Evolutionary developmental biology of bitterling fish

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Chapter 1 Introduction and outline of this thesis

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General introduction to bitterlings

The bitterlings are a group of Eurasian freshwater teleost fish with approximately 75 species (Nelson et al., 2016). Phylogenetically, the bitterlings are a monophyletic group belonging to the family Acheilognathidae (Cypriniformes: Cyprinoidea). According to recent studies, the family is divided into six clusters, namely: *Acheilognathus*, *Tanakia*, *Paratanakia*, *Pseudorhodues*, *Rhodeus* and an unnamed clade (Chang et al., 2014; Cheng et al., 2014; Kawamura et al., 2014). Among them, the genus *Rhodeus* is strongly supported as being monophyletic. As there are no known diagnostic morphological characters to discriminate *Paratanakia* and *Pseudorhodeus* from congeners, we use here the classification and diagnostic key provided by taxonomists (Arai and Yutaka, 1988; Arai et al., 2007; Li and Arai, 2010; Li et al., 2017), and group all bitterling species into three genera: *Acheilognathus*, *Tanakia* and *Rhodeus*.

There are two main hypotheses about the phylogenetic relationships inside the bitterling clade. The original hypothesis is based on the patterns of minute tubercles on the surface of the embryonic skin (Suzuki and Hibiya, 1985a), and on a comparison of the adult cephalic sensory canal system, infraorbital bones, and trunk lateral-line scales (Arai, 2003). It suggests that the basal genus is *Tanakia*, with *Acheilognathus* and *Rhodeus* having evolved within it (Arai, 2003). A more recent alternative hypothesis, supported by molecular data (Chang et al., 2014; Cheng et al., 2014; Kawamura et al., 2014) is that *Acheilognathus* is basal to the *Tanakia-Rhodeus* complex.

Brood parasitism

All bitterling species are brood parasites because they lay eggs in the gills of freshwater mussels (Bivalvia: Unionoida) and rely on the host mussel for the protection of their offspring (Figure 1; Leung, 2014; Mills and Reynolds, 2003; Olt, 1893; Smith, 2016). Other teleosts showing brood parasitism include the snailfish (Liparidae) which oviposit their eggs in the gill chamber of lithodid crabs (Lithodidae;(Gardner et al., 2016)), and the Japanese tubesnout fish (Hypoptychidae) which lay eggs in the peribranchial cavity of ascidians (Ascidiacea; (Akagawa et al., 2004)). Fish that show a preference for brooding in live invertebrates which are termed ‘ostracophils’ (Balon, 1975). Ostracophils are only parasitic during their early developmental stages (as eggs and larvae); their juveniles and adults are free-living.

Brood parasitism in other animals is represented by cuckoos (*Cuculus canorus*) and parasitic ants (*Harpagoxenus sublaevis*; (Davies et al., 1989)). Bitterling fish satisfy the criteria of a brood parasite because they reduce the costs of parental care by exploiting the gills of the mussel, and depend on the mussel for their reproduction (Kitamura, Nagata, Nakajima, & Sota, 2012; Rouchet et al., 2017; Smith, 2016). The parasitism causes the mussel host to suffer a significant fitness cost including reduced growth rate (Smith, Reichard, Jurajda, & Przybylski, 2004), oxygen stress (Spence and Smith, 2013), gill epithelium damage, disrupted water circulation (Reichard et al., 2007) and increased mortality (Smith, Reynolds, Sutherland, & Jurajda, 2000).

The mussel gill is multifunctional. The demibranchs (ctenidia) serve as organs of respiration, filter feeding, blood circulation, and as brooding sites. During the mating season, the female mussels brood their own embryos inside the specialized marsupial gill until the glochidia (mussel larvae) are released into the water (Tankersley, 1996; Tankersley and Dimock, 1992). The released glochidia invade fish and temporally parasitize the gills of fish until free-living stages. However, bitterlings have

evolved resistance to infection by glochidia (Mills and Reynolds, 2003). The oily gudgeon (*Sarcocheilichthys variegatus*), a teleost, also uses freshwater mussels as hosts for its embryos. However, in contrast to the bitterlings, the embryos of this species occupy only the mantle cavity and not the gill chamber (Khlopova and Varaksin, 2009).

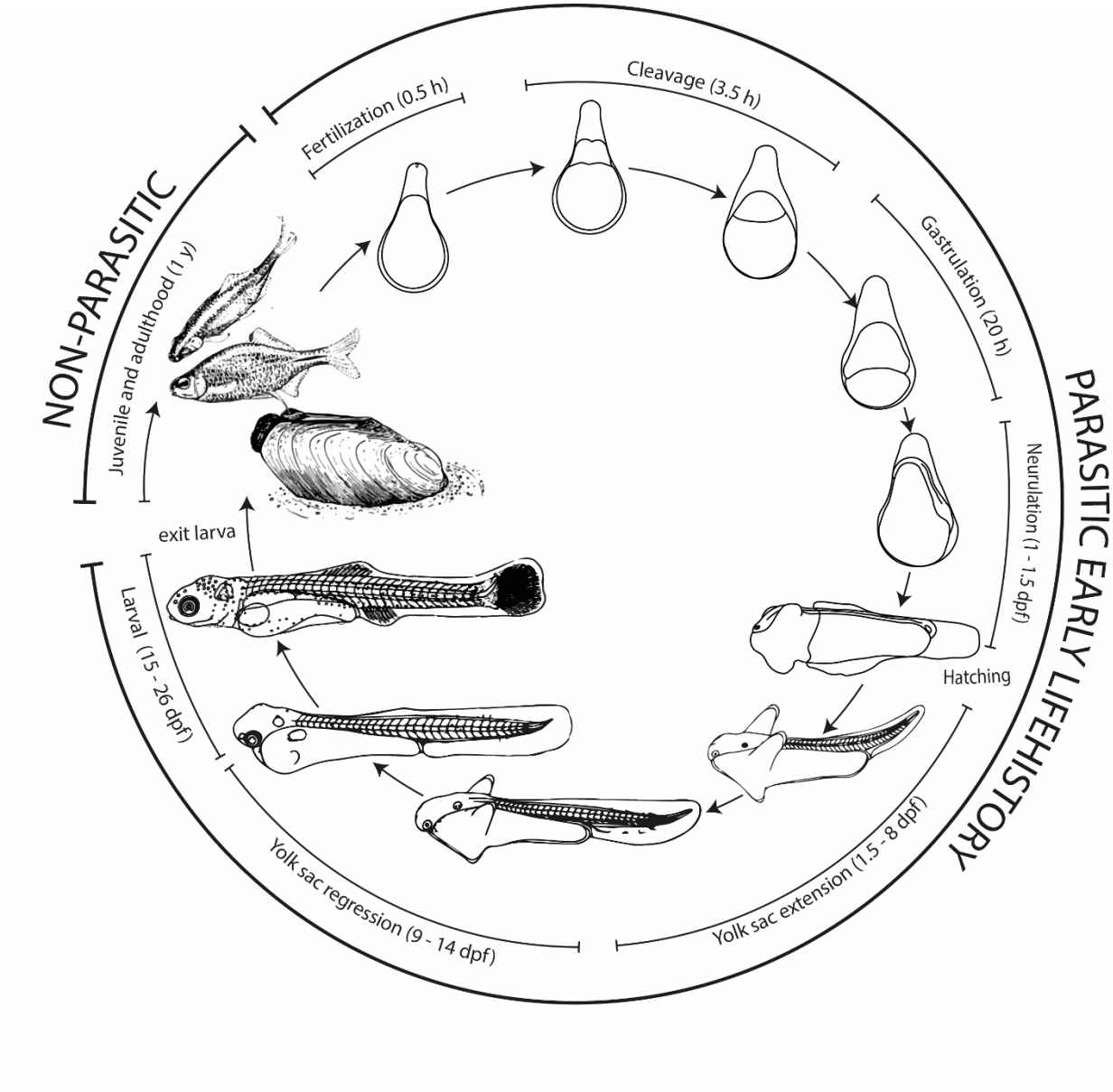

Figure 1 Life history of the bitterling. This figure shows development in the species *Rhodeus ocellatus*. Development progresses clockwise. The parasitic phase takes place inside the mussel while the non-parasitic phase is free-living outside the mussel. Abbreviations: h, hours; dpf, days post fertilization; y, year. Drawn by Wenjing Yi. The figure of adulthood breeding behavior is modified from Boeseman et al. (1938). The figures of periods from hatching until larval period are modified from Kim and Park (1985). Permissions have been granted by the publishers.

The bitterling-mussel interaction as a model system

The distinct bitterling-mussel relationship has been well studied for at least a century (Duyvené de Wit, 1955; Olt, 1893). The reproductive behaviour of *Rhodeus* and its relationship with the mussel were noticed by researchers in Leiden, the Netherlands, including the Nobel Prize winner Nikolaas Tinbergen (Boeseman et al., 1938). Illustrations in that publication presented observations of water

flow, inside the mussel host, that went from the inhalant siphon through the gill chamber to the exhalent siphon (Boeseman et al., 1938). Those authors also observed the behavior of the bitterlings and noted that while the male bitterling inspected and guarded the territory of the mussel host, the female bitterling inserted her ovipositor into the exhalent siphon. Research findings on the European bitterling *Rhodeus amarus* were reviewed by Duyvené de Wit (1955) to motivate worldwide collaboration on studying bitterlings from the aspects of comparative embryology, immunology, behavior, and taxonomy. Smith et al. (2004) reviewed the reproductive ecology of the European bitterling and concluded that the bitterling is a valuable model organism in behavioral, population and evolutionary ecology.

Embryonic adaptations to blood parasitism

In this thesis we study the bitterling from the perspective of evolutionary developmental biology ('evo-devo'). We are particularly interested in the remarkable developmental adaptations that bitterlings show to brood parasitism. The bitterling embryo (Table 1) shows at least two such adaptations: (i) those affecting egg shape or yolk shape, including the remarkable lateral yolk sac extensions (YSEs); (ii) the presence of unicellular tubercles on the epithelium (periderm) that covers the embryo (Fukuhara et al., 1982; Kim et al., 2008; Suzuki and Hibiya, 1984a; Suzuki and Hibiya, 1985b; Suzuki et al., 1986; Suzuki et al., 1989a).

Teleost eggs are usually spherical (Kunz, 2004). Oval and elliptical shapes are often found in bitterlings. The unfertilized eggs of bitterlings are generally ellipsoid. The size and shape vary among genera. (Suzuki, 1958) compared the egg shape of *T. lanceolata*, *A. tabira*, and *R. ocellatus*. According to his measurements, the *T. lanceolata* egg is fusiform, with the highest length/width (l/w) ratio (c. 3.0). *A. tabira* is more rounded (c. 1.6) and *R. ocellatus* is intermediate (c. 2.7). According to (Chi Hong Kim et al., 2006), there are in total 4 types of yolk shape: bulb-like (*Rhodeus*), pear-shaped (*Tanakia*), fusiform (*Tanakia*), and round-oval (*Acheilognathus*).

The shape of the yolk changes during embryonic development in many fish species. In cypriniforms, the initially spherical yolk ball becomes reshaped to form a caudal yolk extension (YE), a cylindrical structure protruding posteriorly from the ball (Virta and Cooper, 2011). According to those authors, the caudal YE is a conserved morphological trait of the order Cypriniformes (carp and minnows). Their experiments with zebrafish (*Danio rerio*) suggested a mechanism underlying formation of the caudal YE: it is likely shaped by the mechanical forces generated by the contractile cell layers of embryonic integument, which exert force on the posterior region of the yolk (Virta and Cooper, 2011). Bitterlings have a caudal YE and two lateral YSEs. It is noticeable that, in *Rhodeus*, the lateral YSEs undergoes the highest level of transformation, forming a pair of prominent wing-like structures on the lateral aspect of the yolk ball (Suzuki, 2004). In *Tanakia*, the lateral YSEs are less developed, having more the appearance of low bulges than projecting wings (Suzuki et al., 1986). *Acheilognathus* has no lateral YSEs (Kim et al., 2018; Suzuki and Jeon, 1991). However, it is still unknown whether the lateral wing-like YSEs shares any homology with the caudal YE.

The teleost epidermis has a complex cellular composition. During development, there are transitory, unicellular hatching gland cells, ion-regulated chloride cells, chromatophores (pigment

Table 1 A summary of yolk sac shape and peridermal features in bitterlings.

Genus	Species	Egg shape	YSEs	EVL tubercles	Ref.
Rhodeus	*ocellatus*	bulb-like	wing-like	hemispheric	(Kim and Park, 1985; Suzuki and Hibiya, 1984b; Ueda et al., 2001)
	atremius	bulb-like	wing-like	hemispheric	(Suzuki and Hibiya, 1984a)
	suigensis	bulb-like	wing-like	hemispheric	(Suzuki and Hibiya, 1984a)
	uyekii	bulb-like	wing-like	hemispheric	(Suzuki et al., 1985)
	sericeus	bulb-like	wing-like	hemispheric	(Aldridge, 1999)
	pseudo-sericeus	bulb-like	wing-like	hemispheric	(Chi Hong Kim et al., 2006; Kim et al., 2008; Suzuki, 2006)
Sinorhodeus	*microlepis*	pear-shape	bulge-like	unknown	(Li et al., 2017)
Tanakia	*limbata*	fusiform	bulge-like	bullet-like	(Suzuki and Jeon, 1988b)
Paratanakia	*himantegus*	fusiform	bulge-like	bullet-like	(Suzuki et al., 1989a)
Pseudorhodeus	*tanago*	pear-shape	bulge-like	bullet-like	(Suzuki et al., 1986)
Acheilognathus	*yamatsutae*	round-oval	none	cone shaped	(Suzuki and Jeon, 1987)
	rhombeus	round-oval	none	cone shaped	(Kim et al., 2018; Suzuki and Jeon, 1991)
	asmussi	round oval	none	cone shaped	(Suzuki and Jeon, 1989)

Note: YSEs, yolk sac extensions; EVL, enveloping-layer of cells; For shape of the EVL tubercles see Figure 2.

cells), mucus glands, an enveloping-layer of cells (EVL) and adhesive glands cells (which are normally covered by the EVL; (Kunz, 2004)). The caudal YE of zebrafish can be divided into four histological compartments (Virta and Cooper, 2011). The innermost compartment is the yolk ball, which is formed by yolk granules. From deep to superficial, the yolk granules are covered successively by: a

yolk syncytial layer (YSL) or periblast; an intermediate epidermal ectoderm; and a surface layer of simple squamous epithelium called the EVL or periderm. These three layers form the walls of the yolk sac at the ventral side of the yolk ball (Kimmel et al., 1990; Webb et al., 2008). The YSL and EVL are lineage-restricted extra-embryonic structures (Kimmel et al., 1990). But the EVL cells can persist beyond metamorphosis and are slowly renewed by proliferation of basal layer keratinocytes (Fischer et al., 2014). The intermediate epidermal ectoderm will develop into the basal layer of the future adult epidermis (Le Guellec et al., 2004).

In studies of different teleost species, the EVL has a variety of synonyms including: periderm, pavement cells, epithelial cells, epithelial envelope, peripheral cells, outermost flattened layer, skin surface, and surface of the yolk sac (Fukuhara et al., 1982). The EVL of zebrafish and other cypriniform fish is furnished with micro-ridges. These are raised, actin-rich structures that help to maintain a layer of mucus on the surface of the fish (Webb et al., 2008). In bitterlings, the surface of the yolk sac wall is also decorated with micro-ridges. But at the region of the lateral and caudal YSEs these surface cells have a specialized apical surface. The central part of the apex of the cell protrudes as a unicellular tubercle.

The location and micro-ridge structure of the so-called 'nicellular tubercles on surface of the yolk sac' leads us to the hypothesis that these surface cells may be specialized EVL cells rather than other cell types found in the developing epithelium. Based on scanning electron microscopy (SEM), there are three types of EVL tubercles (Figure 2): (i) 6.5 - 17.5 µm in height and hemispheric (Kim et al., 2008; Suzuki, 2006); (ii) 15 - 25 µm in height and bullet-like (Suzuki and Hibiya, 1985b; Suzuki et al., 1986; Suzuki et al., 1989a); and (iii) approximately 20 µm in height and conical (Suzuki and Jeon, 1988a).

In summary, the following traits occur in three combinations depending on the bitterling genus: (i) bulb-like eggs with wing-like YSEs and hemispherical EVL tubercles (*Rhodeus*); (ii) fusiform or pear-shaped eggs with bulge-like YSEs and bullet-like EVL tubercles (*Tanakia*); and (iii) round-oval eggs, with no YSEs but with cone -shaped EVL tubercles (*Acheilognathus*).

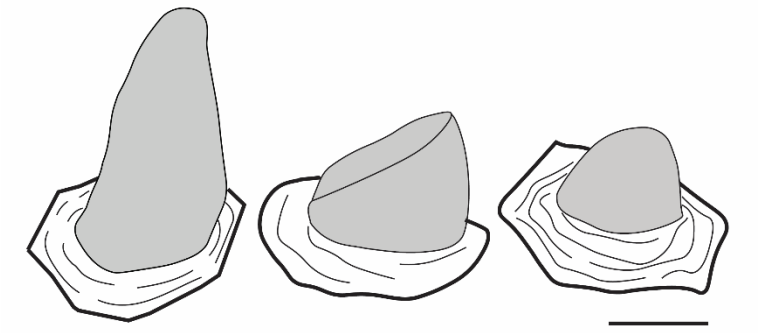

Figure 2 Tubercles of the enveloping-layer of cells (EVL) of bitterling species. From left to right: bullet-like, *Paratanakia himantegus* (Suzuki et al., 1989a); cone shaped, *Acheilognathus rhombeus* (Suzuki and Jeon, 1991); and hemispheric, *Rhodeus uyekii* (Suzuki et al., 1985). The grey colour indicates the protruded tubercles; the narrow lines are the micro-ridges; the broad, dark line is the cell boundary. Scale bar = 10 µm. Drawing by Wenjing Yi based on the references cited.

Host mussel species

We have summarized above the varying developmental adaptations in bitterlings. Previous studies indicated that these differences are related to differences in gill architecture among the mussel host species (Liu et al., 2006). In general, bitterlings spawn inside freshwater bivalve mollusks of the family Unionidae (Bivalvia: Paleoheterodonta: Unionoidea: Unionacea). Some *Rhodeus* individuals have been reported to use mollusks of the family Margaritiferidea as hosts (Smith et al., 2004). A newly-described bitterling species, *Sinorhodeus microlepis*, has been found exploiting the Asian clam *Corbicula fluminea* of the family Corbiculidae (Bivalvia: Heterodonta: Veneroidea: Corbiculoidea). Its larvae were found in the gills of the clam (Dillon, 2000; LemaireGony et al., 1997; Li et al., 2017).

Bivalve mollusks can be divided into four groups according to the complexity of their gill architecture: protobranchia, filibranchia, eulamellibranchia and septibranchia (Lang and Hescheler, 1900). The host mussels for all bitterlings belong to the eulamellibranch group. Eulamellibranchia have the highest degree of gill structural complexity. The lamellae are joined by bars of connective tissue called the interlamellar junctions (septa). The gill filaments are firmly connected by interfilament junctions, and the entire gill has the appearance of a perforated, leaf-like tissue (LemaireGony et al., 1997; McElwain and Bullard, 2014; Medler and Silverman, 2001). The gill chamber between the ascending and descending lamellae is therefore divided by interlamellar junctions into water tubes aligned in parallel.

Reichard et al. (2007) tested interspecific differences among bitterling species in host preference among the eulamellibranch bivalves. They divided bivalves into four types according to the complexity of the water tube: (i) gill without true water tubes or septa (Ableminae); (ii) gill with water tubes and perforated septa (Unioninae); (iii) gill with water tubes and non-perforated septa (Unioninae); (iv) gill with tripartite water tubes and non-perforated septa (Anodontinae). Their results suggest that the host preference of bitterlings is related to gill-structure complexity. For example, *R. ocellatus* embryos, with a well-developed wing-like pair of YSEs, are able to parasitize all mussel species listed above. However, they show a preference for *Anodonta globosula* (type iv), which has the most complex gill type (Reichard et al., 2007).

Liu et al. (2006) mapped bitterling host preference for various East Asian freshwater mussel species of the family Unionidae. In general, *Acheilognathus* and *Tanakia* showed a preference for mussels with a relatively simple gill structure (Ableminae), whereas *Rhodeus* showed a preference for Anodontinae and Unioninae, which have a more complex gill structure.

From the above Introduction, we can make the following conclusions. Phylogenetically, we know that the wing-like YSEs is a synapomorphy supporting the robustness of the genus *Rhodeus*. Ecologically, the fitness advantage associated with the wing-like YSEs is possibly an evolutionary adaptation to the environment of the water tube of the mussel gill. In evolutionary terms, the origin of the YSEs is a speciation event that split the bitterling group. However, the origin of the wing-like YSEs still needs to be explained in terms of developmental mechanisms. To this end, we decided to use the most common species in the genus *Rhodeus*, the rosy bitterling (*R. ocellatus*) as our research organism, and to give special attention to the developmental processes underlying the origin of the lateral wing-like YSEs.

Evolutionary developmental biology (evo-devo) of bitterlings

Evolutionary novelty

The discipline of evolutionary developmental biology (evo-devo) brought evolution and developmental biology together at the time of the discovery of developmental genes in *Drosophila* in the late 1970s (Lewis, 1978). Other stages in the progress of evo-devo include the discovery of the deep homology of developmental regulatory genes across the Metazoa (Creuzet et al., 2005; Ekker et al., 1997; Puschel et al., 1992). This was then linked with conserved metazoan body plans to give the concept of the *zootype* (Graham et al., 2014; Nagashima et al., 2009; Riedel-Kruse et al., 2007) —a definition of the animal body plan in terms of developmental gene expression.

Further, studies on the mechanisms of morphogenesis (Soules and Link, 2005) and organogenesis (Drummond and Davidson, 2016; Ng et al., 2005), provided an opportunity to use developmental tool-kit genes (a small group of transcription factors and secreted peptides and proteins that control embryonic pattern formation; Knoll and Carroll, 1999) to investigate evolution and the origin of evolutionary novelties. For example, Saenko et al. (2008) studied eyespots as an example of an evolutionary novelty in the butterfly (*Bicyclus anynana*). Their research showed that wound healing, a fundamental process, might have been co-opted in the evolution of eyespots via the upregulation of expression of genes *Distal-less*, *engrailed*, and *spalt* in scale-building cells.

In this thesis, the first evo-devo question we ask is the following: is the wing-like YSEs of *R. ocellatus* an evolutionary novelty? According to Mayr (1960), not all particular changes of the phenotype represent the emergence of evolutionary novelties. For example, he suggests that changes in size or pigmentation are not considered to be evolutionary novelties because they are quantitative traits. Traits are only considered novelties if they are new characters, structures, or functions. Therefore, the tentative definition of an evolutionary novelty given by Mayr (1960) is “a change that would permit an organism to perform a new function”. What’s more, modification of the incipient structure is favored by natural selection resulting from a change in the environment.

Accordingly, our hypothesis is as follows. The bitterling YSEs represents a novelty in the following two senses: (i) it represents a morphological change, as the surface of the yolk ball protruded as a pair of wing-like structures; and (ii) because of this morphological change, the bitterling showed a gain of function, namely the ability to parasitize the water tube of the mussel gill. This combined morphological and functional change may be linked to the niche shift of embryonic development from the open water body to the enclosed environment of the mussel body.

From phenotype to genotype

The study of taxon-specific novel phenotypes (shared-derived characters or synapomorphies) is an important topic in evo-devo research. For example, Kupffer’s vesicle (KV) is a teleost-specific embryonic character described by Carl Kupffer (Kupffer, 1868). Histologically, KV is a fluid-filled epithelial sac lined by ciliated cells (Brummett and Dumont, 1978). it is an early, transient embryonic character although its precise stage of appearance is species-specific (Kunz, 2004). It is a useful staging character but was formerly considered to be an embryonic ‘organ of ambiguity’ because of its uncertain function in development (Kimmel et al., 1995; Warga and Stainier, 2002).

More recent research has shown that the function of KV may be related to the normal development of body asymmetry, and might therefore be analogous to the node in mice (formerly called Hensen's node), the gastrocoel roof plate in *Xenopus*, and Hensen's node in the chick (Essner et al., 2002). These regions in all species examined show expression of a dynein gene (*Lrd*, left-right dynein heavy chain). In zebrafish, *lrdr1* (left-right dynein-related1) is first expressed in the dorsal forerunner cells. These cells give rise to KV during the early somitogenesis period. Essner et al. (2002) observed that the node monocilia in KV are motile, and these cilia create a directional fluid flow inside KV. Furthermore, morpholino knockdown of *lrdr1* induces disruption of fluid flow inside KV and perturbs left-right development, suggesting that KV is 'an embryonic orogan of asymmetry' (Essner et al., 2005).

The wing-like YSEs of the bitterling is another transient 'embryonic organ of ambiguity' in our view. We aim to uncover the developmental mechanism of YSEs formation. However, to identify candidate genes for a novel phenotype in a non-model organism like the bitterling YSEs is difficult. In the case of the bitterling *R. ocellatus*, there are no genomic data, and no developmental molecular studies have been done. However, the ZFIN (The Zebrafish Information Network) database (Howe et al., 2013a), and the zebrafish reference genome sequence (Howe et al., 2013b) do provide us with mutant phenotypes related to caudal YE development, and therefore a list of candidate genes. For example, the zebrafish caudal YE is shaped by the contraction force of the embryonic integument. One of the contractile cell layers of the integument is the EVL. Previous studies of zebrafish provided reliable EVL related developmental genes (Chang and Hwang, 2011; Eisenhoffer et al., 2017; Fischer et al., 2014; Imboden et al., 1997; Xiong et al., 2014). In zebrafish, the keratin genes *cyt1*, *cyt2*, *krt4*, *krt8* and *krt18* are normally expressed in the EVL whereas the epithelial stem cell marker p63 is specifically expressed in the basal layer. Using such molecular markers can help us to study bitterlings tubercle EVL cells on the ridges of wing-like YSEs and identify the homologies of those cells.

Wholemount *in situ* hybridization (WISH) is the method we use to screen candidate genes and to profile gene expression patterns in the bitterling *R. ocellatus*. With mRNA probes, WISH allows the reliable visualization of gene expression during embryo development (Thisse et al., 2004). The expression of orthologous genes allows us to formulate testable hypotheses about mechanisms underlying the development of the wing-like YSEs.

Bitterling developmental staging systems

The next question is how to compare bitterling and zebrafish developmental mechanisms and gene expression in an evolutionary context. The zebrafish is closely related to the bitterlings but is not a brood parasite. Spatial and temporal differences in gene expression, if they exist between the zebrafish and bitterling, could result from interspecific differences arising during the course of evolution. Therefore, we need to have a standard staging series as the basis of comparisons between the two species (Jeffery et al., 2002a; Richardson et al., 2001). One approach to making the dynamic process of embryonic development comparable across species, is to adopt a standard event system to study embryos at stages that are presumed to be comparable (Werneburg, 2009). Another option would be to try to stage bitterling or other teleost embryos using the widely-used zebrafish staging system of (Kimmel et al., 1995).

Kimmel et al. (1995) published what would become the gold standard for zebrafish studies. Those authors use numerical staging characters, believing them to be more accurate, reproducible, and easily compared across species. As is well-known, using 'days' or other time intervals to define a developmental stage is problematic because: (i) the stage at a particular day of development varies with temperature (ii) different species develop at different rates and so, for example, 5 days in the zebrafish is not equivalent in stage to 5 days in a bitterling. Homologous morphological characters such as somite count are not subject to these objections (Jeffery et al., 2002b). That is why each zebrafish stage was based by Kimmel on an identifiable morphological character, combined with a numerical index (for example, 18-somites, and prim-6 etc., where prim = primordium of the lateral line). Later, a postembryonic staging series of the zebrafish, based on externally visible anatomical characters (Parichy et al., 2009) further extended zebrafish staging. Unfortunately, the available bitterling staging systems have not been optimized in this way.

Another problem facing us here is that several key studies of bitterling development are written in languages other than English. For example, two publications on the embryonic developmental stages of the rosy bitterling (*R. ocellatus*) by Uchida (1939) and Nikolsky (1963) are in Japanese and Russian, respectively. Later, Kim and Park (1985) published another study in the Korean language, with English used only in the Abstract and figure legends. Suzuki and Jeon (1988) published another description of the Korean subspecies *R. ocellatus ocellatus*, which covered embryonic, larval, and juvenile development, and also wrote it in Korean.

The most recent English-language description of the developmental stages of the species *R. ocellatus* was given by Nagata and Miyabe (1978). Their study provides us with illustrations of all 30 stages. However, the nomenclature they used is no longer consistent with modern teleost staging systems. For example, one of their criteria is 'embryonic-fin appearing stage', and four other stages based on how far the so-called embryonic fin extends to the cranial region. However, what they call the 'embryonic fin' is actually the median fin fold. Their other criteria, such as 'the formation of the embryonic-fin extends gradually to the more anterior region' is vague and not as clear as if they had said something like: 'extends from the level of somite-15 to somite-10'.

In view of these and other issues, there is a pressing need to produce a new character-based bitterling staging series. Furthermore, the lateral wing-like YSEs appears during the early somite period, persists during development of sensory organs such as ears and eyes, and regresses in the time-window of pectoral fin development. Therefore, the new bitterling staging system should also include the morphogenesis of somites, the lens and optic cups, the otic vesicle, and pectoral fin bud, across several stages. As we show, we can then use this new staging system to study heterochrony.

Sequence heterochrony and transcriptional heterochrony

Heterochrony is any change in developmental timing during evolution (Richardson, 1995). During embryonic development, the embryo has an ever-changing morphology. Changes in embryo morphology can be defined in terms of the occurrence of discrete developmental events such as the appearance of the lens placode, the appearance of the first somite etc.; or they can be defined morphometrically, that is by measuring continuous variables such as the size of a particular part. The order of events is called the 'developmental sequence'. Shifts in the sequence of developmental events during evolution are known as sequence heterochrony (Bininda-Emonds et al., 2002).

Aldridge (1999) found that a discrete event (hatching) occurred earlier in the European bitterling *R. sericeus* compared to another teleost, the common carp (*Cyprinus carpio*), when ages were inferred from body length. On the other hand, retinal pigmentation and melanophore differentiation occurred relatively late in the bitterling. The possible explanation for these heterochronic shifts is that it is related to brood parasitism. The early hatching facilitates direct exposure to dissolved oxygen, while late pigmentation may be because there is no need for camouflage in order to avoid prey in the early stages when the fish is inside the dark interior of the mussel.

These observations led us to ask the following question: is the evolution of brood parasitism associated with changes in developmental timing (sequence heterochrony)? To answer this question, we need to compare the developmental sequences of the bitterling with non-parasitic species to detect sequence heterochrony in a phylogenetic context. For this purpose, we will use the zebrafish (*Danio rerio*). We chose this species because it is well-studied and it belongs to the Cyprinidae Family, a sister-taxon with bitterling species (Mayden et al., 2009). This phylogenetic relation is interesting in two respects. First, if the phylogenetic topology is congruent with heterochrony data, it could mean that there is a phylogenetic signal contained in the developmental sequences. Conversely, if the phylogeny and ontogeny are not congruent, the conflict may indicate adaptation to environmental pressures.

One of the forms of heterochrony we want to study is *transcriptional heterochrony*: a change in the timing of initiation or silencing of gene expression (Richardson, 2012; Richardson et al., 2009). Temporal shifts in the expression of developmental regulatory genes are known to be potential mechanism of phenotypic change. For example, Bickelmann et al. (2012) analyzed the temporal and spatial expression profile of *Sox9* (an early marker of chondrification) in developing forelimbs and hindlimbs of the fossorial talpid mole (*Talpa occidentalis*), a terrestrial shrew (*Cryptotis parva*) and the terrestrial mouse (*Mus musculus*). 'Terrestrial' in this context means dwelling above the ground. The spatial distribution of *Sox9* expression domains was similar in all species investigated. But in the mole, *Sox9* expression was advanced in the forelimb compared to the hind limb, in contrast to synchronous expression in the shrew and mouse. This transcriptional heterochrony may account for the enlarged hands in talpid moles, an adaptation for digging.

Advanced imaging techniques for studying bitterlings development

Three-dimensional (3-D) imaging of embryos is a valuable tool for helping us understand development. One example of this is the ongoing effort to develop a 3-D embryo atlas of the zebrafish. Verbeek et al. (1999) developed a 3-D digital atlas of zebrafish embryos by reconstructing serial histological sections into 3-D images. Histological sectioning has the advantages of high optical resolution, and the ability to use a range of histochemical and other stains (Copper et al., 2018). The disadvantage of histology is that embedding and sectioning are time-consuming and destructive to the samples.

A relatively new and non-destructive technology for the study of developmental anatomy is X-ray micro-computed tomography (microCT) (Metscher, 2009a; Metscher, 2009b). In the area of developmental biology, Metscher (2009a) pioneered a protocol for staining soft tissue, and opened up its application to the study of development. With one-step contrast staining, microCT can produce high-contrast, high-resolution images of embryos. The image dataset, which contains digitally

generated 'virtual' sections along all defined anatomical axes of the scanned sample, can be used to give two-dimensional (2-D) histological information. After 3-D volume rendering, the data can be used to visualize anatomical structures. Finally, microCT data can be annotated in 2-D to produce a 3-D model. This method has already been widely used to study mouse development. For example, Wong et al. (2012) created a 3-D mouse embryo atlas which established a baseline of mouse embryo phenotype assessment. (http://www.mouseimaging.ca/technologies/mouse_embryo_atlas.html). Recently, a new zebrafish atlas, Pan-Cellular Tissue Tomography (PANCETTO), has been under construction by the Bio-Atlas team using synchrotron tomography to extend non-destructive methods to a cellular resolution (http://zfatlas.psu.edu).

Here, we chose to use microCT to visualize the developmental morphology of the bitterling because this method is more time-efficient than conventional histology, it is non-destructive, and they are optimal for our specimen size (150 um to 3 cm). By providing a 3-D view of bitterling development, we hope this research will serve as a navigator and a foundation for further evo-devo research on regional development and comparative embryology.

Advancing the bitterling as an evo-devo model system

With this research, we aim to establish bitterling as a new evo-devo model system. Since bitterlings have such a specialized ecology, they could also be developed into an eco-evo-devo system. Eco-evo-devo is the integrated study of ecology, evolution and development (Gilbert et al., 2015). There are many other evo-devo model species among teleosts. A good example is the blind Mexican cavefish (*Astyanax mexicanus*), a unique species whose surface-dwelling population has normal paired eyes, whereas the cave population is blind due to eye regression. This species has emerged as a good model organism to study eye regression as an adaptive trait driven by natural selection (Krishnan and Rohner, 2017; Yamamoto et al., 2004). The Japanese flounder (*Paralichthys olivaceus*) has a highly-derived asymmetric body morphology and extensive craniofacial transformation, providing a useful model for studying the evolutionary origin of asymmetry and the mechanisms of body shape transformation in vertebrates (Shao et al., 2017). The goldfish (*Carassius auratus*), with morphologically divergent domesticated strains, is a particularly advantageous model organism with which to address how artificial selection and developmental mechanisms are related, and how developmental robustness and genetic diversity are related (Ota and Abe, 2016; Tsai et al., 2013).

There are some promising attempts to link evolution and development with ecology to form the discipline of eco-evo-devo, or ecological evolutionary developmental biology (Abouheif et al., 2014; Gilbert et al., 2015). Eco-evo-devo reminds us of the statement by Lee Van Valen that 'Evolution is the control of development by ecology' (Van Valen, 1976). We could paraphrase Van Valen by asking: how does the environment influence development via natural selection to generate novel phenotypes? Here are two examples. Brown planthoppers (*Nilaparvata lugens*) are insects with short-winged and long-winged dimorphism influenced by environmental factors. This dimorphism has been related to the activity of two insulin receptors (InR1 and InR2) on wing bud growth (Xu and Zhang, 2017). The bichir fish (*Polypterus senegalus*) when raised on land, displays behavioral changes that emulate terrestrial locomotion, and anatomical changes that mirror the stem tetrapods of the Devonian period. This suggests that the bichir is a potential evo-devo model of developmental plasticity, which can further be integrated into the study of macroevolutionary change (Standen et al., 2014).

Bitterling have a remarkable brood parasite relationship with bivalves. This gives us an opportunity to use bitterlings model to study adaptation, co-evolution, and developmental plasticity, all in the context of parasitism and the interaction with the host. Bitterling embryos live in a complex environment influenced by both abiotic and biotic environmental factors. The host bivalves can dramatically narrow the space occupied by bitterling embryos by contracting their gill muscles, something which they do normally to regulate the dimensions of their water tube (Medler and Silverman, 2001). This pumping process can even eject embryos before the end of the parasitic period (Kitamura, 2005; Rouchet et al., 2017). When stressed or disturbed, bivalves retract their siphon, close their shell and bury themselves into sediments; therefore, the embryos are forced into a temporarily hypoxic environment (Aldridge, 1999; Smith et al., 2004). Interspecies differences in host preference, and species-specific embryonic adaptation, are potentially interesting evo-devo topics. Furthermore, host-parasite interaction is an ecological factor that influences bitterling development. In summary, the bitterling is a promising candidate model organism for eco-evo-devo research.

Aims of the thesis

First, we develop the bitterling as a unique, well-studied model organism in the area of the evolutionary ecology of brood parasitism. The bitterling-mussel relationship, interspecific mussel host preference, and mussel gill structure are studied in detail, to help understand the developmental adaptation of bitterling embryos in response to their mussel hosts. Further, the study of bitterlings is necessary to provide a better understanding of teleost developmental evolution, teleost evolutionary novelties and their developmental origin. Finally, bitterlings are interesting as evo-devo and eco-evo-devo model organisms. (**This Chapter**)

We revise and expand on the previously-published time-based staging systems by making producing new, character-based systems that are compatible with the widely-used zebrafish staging system. By using microCT, we identify a series of anatomic characters as development landmarks. This allows us to: (i) produce the first 3-D atlas of bitterling embryonic development (ii) update the normal developmental stages of *R. ocellatus* (iii) explore the development of the central neural system in detail. In all of these studies we produce digital 3-D models. (**Chapters 2 and 3**)

We also investigate the early brain regionalization of the rosy bitterling (*Rhodeus ocellatus*) by studying the expression pattern of marker genes *dlx2a*, *fgf8a*, *msx3*, *pax6a*, *shha*, and *sox9b* using whole-mount *in situ* hybridizations (WISH). This allows us to determine the formation of brain subdivisions. To test our hypothesis that the bitterling shows transcriptional heterochrony associated with brood parasitism, we investigate the compare the bitterling gene expression patterns with the zebrafish. (**Chapter 4**)

To study the morphogenetic process of *blastokinesis* in the bitterling embryo, and its possible relation to brood parasitism, we focus on early pre-hatching development and the hatching event of the rosy bitterling (*Rhodeus ocellatus*). We profile the expression of developmental regulatory genes *fgf8a*, *msx3*, *krt8* and *ctslb* by whole-mount *in situ* hybridization (WISH). This allows us to visualize morphogenetic movements during gastrulation and neurulation, and the process of body elongation during somitogenesis. We also analyze the hatching event by time-lapse photography, histology and microCT to explore the phenomenon of blastokinesis and its possible adaptive significance. (**Chapter 5**)

Finally, we summarize and discuss the preceding chapters, and highlight the overarching conclusions of the work described in this thesis. (**Chapter 6**)

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