of the tail [Fig. 2(d)].

Development at the base of the gill in the exhalant cavity

At 8·15 ± 0·09 mm (n=136) the embryos can swim if removed from the host. The lateral process of the yolk sac is lost [Fig. 2(e)], enabling the embryos to free themselves from the interlamellar space and migrate to the exhalant cavity at the gill base (Fig. 3). The head has straightened out and the lower jaw is visible.

Development external to the mussel

The yolk sac is absorbed gradually through development (Fig. 4), and has been absorbed completely at 10·53 ± 0·08 mm (n=27) [Fig. 2(f)]. At this length the bitterling leave the mussel and commence exogenous feeding. Larvae may emerge prematurely from mussels if temperatures are particularly high (above 25° C), in which case the yolk sac may still be evident.

DEVELOPMENTAL REQUIREMENTS

With the exception of one individual, only larvae >5·8 mm continued development *in vitro* outside the mussel when no aeration was provided, while most smaller individuals died [Fig. 5(a); t=11·00, d.f.=136, P<0·001; comparing the lengths of individuals that survived or died]. Aeration allowed a greater

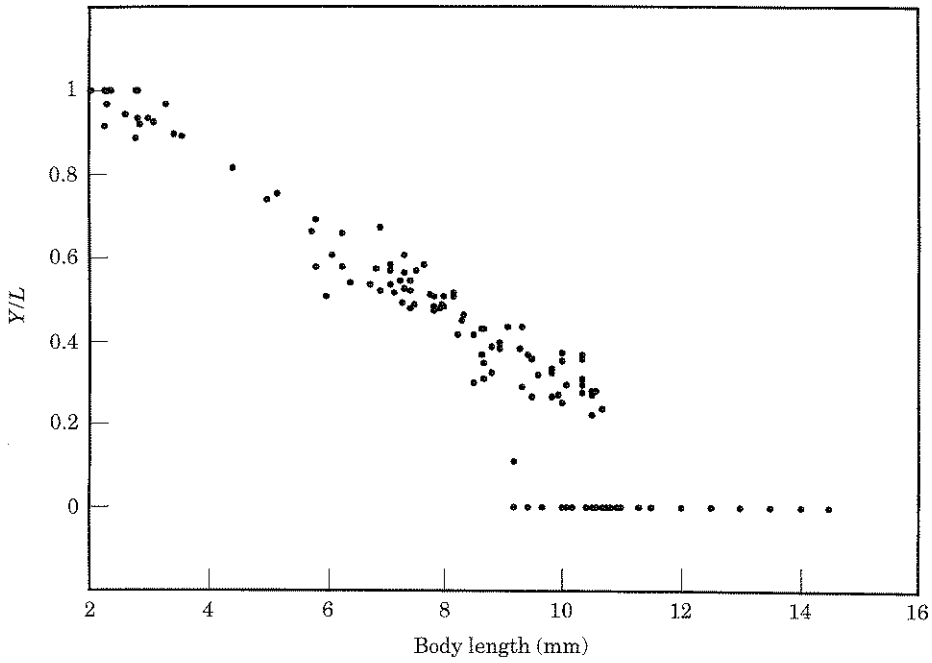

FIG. 4. The change in the length of bitterling yolk sac (S, mm) in relation to total body length (L, mm).

proportion of the smaller larvae to survive [Fig. 5(b)], with 11 of the 23 (48%) experimental individuals (all <7 mm) surviving for at least 3 days when aeration was supplied, compared with eight from 75 (11%) larvae within the same size category surviving without aeration.

DEVELOPMENTAL ABERRATIONS

A number of embryonic bitterling removed from *U. tumidus* collected in Little Mere swelled up during *in vitro* development (Fig. 6), and ultimately they died. Following comparisons with normal embryos, these fish were found to have no pigmented blood system.

DISCUSSION

Bitterling development is atypical of cyprinid fish in many ways (Table I). Reasons for these differences can be investigated by considering the unusual environment in which bitterling embryos develop compared with those for most other fish.

EGG SIZE AND NUMBER

Oxygen diffusion through water is extremely slow compared with diffusion through air and consequently oxygen is often a limiting resource for embryonic development (Kamler, 1992). Bitterling eggs may experience particularly low oxygen conditions for at least three reasons: the bitterling is exposed to water which has already passed across the respiratory surfaces of the mussel, and so is likely to be depleted of oxygen; when stressed or disturbed, mussels close their

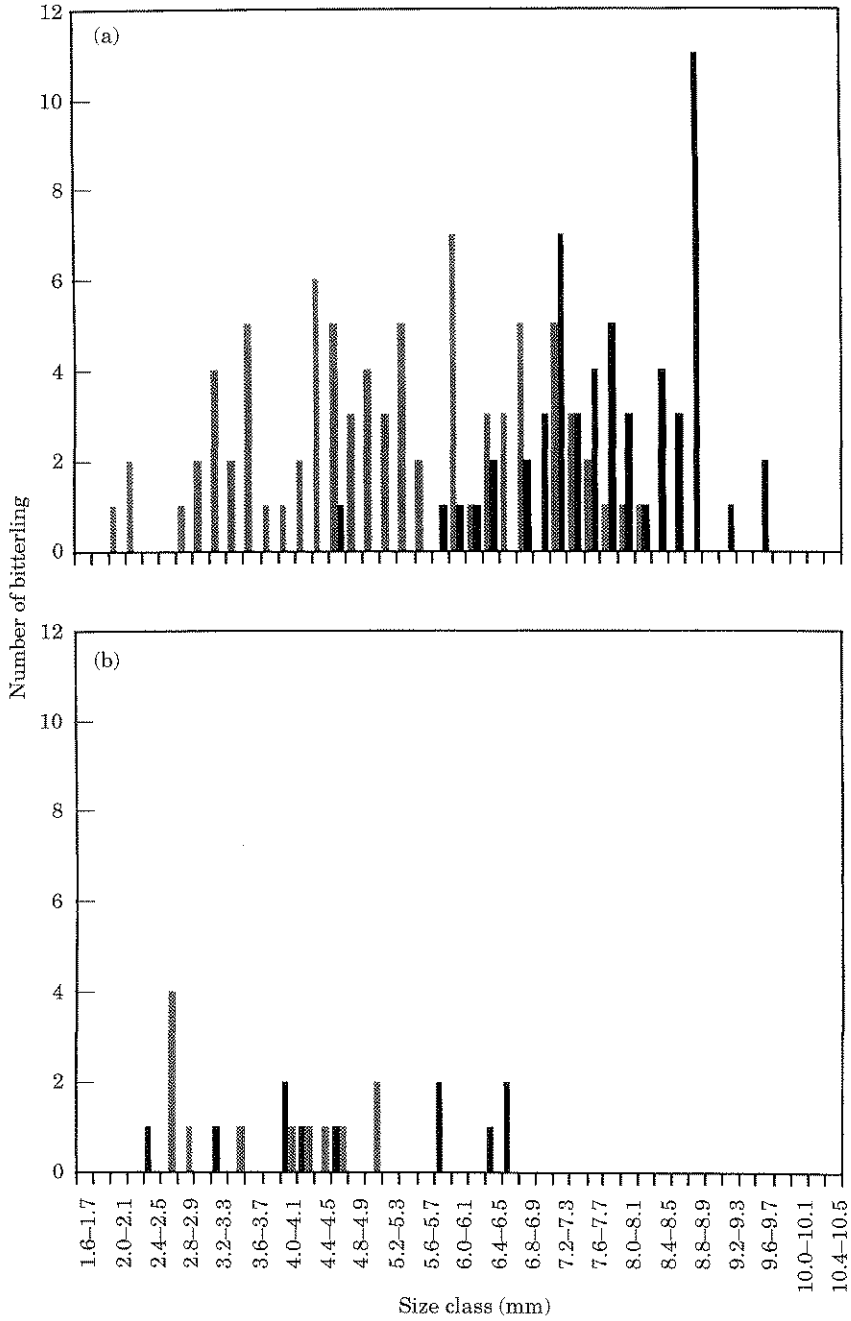

FIG. 5. The number and length of bitterling surviving (solid bars) or dying (stippled bars) 3 days after removal from the host mussel. (a) Without aeration; (b) with aeration (data shown only for fish <7 mm).

valves and therefore their internal environment becomes temporarily hypoxic; and bitterling eggs are unusually large for a cyprinid, so diffusion distances are particularly long.

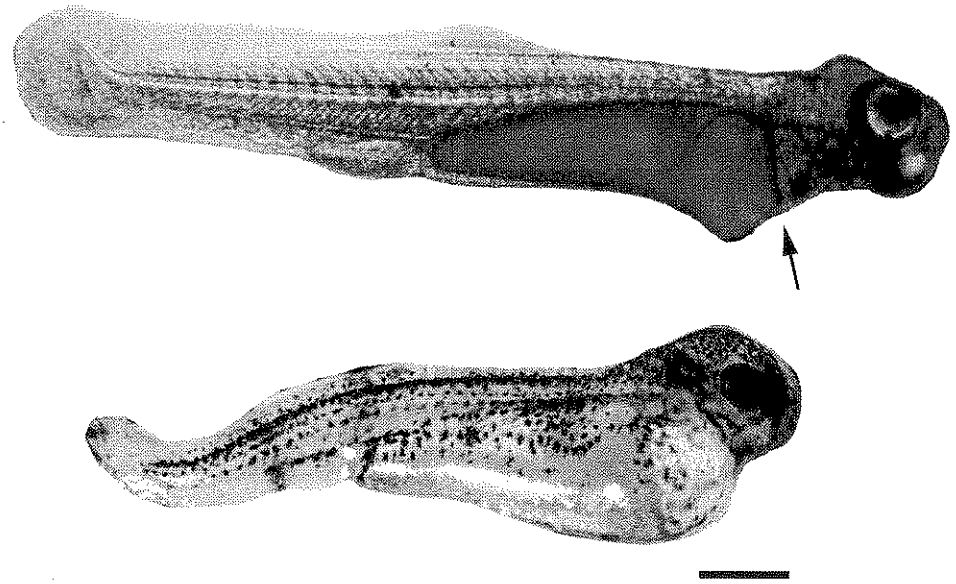

FIG. 6. (a) Normally developed bitterling, with extensive pigmented blood system (indicated by arrow). (b) Abnormally developed bitterling, with swollen ventral surface, stunted tail growth and no pigmentation in the circulatory system. Scale bar=1 mm.

TABLE I. Comparison of early ontogenic stages of bitterling and common carp *Cyprinus carpio* L., embryos

	Common carp*	Bitterling
Number of eggs per female	40 000 to 2 000 000†	60‡
Egg shape	Spherical	Elliptical
Egg size	1·25 mm	2·59 × 1·74 mm
Days to exogenous feeding	4	28
First movements	2·8 mm	7·4 mm
Eyes first pigmented	2·9–3·5 mm	7·4 mm
Blood first pigmented	3·3 mm	6·4 mm
First melanophores	3·5–5·0 mm	7·9 mm
Hatching	4·7 mm	3·3 mm
First locomotion	6·0 mm	8·2 mm
Yolk sac resorbed	6·7–7·1 mm	10·5 mm

*All data on *C. carpio* from Peňáz *et al.* (1982) except: †data cited in Maitland & Campbell (1992); ‡data from Aldridge (1997).

Most cyprinid eggs adhere to aquatic vegetation in slack waters, where oxygen-depleted boundary layers are less easily replenished, and where dissolved oxygen can exceed 100% in the day, but can fall very low at night. Thus, access to oxygen may be a factor limiting the size of fish eggs. The particularly long diffusion distances experienced by bitterling eggs may mean that some tissues experience relatively hypoxic conditions during development, and this effect may be heightened if the host mussel closes its valves for a prolonged time. Bitterling

are able to tolerate periods of hypoxia by breaking down glycogen to ethanol and carbon dioxide, rather than lactate (Waarde *et al.*, 1993). The bitterling's end products can, unlike lactate, be excreted into the surrounding water, thus bypassing the toxic build-up of metabolic end products found in most other fish. Therefore, possession of the ethanol pathway may be an important predisposition to living within a mussel, and to enabling the production of large eggs.

Why have large eggs been selected for in bitterling? Much work is available on the evolution of life histories, particularly in relation to clutch size (Lessells, 1991). All models of the evolution of clutch size assume that there is a trade-off between the number of progeny produced and the fitness of each of them, because the reproductive effort is divided between all the offspring. When parental care is provided, the extent of provisioning per individual will decline with increased offspring number. Numerous studies have shown that young in larger families suffer from increased mortality rates, extended development, and reduced size and fecundity (Lessells, 1991). Studies on fish populations by Ware (1975) show that mortality rates during early development are lower in species producing larger eggs.

The protection of the larvae provided by a host mussel suggests that bitterling clutch-size dynamics should be comparable with those of other species showing parental care. Indeed, nest-builders, such as three-spined sticklebacks *Gasterosteus aculeatus* L., and mouth-brooders, such as mosquito fish *Gambusia affinis* Baird & Girard, produce relatively few large eggs. It is only in such species, where juvenile mortality is low, that investment in a small number of large eggs will be selected for. The fact that bitterling do not themselves provide protection for their offspring, while all the benefits associated with parental care are afforded, suggests that bitterling can invest more energy directly into their reproductive output than other species. This may enable bitterling to put extra resources into production of more eggs, or into the production of particularly large eggs. Furthermore, by divesting the care of its offspring to mussels, a female bitterling can lay a few eggs in each of a number of mussels, and therefore reduce the risk of losing her entire year's reproductive output should an individual mussel evacuate its gills or die. Generally, such a bet-hedging strategy is not observed in species that exhibit parental care; a three-spined stickleback may lose its entire brood if the nest is found by a predator, or if the nest is disturbed.

The production of large eggs is associated with low mortality primarily because the number of potential predators falls with increasing size. Smaller individuals are also more sensitive to changes in their ambient surroundings. By producing large eggs and developing within a mussel, juvenile bitterling are first exposed to predation risk at 10·5 mm, rather than being exposed from the point at which the egg is laid, as occurs in most other cyprinids.

EGG SHAPE

A spherical shape minimizes an object's surface area to volume ratio and therefore minimizes the rate at which oxygen can diffuse into the egg and the rate that excretory products, such as ammonia and urea, can move out. The elliptical shape of the bitterling egg has a greater surface area to volume ratio, so will

allow relatively fast diffusion rates and its selection probably reflects the hypoxic developmental environment. The lateral processes of the anterior yolk sac may increase surface area further to enhance oxygen diffusion. Also, diffusive rates may be increased in bitterling eggs by the steady flow of water generated by the mussel's ventilation, which would reduce the thickness of oxygen-depleted boundary layers around the embryos. The importance of this effect is illustrated by the observation that larvae <5·4 mm survive *in vitro* only when aerated.

An elliptical egg may confer additional advantages for development within the interlamellar spaces of gill demibranchs: such a shape may help to wedge the egg securely into place after it is ejected from the ovipositor. If an egg fails to become secured, it passes into the exhalant cavity and may be expelled with the mussel's pseudofaeces in the excurrent (D. Aldridge, pers. obs.). Also, its association with the gill surface, where oxygen exchange occurs in the host, will be less intimate within the exhalant cavity than it is between the lamellae so that subsequent development may be impaired. Indeed, the surface area of the egg which is in intimate contact with the gill lamellae will be much higher in an elliptical egg than in a spherical one. Furthermore, since the interlamellar space is relatively narrow, the only possible way to produce a larger egg is to extend it lengthways. Therefore, elliptical eggs may be both a response to unionid gill morphology and a way of increasing the supply of oxygen.

DEVELOPMENT

A large egg weight often corresponds with increased development times (Ware, 1975). This is true not only of fish, such as the bitterling (e.g. 28 days to exogenous feeding compared with 4 days in common carp; Table I), but also of many invertebrates (Kirk, 1997). In general, most distinct stages of cyprinid embryonic development occur in bitterling at a greater body length than they do in common carp (Table I). This may be expected given the relatively large size of bitterling eggs, but a number of ontogenic stages in bitterling occur at relatively short or long body lengths, corresponding to relatively early or late stages in ontogeny. These changes in the timing of development can be interpreted in terms of the unusual environment in which bitterling embryos develop.

The length at which most cyprinids hatch from the chorion ranges from 4 to 5 mm in gudgeon *Gobio gobio* L., and common carp to 7–8 mm in chub *Leuciscus cephalus* L. (Maitland & Campbell, 1992). However, bitterling hatch much earlier in ontogeny (3·3 mm) (Table I). The chorion is a particularly restrictive barrier to oxygen diffusion, as is illustrated by the increased oxygen consumption in embryonic Atlantic salmon *Salmo salar* L., when the chorion is removed (Hayes *et al.*, 1951) and its early loss would enhance the delivery of oxygen to bitterling embryos. Moreover, the primary function of the chorion is to provide protection to the developing embryo but such protection is less important to bitterling than to cyprinids developing in open water. The importance of maximizing access to oxygen in bitterling embryos may also explain the relatively early formation of blood pigmentation (Table I).

Developing within the protective environment of the mussel removes the risk of direct predation on bitterling embryos and may explain the relatively late development of eyes and melanophores (Table I); predator detection and cryptic

coloration are likely to serve a greater function in the early ontogeny of free-living embryos.

The length at which bitterling migrate from the confines of the gill lamellae corresponds with the length that bitterling become able to move, which in turn corresponds with the length at which bitterling become able to develop *in vitro* without aeration. The ability to move would enable larvae periodically to replace oxygen-depleted boundary layers with oxygenated water. The finding that smaller immobile larvae can develop only with aeration suggests that, until the ability to move has been acquired, bitterling larvae are entirely dependent on the host's gills for oxygenation. By moving into the exhalant cavity, the larger larvae lose their direct contact with the gill surface, and so oxygen supply may be reduced, but there is more space for continued development.

The stage at which bitterling leave the host is very distinct (10·5 mm from all mussel species), suggesting that the juveniles swim out from the host actively, rather than being ejected by the mussel. In all cases, emergent juveniles had absorbed their yolk sacs. The absorption of the yolk sac may be critical in triggering the emergence of larvae for two reasons. First, the fish must commence exogenous feeding. Because the fish develop within the exhalant cavity, the only potential food resources available will be the pseudofaeces expelled by the mussel, which are unlikely to be nutrient-rich (Bayne & Newell, 1983, and references therein), so food resources must be sought external to the host. Second, activity of the bitterling increases markedly once the yolk sac has been consumed and this will lead to an increase in oxygen requirements. Thus, if oxygen concentrations are relatively low within the host compared with outside it, this may promote emergence.

Under natural conditions, it is unclear how commonly embryonic bitterling develop without a pigmented blood system. Developmental aberrations may be triggered by exposure to *in vitro* conditions or it may be that the embryos are of a mutant form that is particularly abundant in a population founded from a small gene pool. It is possible that the absence of a fully functioning vascular system reduced the osmoregulatory abilities in these fish and this resulted in an influx of water and the subsequent swelling.

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