Evolutionary developmental biology of bitterling fish

Yi, W.

Chapter 6 Summary and discussion

A tribute to landmark researches

Compared with modern results the wonder is, not that these early workers made mistakes, but that they made so few. (De Beer, 1937: p. 14)

Wenjing Yi, Michael K. Richardson

Institute of Biology, University of Leiden, Sylvius Laboratory, Sylviusweg 72, 2333BE, Leiden, the Netherlands.

Manuscript in preparation

In this chapter, we summarize the results of this thesis while reviewing previous landmark researches, and discuss how the results of this thesis connect scattered fragments from the past. The bitterlings, a special group of species that have drawn the attention of biologist, have been known for more than a century (Boeseman et al., 1938; Chang, 1948; Chang and Wu, 1947; Duyvené de Wit, 1955; Kitamura et al., 2012; Methling et al., 2018; Mills and Reynolds, 2003; Olt, 1893; Reichard et al., 2007; Rouchet et al., 2017; Smith, 2016; Wiepkema, 1962). Among all the biologist that focussed on bitterlings, Olt (1893) was the first to describe the developmental stages of the European bitterling embryo (*Rhodeus amarus*). His illustrations of the changing shapes of the yolk are still regarded as classics. **Chapter 2** of this thesis is a tribute to Olt's staging series (Olt, 1893) and to the work of Kim & Park (1985) and Nagata & Miyabe (1978).

I want to emphasize again the importance of staging descriptions for understanding development. Franz Keibel set up a paradigm for embryonic research by presenting normal plates, tables, and stages (Hopwood, 2007; Keibel, 1895). These provide detailed developmental data in a standard way that helps in the analysis of differences between ontogeny and phylogeny (Bininda-Emonds et al., 2002). The staging system is a way to organizing embryonic development and is the cornerstone of developmental and evolutionary research (Iwamatsu, 2004; Kunz, 2004; Richardson and Keuck, 2002; Wong et al., 2015). Development is a dynamic process. As Wilhem Roux wrote, 'Develompent is Change' (Roux, 1894). International cooperation and cross-disciplinary collaboration are possible only when staging characters are established, providing a specific time window for cross-species comparative studies (Kimmel et al., 1995; Signore et al., 2009; Werneburg, 2009). Our complete stage series of the bitterling species *R. ocellatus* In **Chapter 2** is a response to the call of Duyvené de Wit (1955) for realizing a broad research scheme including comparative embryology, endocrinology, ethology and taxonomy.

In **Chapter 3**, we described the neuroanatomy of bitterling for the first time, filling the gaps in the previous embryonic research in various bitterling taxa. Combined with the molecular analysis of brain early development in **Chapter 4**, brain development in the rosy bitterling is compared with that in the zebrafish. We found that there is a timing difference between head development and trunk development in the rosy bitterling vs. the zebrafish. Compared with previous bitterling embryonic research that focussed on phylogeny and classification (Kim, 2020; Suzuki, 2006; Suzuki and Hibiya, 1984a; Suzuki et al., 1989b), I have introduced the zebrafish (*Danio rerio*) as a comparison species to study developmental heterochrony. By taking advantage of the knowledge of the genetic background of the zebrafish (Kudoh et al., 2001; Thisse and Thisse, 2014; Thisse et al., 2004), and its development (Mork and Crump, 2015; Virta and Cooper, 2011; Whitfield et al., 2002), I have tried to provide an insight to the conserved aspects of teleost development while highlighting synapomorphies of the bitterling.

Olt (1893) mentioned that the embryos that he found in the mussel gills were, without exception, oriented with their heads towards the blind end of the gill, and their tails oriented towards the gill duct. In this way, the bitterling can remain safely in the gill by means of its wing-like YSEs. Olt (1893) believed that the embryo's orientation is caused by gravity; by contrast, Chang & Wu (1947) refuted the gravity hypothesis through experiments. The research of (Chang and Wu, 1947) is an important landmark because it pioneers the study of the morphogenesis of *R. ocellatus*. Those authors

proposed that blastokinesis is the reason for embryo's rotation in the chorion. The rotation occurs in the same way, no matter how the influence of gravity changes.

In **Chapter 5**, I studied blastokinesis by means of molecular markers: *fgf8a*, a marker of the embryonic shield; and msx3, a marker of the neural ectoderm boundary. My conclusion is that bitterling-specific blastokinesis is convergent with insect blastokinesis. Compared to the well-known blastokinesis of insects (Panfilio, 2008; Panfilio et al., 2006), the direction of embryo displacement during bitterling blastokinesis is reversed. Thus, there is a 'backflip' in the milkweed bug (*Oncopeltus fasciatus*) vs. a 'frontflip' in the rosy bitterling. The bitterling-specific blastokinesis is essentially a convergent extension process (D'Amico and Cooper, 2001; Tada and Heisenberg, 2012). It is noticeable that the convergent extension migration of cells in the rosy bitterling takes place on an irregular yolk mass shaped like an inverted balloon. Therefore, I speculate that the special features of bitterling blastokinesis are related to changes in the axial orientation of the cells as they migrate over the irregular yolk mass.

In the future, my research outlook will be: 1) to introduce more closely-related species to the species pool, including *Tanakia* and *Acheilognatus* sp. For these sister groups, thorough embryonic research is necessary to facilitate comparative studies and help us answer how they have adapted to their brood parasitic life history. One thing that needs special investigation is the molecular regulation mechanism of YSEs development; 2) to trace cell migration *in toto* during the blastokinesis period, and at the same time manipulate the expression of genes that control the axial migration of cells using gene editing.

Techniques to study bitterling development

In this thesis, I applied a variety of techniques to study the development of a single species. Therefore, it is necessary to summarize the advantages of different techniques and integrate them into a combined protocol for future studies. I recommend time-lapse video as the first step of any embryonic research. It is useful for the recording of dynamic processes, for example heart rate, tracking body movements and tracing the establishment of the blood circulation (**Chapter 2**). More importantly, tracking the hatching moment of the rosy bitterling in real time helped me hypothesise that the hatching process is mechanical rather than enzymatic. In addition, by regularly recording embryo dynamics before hatching, I became aware of the body rotation movements inside the chorion, a part of the bittlerling-specific blastokinesis, which is otherwise easily overlooked (**Chapter 5**).

MicroCT is helpful for the observation of external morphology and morphological staging characters. Numerically indexed characters such as somite number (from counting the somite/myotome boundaries) and prim number (by discerning the leading, posterior end of the posterior lateral line primordium during its caudal migration) were only observable with the help of MicroCT (**Chapter 2**). The three-dimensional (3-D) images obtained by microCT are like a spatial navigation system, and are extremely useful for anatomical analyses of the complex brain structures. To gain insight into the morphogenesis of the bitterling brain, I analyzed the formation of the brain ventricular system in three-dimension from stage *1-ovl* to *long-pec* (**Chapter 3**). Furthermore, microCT has the capacity to indicate the location of proliferative zones. It provides an updating of traditional modalities (e.g., histology) for future comparative studies of the teleost brain.

I also note the limitations of microCT. First of all, the virtual sections have a limited resolution, much less than that of conventional histological sections (Figure 1).). Secondly, tissue specific staining is not currently feasible in routine microCT.

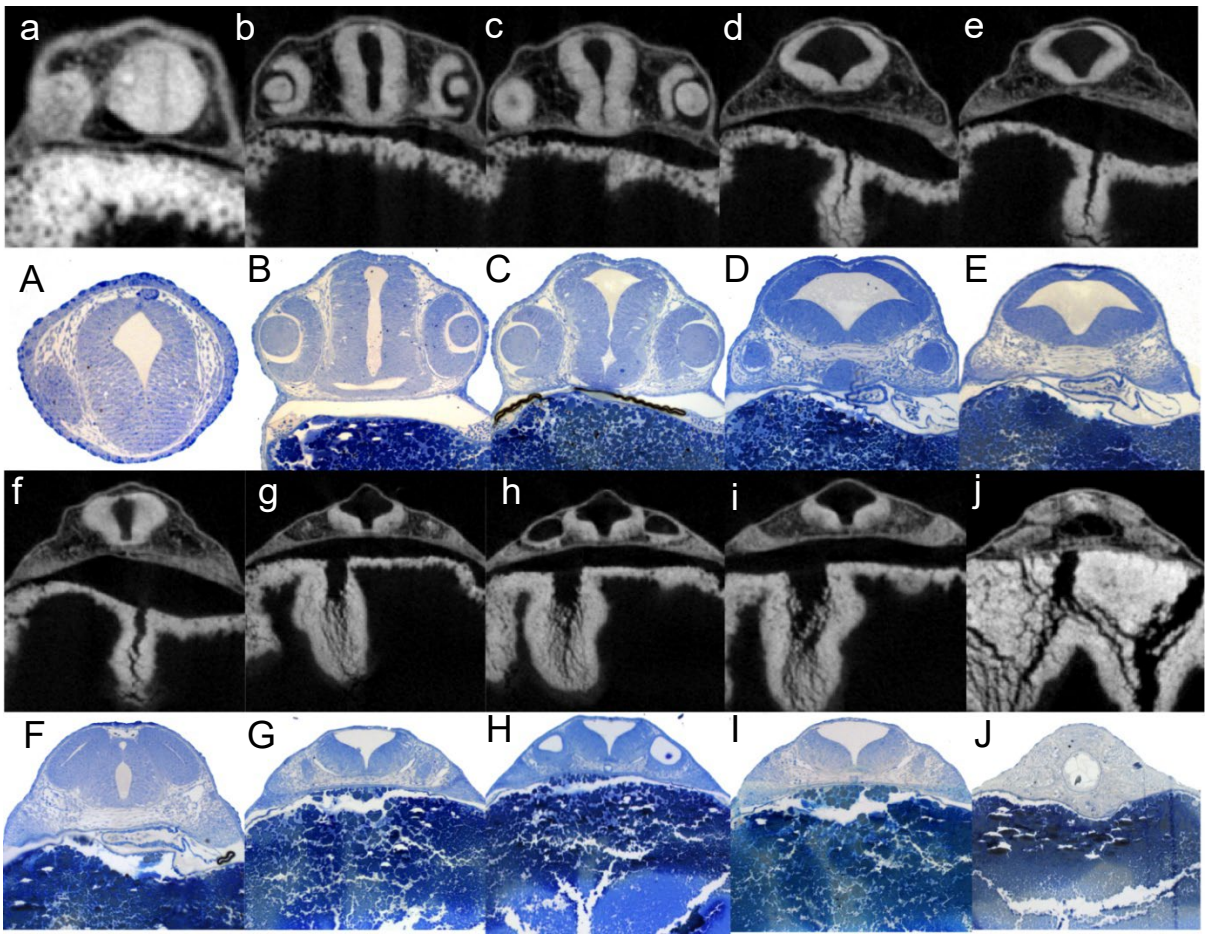

Figure 1 *Rhodeus ocellatus*, virtual microCT sections compared to histological sections. a to j: microCT virtual sections, 78.5 hpf. A to J: epon embedded sample stained with toluidine blue, 3 dpf. Transverse sections from rostral to caudal at the level of olfactory placode (a and A), optic stalk and forebrain ventricle (b and B), optic cup (c and C), midbrain ventricle (d, e and D, E), midbrain hindbrain boundary (f and F), hindbrain ventricle (g and G), otic vesicle (h and H), notochord (i and I), and myotome (j and J).

Nonetheless, microCT is time-efficient, non-destructive and the counter staining using PTA is reversible and does not preclude subsequent histological staining (Keklikoglou et al., 2019). In terms of technical procedures, CT scanning is highly recommended as the next step of *in vivo* research, to be used before routine histology or other destructive methods. Our study demonstrates the value of microCT in developmental biology. For species, like the rosy bitterling, that were previously difficult to study because of limited material, microCT scans provide a wealth of morphological data and readily yield 3-D information. In addition, microCT has the capacity of visualizing and analyzing specimen digitally, which facilitate data sharing and the reuse of the digital data for comparative studies (Davies et al., 2017).

Wholemount *in situ* hybridization (WISH) is the technique I used to study temporal and special gene expression patterns during bitterling development. In **Chapter 4**, the brain segmental boundaries are marked by discrete gene expression domains the early embryo. At these early stages,

boundaries are not discernible using microCT or histology. For example, the midbrain-hindbrain boundary (MHB) is distinctly marked by *fgf8a* expression in the early embryo at 30 hpf (hatching). The initial migration of the neural crest cells marked by the *dlx2a* expression, began at 50 hpf. These genoarchitectonic boundaries are based on highly conserved gene expression patterns, which are related directly to the causal mechanisms that create the relevant morphological subdivisions (Puelles and Ferran, 2012; Schredelseker and Driever, 2020). WISH therefore provides an opportunity to understand the causal mechanisms from genomic control to the boundaries that were defined by gross morphology (e.g., ventricular sulci and cytoarchitecture).

The molecular marker method also provides an opportunity for understanding the blastokinesis (**Chapter 5**), and early embryonic development of the bitterling *R. ocellatus*. The expression data suggest that blastokinesis in bitterlings is based on the convergent-extension movements of the blastoderm cells during gastrulation and neurulation. Our study can potentially identify candidate genes that regulate blastokinesis, but functional studies are needed to make the identification definitive. My hypothesis is that blastokinesis is morphogenetic movement which results from collective cell migration on the anterior-posterior and dorsal-ventral axes. In most cases, the hatching embryo is located with its head towards the vegetal side, as a result of the cell migration. Heterochrony (a change in developmental timing) can potentially modify blastokinesis; by prolonging or delaying convergent extension movement, it is possible that the embryo could hatch from the animal pole of the chorion or the head could only migrate halfway.

In summary, innovative research techniques have brought us new perspectives and have updated our understanding of the development of bitterlings. In the future, new techniques such as *in toto* imaging (Bassi et al., 2015), which is capable of quantitative analysis of cell shape changes and the orientation of cell divisions, will provide an opportunity to understand the regulation of blastokinesis. However, embryonic development is dynamic and complex process. Before observing embryos at single-cell resolution, I recommend starting with time lapse observations and constructing a 3D model in order to form a global view.

Summary of the comparison with zebrafish development

In **chapters 2, 3 and 4**, we compared heterochrony (changes in development timing) and transcriptional heterochrony (changes in the timing of gene expression) between the rosy bitterling and the zebrafish. The comparison indicated evolutionary adaptations related to the bitterling's brood parasitic lifestyle. These adaptations are summarized as follows:

I identified developmental delays in retinal pigmentation and pectoral fin development (in *R. ocellatus* compared to the zebrafish *D. rerio*). Possible explanations for these delays are: 1) the bitterling embryos and larvae develop in a dark, enclosed and sheltered environment, with no need for retinal photosensitivity. This is comparable, perhaps to the lack of retinal pigmentation in cave fish (Yamamoto et al., 2004). 2) **the** motility of bitterling is restricted while they are developing in the gill water-tube of their host mussel, and the fin is therefore effectively functionless.

A developmental advance is conspicuous in the development of the inner ear. The morphogenesis of the semicircular canals, the separation of the lagena from the sacculolagenar pouch and the formation of the asteriscus otolith are all pre-displaced in bitterling development. This

predisplacement may be related to embryonic development in a dark environment where hearing is more useful than vision. This in turn would also explain why visual developent appears to be delayed in the bitterling.

For transcriptional heterochrony, If I just compare the expression patterns and timing in the brain region, there are comparable developmental stages in the bitterling and zebrafish. But if the comparison expands to the whole embryo, including the pectoral fin bud and myotomes, such comparable developmental stages do not exist. It is obvious that the development of pectoral fins lags behind the development of the brain in both species. My explanation is that development is modular; each module has its independent rhythm or autonomous growth. Just as there are different time zones on the earth, the time zone of the brain region is several hours earlier than the trunk region. Even the ticking of the clock may be faster or slower. The high level of timing changes (heterochrony) between developmental modules is an important evolutionary mechanism that has been shown to underlie phenotypic evolution (Bininda-Emonds et al., 2002; Bininda-Emonds et al., 2003; Olaf R. P. et al., 2003; Richardson, 1995).

References

Bassi, A., Schmid, B. and Huisken, J. (2015). Optical tomography complements light sheet microscopy for in toto imaging of zebrafish development. *Dev.* **142**, 1016–1020.

Bininda-Emonds, O. R. P. P., Jeffery, J. E., Coates, M. I. and Richardson, M. K. (2002). From Haeckel to event-pairing: the evolution of developmental sequences. *Theory Biosci.* **121**, 297–320.

Bininda-Emonds, O. R. P., Jeffrey, J. E. and Richardson, M. K. (2003). Is Sequence Heterochrony an Important Evolutionary Mechanism in Mammals? *J. Mamm. Evol.* **10**, 335–361.

Boeseman, M. J., Van der Drift, J., Van Roon, J. M., Tinbergen, N. and Ter Pelkwijk, J. J. (1938). De bittervoorns en hun mossels. *Levende Nat.* **43**, 129–136.

Chang, H. W. (1948). Life history of the common Chinese bitterling, Rhodeus ocellatus. *Sinensia* **19**, 12–22.

Chang, H. W. and Wu, H. W. (1947). On the blastokinesis occurring in the egg of the common Chinese Bitterling, Rhodeus ocellatus. *Sinensia* **17**, 15–22.

D'Amico, L. A. and Cooper, M. S. (2001). Morphogenetic domains in the yolk syncytial layer of axiating zebrafish embryos. *Dev. Dyn.* **222**, 611–624.

Davies, T. G., Rahman, I. A., Lautenschlager, S., Cunningham, J. A., Asher, R. J., Barrett, P. M., Bates, K. T., Bengtson, S., Benson, R. B. J., Boyer, D. M., et al. (2017). Open data and digital morphology. *Proc. R. Soc. B Biol. Sci.* **284**, 20170194.

De Beer, G. R. (1937). *The development of the vertebrate skull*. Oxford: Clarendon Press.

Duyvené de Wit, J. (1955). Some results of investigations into the European Bitterling, Rhodeus amarus BLOCH. *Japanese J. Icthyology* **4**, 94–104.

Hopwood, N. (2007). A history of normal plates, tables and stages in vertebrate embryology. *Int. J. Dev. Biol.* **51**, 1–26.

Iwamatsu, T. (2004). Stages of normal development in the medaka Oryzias latipes. *Mech Dev* **121**, 605–618.

Keklikoglou, K., Faulwetter, S., Chatzinikolaou, E., Wils, P., Brecko, J., Kvaček, J., Metscher, B. and Arvanitidis, C. (2019). Micro-computed tomography for natural history specimens: a handbook of best practice protocols. *Eur. J. Taxon.* 1–55.

Kim, H. S. (2020). Minute tubercles in bitterling larvae: Developmental dynamic structures to prevent premature ejection by host mussels. *Ecol. Evol.* **10**, 5840–5851.

Kim, Y. U. and Park, Y. S. (1985). Egg development and larvae of the rose bitterling Rhodeus ocellatus (KNER). *Korean J. Fish. Aquat. Sci.* **18**, 586–593.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253–310.

Kitamura, J., Nagata, N., Nakajima, J. and Sota, T. (2012). Divergence of ovipositor length and egg shape in a brood parasitic bitterling fish through the use of different mussel hosts. *J. Evol. Biol.* **25**, 566–573.

Kudoh, T., Tsang, M., Hukriede, N. A., Chen, X., Dedekian, M., Clarke, C. J., Kiang, A., Schultz, S., Epstein, J. A., Toyama, R., et al. (2001). A gene expression screen in zebrafish embryogenesis. *Genome Res.* **11**, 1979–1987.
Kunz, Y. W. (2004). *Developmental Biology of Teleost Fishes*. Dordrecht: Springer Netherlands.
Methling, C., Douda, K., Liu, H., Rouchet, R., Bartáková, V., Yu, D., Smith, C. and Reichard, M. (2018). Energetic costs in the relationship between bitterling and mussels in East Asia. *Biol. J. Linn. Soc.* **125**, 750–759.
Mills, S. C. and Reynolds, J. D. (2003). The bitterling-mussel interaction as a test case for co-evolution. *J. Fish Biol.* **63**, 84–104.
Mork, L. and Crump, G. (2015). Zebrafish Craniofacial Development. In *Current Topics in Developmental Biology*, pp. 235–269.
Nagata, Y. and Miyabe, H. (1978). Development Stages of the Bitterling, *Rhodeus ocellatus ocellatus* (Cyprinidae). *Mem. Osaka Kyoiku Univ. III, Nat. Sci. Appl. Sci.* **26**, 171–181.
Olaf R. P., B.-E., Jonathan E., J. and Richardson, M. K. (2003). Inverting the hourglass: quantitative evidence against the phylotypic stage in vertebrate development. *Proc. R. Soc. London. Ser. B Biol. Sci.* **270**, 341–346.
Olt, A. (1893). Lebensweise und Entwicklung des Bitterlings. *Zeitschrift für wissenschaftliche Zool.* **55**, 543–575.
Panfilio, K. A. (2008). Extraembryonic development in insects and the acrobatics of blastokinesis. *Dev. Biol.* **313**, 471–491.
Panfilio, K. A., Liu, P. Z., Akam, M. and Kaufman, T. C. (2006). Oncopeltus fasciatus zen is essential for serosal tissue function in katatrepsis. *Dev. Biol.* **292**, 226–243.
Puelles, L. and Ferran, J. L. (2012). Concept of neural genoarchitecture and its genomic fundament. *Front. Neuroanat.* **6**, 47.
Reichard, M., Liu, H. and Smith, C. (2007). The co-evolutionary relationship between bitterling fishes and freshwater mussels: insights from interspecific comparisons. *Evol. Ecol. Res.* **9**, 239–259.
Richardson, M. K. (1995). Heterochrony and the phylotypic period. *Dev Biol* **172**, 412–421.
Richardson, M. K. and Keuck, G. (2002). Haeckel's ABC of evolution and development. *Biol. Rev. Camb. Philos. Soc.* **77**, 495–528.
Rouchet, R., Smith, C., Liu, H. Z., Methling, C., Douda, K., Yu, D., Tang, Q. Y. and Reichard, M. (2017). Avoidance of host resistance in the oviposition-site preferences of rose bitterling. *Evol. Ecol.* **31**, 769–783.
Schredelseker, T. and Driever, W. (2020). Conserved Genoarchitecture of the Basal Hypothalamus in Zebrafish Embryos. *Front. Neuroanat.* **14**, 3.
Signore, I. A., Guerrero, N., Loosli, F., Colombo, A., Villalón, A., Wittbrodt, J. and Concha, M. L. (2009). Zebrafish and medaka: Model organisms for a comparative developmental approach of brain asymmetry. *Philos. Trans. R. Soc. B Biol. Sci.* **364**, 991–1003.
Smith, C. (2016). Bayesian inference supports the host selection hypothesis in explaining adaptive host specificity by European bitterling. *Oecologia* **183**, 1–11.
Suzuki, N. (2006). Egg and larval development of the bitterling, Rhodeus pseudosericeus (Cyprinidae). *Japanese J. Ichthyol.* **53**, 47–54.
Suzuki, N. and Hibiya, T. (1984). Development of Eggs and Larvae of Two Bitterlings, Rhodeus atremius and R. suigensis (Cyprinidae). *Japanese J. Ichthyol.* **31**, 287–296.
Suzuki, N., Umezawa, K., Yabe, T. and Murai, H. (1989). Development of the Bitterling, Paracheilognathus-Himantegus (Cyprinidae), with a Note on Minute Tubercles on the Skin Surface. *Japanese J. Ichthyol.* **36**, 318–326.
Tada, M. and Heisenberg, C.-P. (2012). Convergent extension: using collective cell migration and cell intercalation to shape embryos. *Development* **139**, 3897–3904.
Thisse, B. and Thisse, C. (2014). In Situ Hybridization on Whole-Mount Zebrafish Embryos and Young Larvae. In *Methods in Molecular Biology*, pp. 53–67.
Thisse, B., Heyer, V., Lux, A., Alunni, V., Degrave, A., Seiliez, I., Kirchner, J., Parkhill, J. P. and Thisse, C. (2004). Spatial and temporal expression of the zebrafish genome by large-scale in situ hybridization screening. *Methods Cell Biol* **77**, 505–519.
Virta, V. C. and Cooper, M. S. (2011). Structural components and morphogenetic mechanics of the zebrafish yolk extension, a developmental module. *J. Exp. Zool. Part B Mol. Dev. Evol.* **316 B**, 76–92.
Werneburg, I. (2009). A Standard System to Study Vertebrate Embryos. *PLoS One* **4**, e5887.
Whitfield, T. T., Riley, B. B., Chiang, M.-Y. and Phillips, B. (2002). Development of the zebrafish inner ear. *Dev. Dyn.* **223**, 427–458.
Wiepkema, P. R. (1962). An Ethological Analysis of the Reproductive Behaviour of the Bitterling (*Rhodeus Amarus* Bloch). *Arch. Néerlandaises Zool.* **14**, 103–199.

Wong, M. D., Van Eede, M. C., Spring, S., Jevtic, S., Boughner, J. C., Lerch, J. P. and Mark Henkelman, R. (2015). 4D Atlas of the Mouse Embryo for Precise Morphological Staging. *Dev.* **142**, 3583–3591.
Yamamoto, Y., Stock, D. W. and Jeffery, W. R. (2004). Hedgehog signalling controls eye degeneration in blind cavefish. *Nature* **431**, 844–847.