Adaptations to the Brood Care Paradigm in the Shell-dwelling Cichlid *Lamprologus ocellatus*

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Dissertation zur Erlangung des naturwissenschaftlichen Doktorgrades „Doctor rerum naturalium“ (Dr. rer. nat.) an der Fakultät für Biologie der Ludwig-Maximilians-Universität München

Munich, June 2024

Diese Dissertation wurde angefertigt
unter der Leitung von Prof. Dr. Herwig Baier
im Bereich von Max Planck Institute für Biological Intelligenz
an der Ludwig-Maximilians-Universität München

Erstgutachter: Prof. Dr. Herwig Baier

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Tag der Abgabe: 12.06.2024

Tag der mündlichen Prüfung: 04.02.2025

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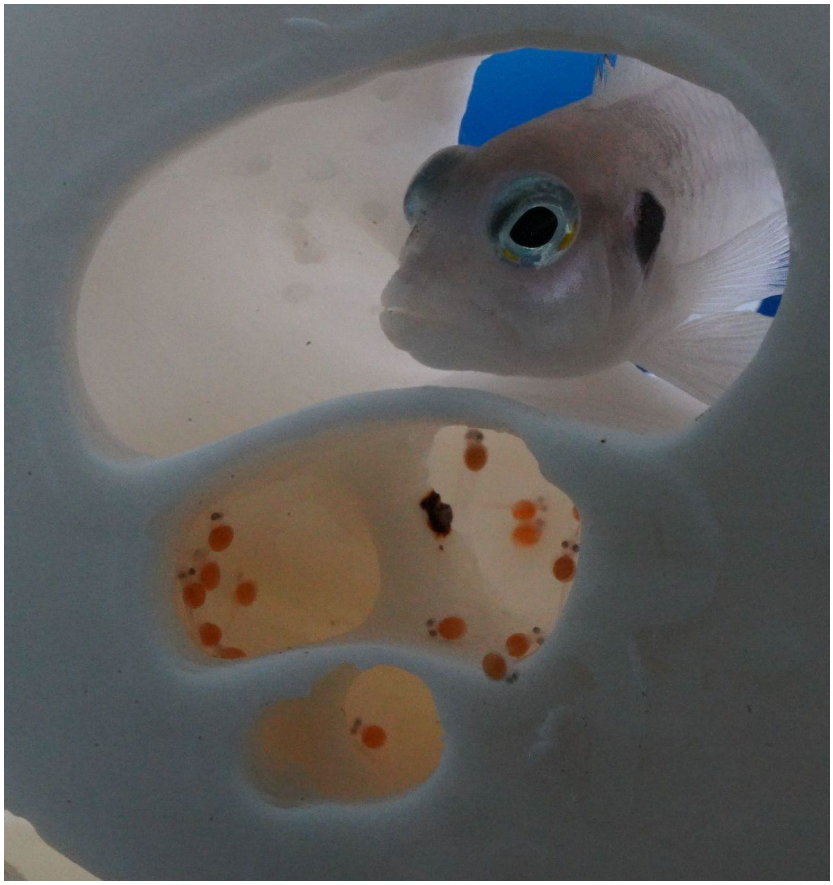
München, den 22.02.2025

Ash Parker

Dedicated to my mom,
who parented me with utmost care

In the end, we will conserve only what we love; we will love only what we understand; and we will understand only what we are taught.

- Baba Dioum



Parts of this thesis have been published in:

Parker, A. V., Stemmer, M., Grätsch, S., Dorigo, A., Ramirez, O. R., Adel, A., ... & Baier, H. (2025). **Intrinsic timing of brood care in shell-dwelling cichlids**. Current Biology, 35(3), 672-680.

Summary

The evolution of parental care in animals is influenced by ecological, evolutionary, and environmental factors. Birds and mammals are typically studied for their elaborate and prolonged care, but systems where parental care evolved independently, such as in cichlids, offer valuable insights into the diversity of these behaviours. Cichlids exhibit a wide range of parental strategies, making them an excellent model for studying the evolution of parental care. This thesis focuses on *Lamprologus ocellatus*, a small shell-dwelling cichlid with a unique brood care strategy where females lay eggs in abandoned snail shells, providing a protected environment until the larvae become free-swimming.

I developed a laboratory paradigm to observe and analyse interactions between mother and larvae within 3D-printed 'window' shells. This approach enabled detailed monitoring of the spatial distribution of fry and mother, and continuous recording of parent-offspring interactions over an 11-day period, from fertilisation to late-larval stages. The mother actively cares for the offspring, leading up to their emergence from the shell at 9 days post-fertilisation. Remarkably, this emergence time coincides with a switch in phototaxis behaviour from dark preference to seeking light in the larvae. We were able to delay larvae emergence by using a foster mother whose biological offspring were younger, causing a conflict between the larvae's natural emergence time and the foster mother's intrinsic timer. This study shows that larval and maternal behaviours in *L. ocellatus* are governed by independent internal timing mechanisms, usually synchronised but can conflict through experimental manipulations. Comparing the brood care strategies of shell-dwellers like *L. ocellatus* to other parental care strategies in Lake Tanganyika—ancestral substrate brooding and derived mouthbrooding—I found shell-dwellers have adapted to a unique niche, exhibiting parallels with both groups. Convergent strategies shared with mouthbrooders, such as mating strategies and clutch size constraint, may arise from larvae growing up in confined spaces. Conversely, traits reminiscent of substrate brooders, such as smaller egg sizes, larval anatomy and behaviours, and maternal interactions highlight the influence of evolutionary history. In conclusion, this thesis provides the first comprehensive delineation of the brood care paradigm of shell-dwelling cichlids, shedding light on the behavioural adaptations and development of *L. ocellatus*. By introducing *L. ocellatus* as a novel model organism, this research offers valuable insights into the mechanisms of parent-offspring coordination during brood care, contributing to our understanding of the evolution of parental care in animals.

Acknowledgements

First and foremost, I would like to thank Herwig for hiring me and trusting me with this exciting project in the lab, as well as for funding it. I am equally grateful to Manuel Stemmer, with whom I worked closely in establishing the cichlids as a new model in the lab. His expertise in fish work and construction was instrumental to my project. Special thanks also to Krasimir, Karin, and the Tecniplast team for leading the construction of the cichlid facility, allowing us the freedom to design optimal habitats. This project could not have started without our close collaborator, Alex Jordan, who provided many fish and invaluable knowledge.

I am most grateful to the incredible Team Cichlid: Swanni, Manuel, Alessandro, Oriol, Abdel, and Helene. From weekly meetings, coding support, building software and setups, to advice and proofreading manuscripts, your dedication went above and beyond. Thanks to Joel for helping develop the essential tile plot for my project, and to IMPRS-LS and my TAC team—Manfred Gahr, Alex Jordan, Nicolas Gompel, and Herwig—for their guidance and suggestions.

I would also like to thank the past and present members of the Baier lab. The Caterpillar Lunch Club (Swanni, Enrico, Laurids, Lukas, and Robert) provided much-needed healthy meals. Thanks to Enrico, Irene, and Krasimir for handling orders, Anne for support and to Martin P and Greg for their endless patience and help. The input from the entire Baier Lab during meetings significantly shaped this project. The camaraderie and fun times with colleagues, such as the quiz lunch crew (and uncle Ian), Max-Planckers and cooking-competition team made the lab an enjoyable place to work. A special thank you to Qing, my wonderful office mate and friend, for her wisdom, humour, and support. Throughout the writing of this thesis I greatly appreciated the editing efforts of Qing, Martin P, Swanni, Alessandro, Manuel, the weekly walks with Amalia, and the nutritional support from Jasmine and Lachie.

Finally, I want to acknowledge my life outside the lab which was instrumental to my happiness and stability during my PhD. My close friends TJ, Swanni, Katja, Leo, Shachar, Manu and Maria, the psychoanalytic crew, Rohan, Shriya, Qing, Knut, Ben, Joel, Lachie and Jas who supported me from near and far at various stages of my PhD. Parkrun and the SRC community made Munich a home and gave a feeling of belonging. Most importantly, I thank my mom, my rock and biggest supporter, who has always been impressed by even my smallest achievements and been on the other side of the phone in all the highs and lows.

Contents page

List of figures

Acronyms

°C	Degrees celsius
3D	Three dimensional
cm	Centimeter
CSV	Comma separated value
dpf	Day/s post fertilisation
Fig.	Figure
L.	Lamprologus
m	metres
m²	Metres squared
mA	Milliamps
mAP	Mean average precision
ml	Mililitres
mm	Millimeters
NIR	Near infrared
nm	nanometer
no.	Number
ROI	Region of interest
sd	Standard deviation
v	version
YOLO	You only look once
µS	microSiemens

Chapter 1: Introduction

1.1 Parental Care in Animals

Organisms exhibit remarkable parental care diversity across the animal kingdom, from minimal efforts to highly elaborate strategies aimed at enhancing offspring survival. Parental investment can take many forms (Balshine, 2012; Smiseth et al., 2012): some species just provide initial nourishment through larger yolks or placenta-like structures (Fig. 1A), while others build nests or select safe sites for egg-laying to protect their offspring from predators and harsh environments (Fig. 1B-E). Furthermore, some parents remain with their eggs or offspring to guard them (Fig. 1C-G), while others continue to provide food, care and teaching long after hatching or birth (Fig. 1E-G). This care can be provided by the mother (Fig. 1B, C & F), the father (Fig. 1D), both parents (Fig. 1E), or even cooperatively with the help of non-parental group members (Fig. 1G), highlighting the complexity and adaptability of parental strategies across different species.

Figure 1: Diversity of parental care across the animal kingdom. A) Common garden snail (*Cornu aspersum*) laying yolk-filled eggs to nourish developing embryos. **B)** Cape rain spider (*Palystes superciliosus*) nest created from silk and leaves by the mother and suspended in shrubbery to protect the eggs within. **C)** *Lamprologus ocellatus* female guarding her eggs inside a nest created around an abandoned snail shell. **D)** Plain midshipman (*Porichthys notatus*) male guarding his orange and yellow eggs laid on the underside of a rock. **E)** Male greater frigatebird (*Frigata minor*) standing guard over his chick sitting atop their nest. **F)** Galápagos Sea Lion (*Zalophus wollebaek*i) female playing with her pup. **G)** African elephant (*Loxodonta africana*) herd consisting of a matriarch and her female relatives collectively caring for the younger elephant calves. Photographs taken by Pippa Parker (A & B), myself (C & G), and Konstantin Holzner (E & F).

Parental care in most species likely involves a mixture of fixed-action patterns and context-dependent behaviours, enabling parents to instinctively respond to certain stimuli while also adapting their strategies based on environmental conditions and the specific needs of their offspring. Tinbergen's classic experiment with geese demonstrated a fixed-action pattern component of parental behaviour (Tinbergen, 1951), showing that when provided with any egg-like object outside the nest, geese (*Anser anser*) will engage in a stereotyped motion to roll this 'egg' back into place. However, parents often have to balance many competing demands such as feeding, guarding and cleaning while adapting to external factors, both social (eg. offspring begging, partners involvement) and non-social (eg. food, predators, environment; Royle et al., 2014). For example, Westneat et al. (2011) showed that in house sparrows (*Passer domesticus*), male nest-visiting rate changed depending on brood size and time of day, while that of females adapted feeding as the nestling aged. For both sexes, visiting rates adapted to that of their partner's. This adaptability is crucial, particularly as the demands on parents change as their offspring grow and develop (Kelly et al., 2017).

1.1.1 Factors Influencing Parental Care Evolution

The evolution of parental care is shaped by a complex interplay of factors that contribute to the diversity and adaptability of care strategies across species. Initially, the transition from no care to some form of parental care is driven by high offspring need, which arises in extreme environments, such as those with high levels of environmental stress and predation (Klug & Bonsall, 2010; Reyes et al., 2016). Parental care is more likely to evolve under these conditions because offspring survival is significantly enhanced by the presence of care. The extent and type of care provided are then influenced by evolutionary history, reproductive strategies, and environmental factors. Understanding these influences helps to inform how parental care has evolved to optimise offspring survival and reproductive success.

1.1.1.1 Evolutionary history

The evolutionary history of a species or group greatly shapes its parental care strategies. For example, all mammals and almost all bird species practise extensive parental care, a trait long embedded in their evolutionary lineage. This care includes gestation and lactation in mammals and incubation and feeding in birds (Balshine, 2012). In contrast, parental care has evolved independently multiple times in other lineages, such as reptiles, amphibians, fishes, and

invertebrates, often driven by specific ecological pressures rather than a shared ancestral trait (Balshine, 2012). In ray-finned fishes alone, phylogenetic analyses suggest that the independent emergence of parental care has occurred at least 33 times (Mank et al., 2005). A long-standing evolutionary history may impose constraints on the evolution of parental care strategies. For example, in mammals, the necessity of milk provision by the mother ensures that females must always play a role in parental care for a significant period. In contrast, species that have only recently evolved parental care have a broader array of options and extents for providing care.

1.1.1.2 Reproductive strategy

Differences in parental care among species are intricately linked to their mating systems and reproductive strategies (Balshine, 1991; Kuwamura, 1986; Trivers, 1972). Species that produce many offspring often exhibit less intensive parental care per individual, focusing on quantity over quality (Pianka, 1970). In contrast, species with fewer offspring invest significantly more in each, providing extensive care to ensure survival. Monogamous species usually display biparental care, where both parents contribute to raising the young, a strategy typically seen in bird species (Balshine, 1991; Tumulty et al., 2014). Polygynous systems, on the other hand, might see reduced paternal care, with males investing more in mating opportunities than in offspring rearing.

Generally, maternal care is more common across taxa, especially in mammals and reptiles, whereas paternal care is prevalent in amphibians and more common in fishes (Balshine, 2012; Royle et al., 2016). Factors like anatomy, adult mortality rates, adult sex ratios and reproductive success can all shape sex-specific behaviours and influence which sex takes over parental care (Balshine, 1991; Royle et al., 2016; J. M. Smith, 1977). Across evolutionary history, transitions between mating strategies and care roles are relatively common (Klug et al., 2013). For instance, in ray-finned fishes, transitions between maternal, paternal, and biparental care have arisen up to nine times (Mank et al., 2005). Despite evolutionary history constraints on mammalian parental care, primates have experienced up to 23 transitions from maternal to biparental care and 3–8 transitions from biparental to maternal care (Reynolds et al., 2002). This evolutionary link between mating behaviour and parental care is a feedback relationship; mating opportunities dictate mating strategy, influencing parental care patterns, which in turn affect future mating opportunities (Székely et al., 2000).

1.1.1.3 Ecological influences

Ecological factors, including predation pressure, food availability, and habitat conditions are influential in shaping parental care behaviours. High predation risk drives the evolution of protective behaviours such as guarding or brooding to improve offspring survival (Smiseth et al., 2012). For example, researchers showed that a subpopulation of long-tailed skink *(Eutropis longicaudata*), a species not normally practising parental care, has evolved egg-guarding in response to the presence of egg-eating snakes (Pike et al., 2016). Food availability also impacts parental investment; in resource-rich environments, parents can afford to invest more in fewer offspring, while in resource-poor environments, producing numerous offspring with minimal care may be advantageous (Lavery & Kieffer, 1994; Townshend & Wootton, 1985).
Habitat conditions and resource availability influence nest-building and parental care strategies. For example, breeding pool size has been shown to drive the evolution of biparental care and monogamy in two Peruvian poison frog species (*Ranitomeya imitator* and *R. variabilis).* Small pools, inhabited by *R. imitator*, necessitate both parents' involvement to ensure tadpole survival, leading to the evolution of monogamy (J. L. Brown et al., 2010). Additionally, harsh environmental conditions, such as hypoxia and desiccation, influence the evolution of parental care behaviours. A notable example is the bromeliad crab (*Metapaulias depressus*), where females deposit snail shells into bromeliad pools, in which they spawn, to neutralise low pH levels and increase calcium carbonate in the water, thus enhancing offspring development (Diesel, 1989).

1.1.1.4 Parent-offspring interactions

"Parental care is a co-evolutionary game played by the whole family" (Royle, 2016). This quote highlights the interplay between parental and offspring behaviours that shapes the evolution of care strategies, naturally affecting both parental and offspring fitness. This mutual dependency creates a dynamic where changes in one party drive adaptations in the other (Royle 2016). Both parents and offspring act as agents and targets of selection, creating a complex evolutionary path where offspring growth and survival hinge on parental resource provision, which in turn may depend on the intensity of offspring signalling (Lock, 2004).

Studies in parental care have largely focused on the adaptations and behaviours of parents, neglecting those benefiting offspring. To fully understand how evolution has built and sculpted this paradigm in nature, one should look at both sides of the system. The co-ordination of parent and offspring behaviours is an important factor for the successful production of the next

generation in a species (Lock et al., 2004; Mousseau & Fox, 1998). For example, in the burying beetle (*Nicrophorus vespilloides*) offspring have co-evolved begging behaviours alongside the parental behaviour of direct food provisioning via regurgitation, the latter behaviour reliant on the offspring's cue (Lock et al., 2004). Another excellent scenario where interplay between parents and offspring is required is fledging or emergence, the transition of altricial young from the protective nest to the outside world (Jones et al., 2020; Naef-Daenzer & Grüebler, 2016).

This transition from the nest has been extensively studied in avian species, with long-standing debates among scientists about the influence of parents on the timing of offspring fledging (Johnson et al., 2004; Jones et al., 2020; Naef-Daenzer & Grüebler, 2016; Nilsson & Svensson, 1993). Parents may benefit from their young fledging early, reducing the time and care needed for the next brood (Johnson et al., 2017). However, extending the nest period can enhance offspring fitness. Currently in songbirds, two hypotheses are debated among scientists: the parent manipulation hypothesis, where parents and offspring are in conflict over the optimal fledging age (Jones et al., 2020; Martin et al., 2018), and the nestling choice hypothesis, that suggests fledging occurs when offspring reach a developmental threshold (Johnson et al., 2004, 2017; Nilsson & Svensson, 1993). Notably, research on this topic in non-avian species remains limited.

1.2 Parental Care in Cichlids

One non-avian system extensively studied for its parental care is the cichlid species flock of the family Cichlidae within the order Perciformes (Balshine & Abate, 2021; M. H. A. Keenleyside, 1991; Sefc, 2011). Over 3000 species of cichlid are found in tropical and subtropical freshwater systems across Africa, Central and South America, and parts of Asia (Koblmüller et al., 2008). The greatest diversity of cichlids is found in East Africa's Great Lakes—Victoria, Malawi, and Tanganyika—where they have undergone adaptive radiation (Fig. 2; Ronco et al., 2021; Salzburger & Meyer, 2004). This has resulted in a wide variety of species with diverse morphologies and behaviours, making these lakes key areas of study in evolutionary biology, ecology, and ethology (Kocher, 2004).

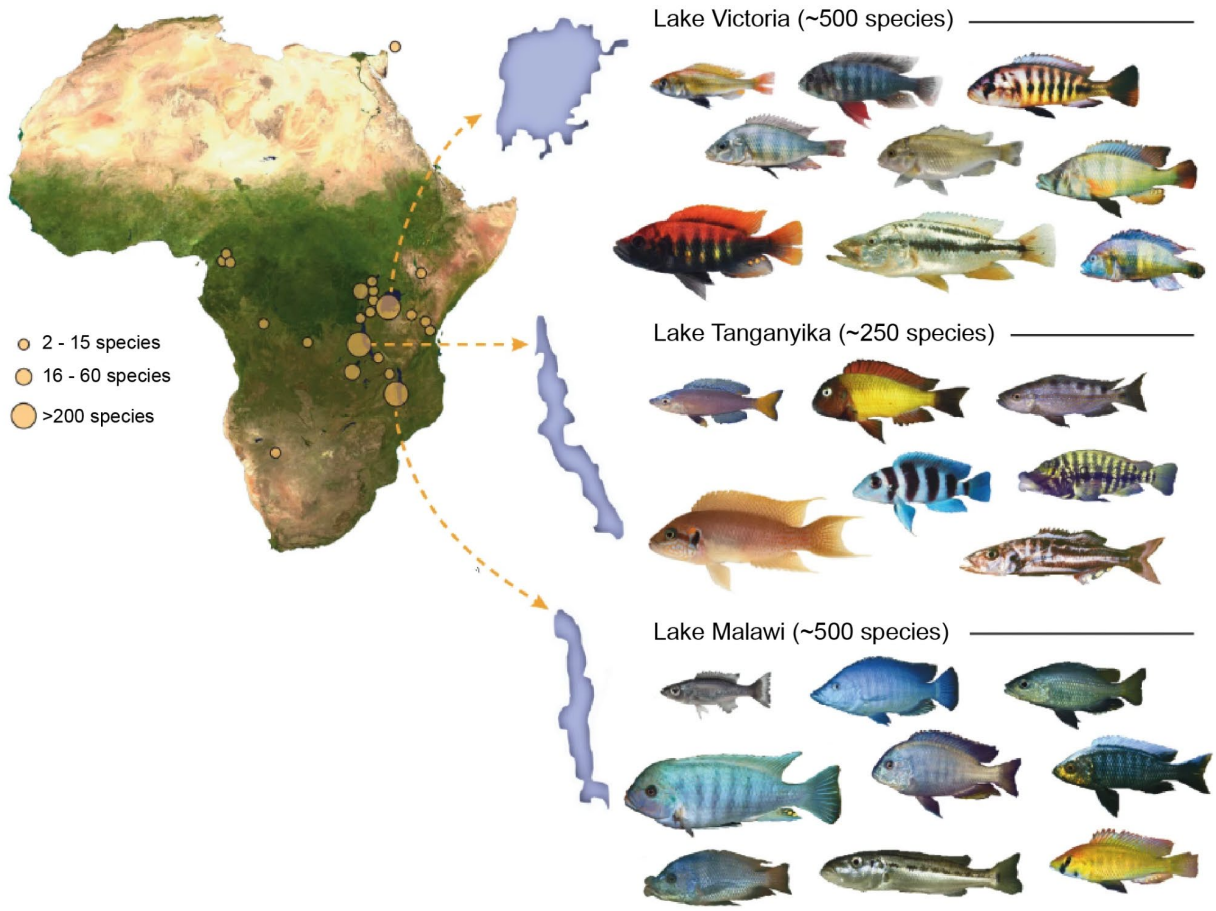


Figure 2: Map of Africa showing the East African Great Lakes and the diverse cichlids within them. Cichlid species pictured were selected to represent the major ecotypes from each lake, with varying feeding strategies. Photos taken by Ad Konings, Oleg Simakov, Frans Witte, Walter Salzburger, Oliver Selz and Marcel Haesler. Figure adapted from Brawand et al. (2014) (Open access: CC BY-NC-SA 3.0 DEED).

However, it is the cichlids' universal and extensive investment in their offspring that first drew behavioural scientists to these fishes (Baerends & Baerends-Van Roon, 1950; Balshine & Abate, 2021; Sefc, 2011). The diversity in parenting behaviour of cichlids matches their phylogenetic diversity, offering a wide range of research opportunities (Koblmüller et al., 2008). Different species care for between 10–12000 offspring, with care duties lasting from 5–249 days (Kolm et al., 2006a; Kuwamura, 1986). These duties involve nest building, cleaning, oxygen provision, protection and transportation among other behaviours to improve offspring fitness. For instance, convict cichlids (*Amatitlania nigrofasciata*) engage in behaviours like fin digging and leaf lifting to increase food availability for their young (Wisenden et al., 1995), while Amazonian cichlids (*Symphysodon spp*), provide offspring with mucus secretions to feed from after hatching (Buckley et al., 2010).

While parental care strategies vary widely within this group, a commonality emerges: females supply nutrient-rich yolk sacs which, while sustaining altricial fry for days to weeks, also constrain larvae mobility (Kuwamura, 1986). To safeguard their vulnerable offspring, parents either engage in substrate brooding, where they provide secure spaces or nests for their offspring, or mouthbrooding, where parents carry offspring in their buccal cavity (Fig. 3). Some species also employ both strategies (Sefc, 2011).

1.2.1 Comparison of Substrate Brooding and Mouthbrooding

Substrate brooders lay their eggs on various solid surfaces, where one or both parents guard and tend to the eggs until hatching. Hatched embryos are often then transferred in the parent's mouth to rock crevices or dug-out pits where further care and protection are provided (Nagoshi, 1987; Nagoshi & Gashagaza, 1988). This method, more commonly used among American cichlids, requires behaviours such as guarding offspring from predators, fanning to oxygenate the eggs and cleaning the nest site to prevent fungal growth (Fujimura & Okada, 2007; Sefc, 2011). In substrate brooders, there is often competition for nesting sites, and their exposed larvae can make the brood more vulnerable to predators (Nagoshi, 1987; Nagoshi & Gashagaza, 1988; Sefc, 2011).

Conversely, mouthbrooding, which is prevalent among African cichlids of the Great Lakes, typically involves one parent, usually the female, carrying fertilised eggs and then the hatched fry inside their buccal cavity until offspring can survive independently (Keenleyside, 1991; Sefc, 2011). This strategy provides several advantages, including brood protection from predators and environmental stressors, and the flexibility to relocate offspring to optimal habitats (Corrie et al., 2008). However, mouthbrooding individuals suffer significant physiological costs such as reduced feeding, altered gut microbiota, and delayed gonadal recovery, impacting the parent's health and reproductive frequency (Corrie et al., 2008; Nagoshi, 1987; Yanagisawa & Sato, 1990).

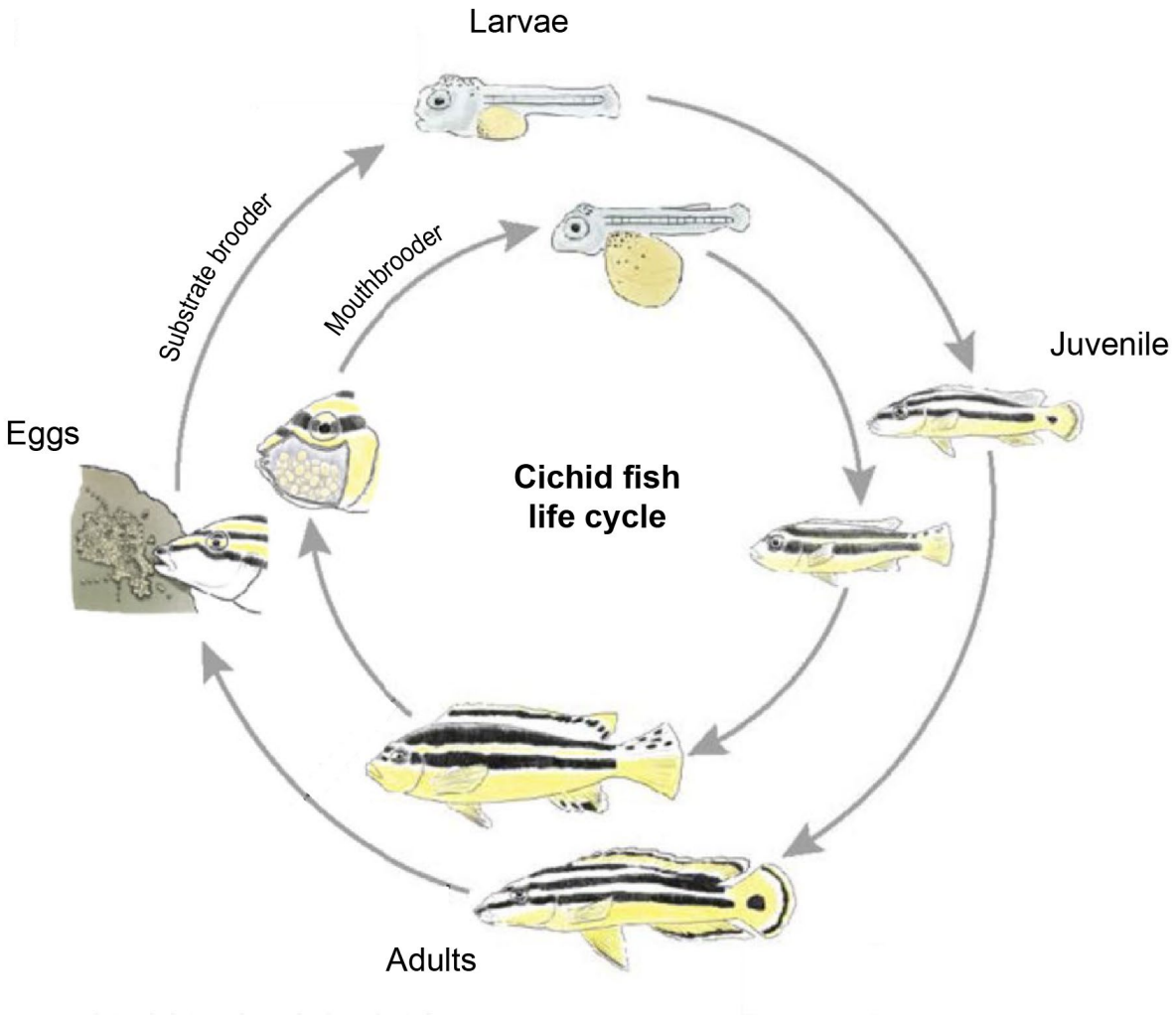


Figure 3: The life cycles of mouthbrooders and substrate brooders. A diagram taken from Santos et al. (2023) illustrates the four stages (eggs, larvae, juvenile and adults) of mouthbrooding (inner circle) and substrate brooding (outer circle) cichlids. The greatest differences are seen in their parental care methods, with mouthbrooders retaining eggs, and later larvae, inside their buccal cavity, while substrate brooders lay their eggs on a surface, to which eggs adhere to until hatching. Later hatched substrate brooding larvae remain attached to the surface by adhesive glands on top of their heads. Image adapted from Santos et al (2023) (open access: CC BY 4.0 DEED)

The ancestral form of cichlid parental care is thought to have involved biparental substrate breeding (Goodwin et al., 1998). Evolutionary pressures have led to a diversification of care strategies, resulting in shifts toward mouthbrooding and female-only care. This transition is thought to have occurred multiple times independently, indicating strong selective pressures that favour the protective advantages of mouthbrooding. These two parental care strategies tend to have distinct reproductive characteristics that are influenced by their unique evolutionary pressures. For instance, mouthbrooders generally produce fewer, larger eggs limited by the capacity of the buccal cavity, and while these eggs take longer to hatch, parents offer little if any

care after the larvae are released (Kolm et al., 2006a; Sefc, 2011; Wassenbergh et al., 2016). In contrast, substrate brooders typically lay smaller, more numerous eggs that, once hatched, are cared for over a longer period of time (Kuwamura, 1986; Nagoshi, 1985; Yanagisawa et al., 1996; Yanagisawa & Sato, 1990).

Substrate and mouthbrooding offspring have also evolved some interesting adaptations to facilitate these divergent care strategies. Studies on American substrate brooding larval development revealed the eggs adhere to surfaces and one another due to a sticky mucus layer or attachment filaments around the chorion (Kratochwil et al., 2015; Meijide & Guerrero, 2000). Additionally, the hatched larvae have adhesive glands on the top of their heads that keep them firmly attached to the substrate protected by their parents. Mouthbrooder eggs and larvae lack these adhesive properties. Research on African mouthbrooding larvae development highlights the rapid progression from the larval to the adult body plan, which omits the prolonged larval period in comparison to substrate brooders, likely a factor related to their reduced post-hatching care (Fujimura & Okada, 2007; Woltering et al., 2018).

1.2.2 The Cichlids of Lake Tanganyika

Of the East African Great lakes, Lake Tanganyika offers a unique perspective on cichlid parental care strategies. As the oldest and deepest of the three lakes, with an age of around 9–12 million years and a maximum depth of 1470 metres, Lake Tanganyika is the largest body of freshwater in Africa by volume (Salzburger et al., 2014). This ancient lake is known for its ecological diversity and unusual water chemistry, featuring high conductivity (~ 660 µS/cm) and alkaline pH levels (8.0–9.0) (Degens et al., 1971; Plisnier et al., 1999).

Home to approximately 240 cichlid species, nearly all of which are endemic, Lake Tanganyika's cichlid population stands out for the incredible morphological and behavioural diversity it holds (Fryer & Iles, 1972; Ronco et al., 2021). In contrast to Lake Malawi and Lake Victoria, which predominantly host mouthbrooders, about one-third of the cichlid species of Lake Tanganyika are substrate brooders (M. H. A. Keenleyside, 1991; Ronco et al., 2021; Watanabe, 2000).

1.2.2.1 Lamprologini shell-dwellers

The Lamprologini tribe constitutes almost the entire portion of substrate brooding cichlids in Lake Tanganyika (Sturmbauer et al., 1994). This group—one of the lake's oldest cichlid lineages—

originated around 15.27 million years ago, with significant diversification occurring around 5.3 million years ago (Schedel et al., 2019; Sturmbauer et al., 2010) and is said to have evolved the lake's most diverse mating strategies and patterns of parental care (Gashagaza, 1991; M. H. A. Keenleyside, 1991).

A number of Lamprologini species exhibit a remarkable adaptation by utilising empty gastropod shells scattered across the lake floor as protection and breeding sites (Bills, 1996; Fryer & Iles, 1972; Haussknecht & Kuenzer, 1991). The lake's unusual abundance of these shells can be attributed to the evolution of thick-shelled gastropods, mostly of the species *Neothauma tanganyicense*, which carry robust exteriors as a defence against shell-crushing predators (West & Cohen, 1996). Additionally, the lake's distinctive ecological conditions—high pH levels and calcium-rich waters—help preserve these shells, ensuring their availability and integrity (Talling & Talling, 1965). The abundance of these shells creates a number of unique microhabitats, from scattered shells in sandy areas to extensive homogenous shell beds (Fig. 4; Sato & Gashagaza, 1997). The nature and distribution of these shells directly influence shell-dweller evolution, affecting physical attributes such as body size, as well as territorial behaviours and reproductive strategies (Koblmüller et al., 2008; Sato & Gashagaza, 1997; Schuetz & Taborsky, 2005; B. Walter & Trillmich, 1994).

These adaptations are particularly evident in the diverse mating systems and parental care strategies of shell-dwellers, from monogamy to various forms of polygamy and even alternative reproductive tactics such as sneaker males (M. H. Keenleyside, 1991; Ota et al., 2012; Sato & Gashagaza, 1997; Yanagisawa, 1987). Within this group, we distinguish between facultative and obligate shell-dwellers. Facultative shell-dwellers use shells when convenient, but may also breed on other substrates. In contrast, obligate shell-dwellers' reproductive cycles depend entirely on the availability of shells, with their entire breeding strategy and parental behaviours intricately linked to these unique habitats. Nesting strategies and shell manipulation are diverse, ranging from multi-species communal shell piles created by giant *Lamprologus callipterus* males (Fig. 5, Schuetz & Taborsky, 2005) to isolated shells actively buried into sandy substrates by dwarf species (Haussknecht & Kuenzer, 1991). The varied parental care periods—from a several days to a few months—can be executed solely by the mother, both parents, or with the assistance of offspring from previous spawns, as seen in *Neolamprologus brichardi* (Sturmbauer et al., 1994; von Siemens, 1990).

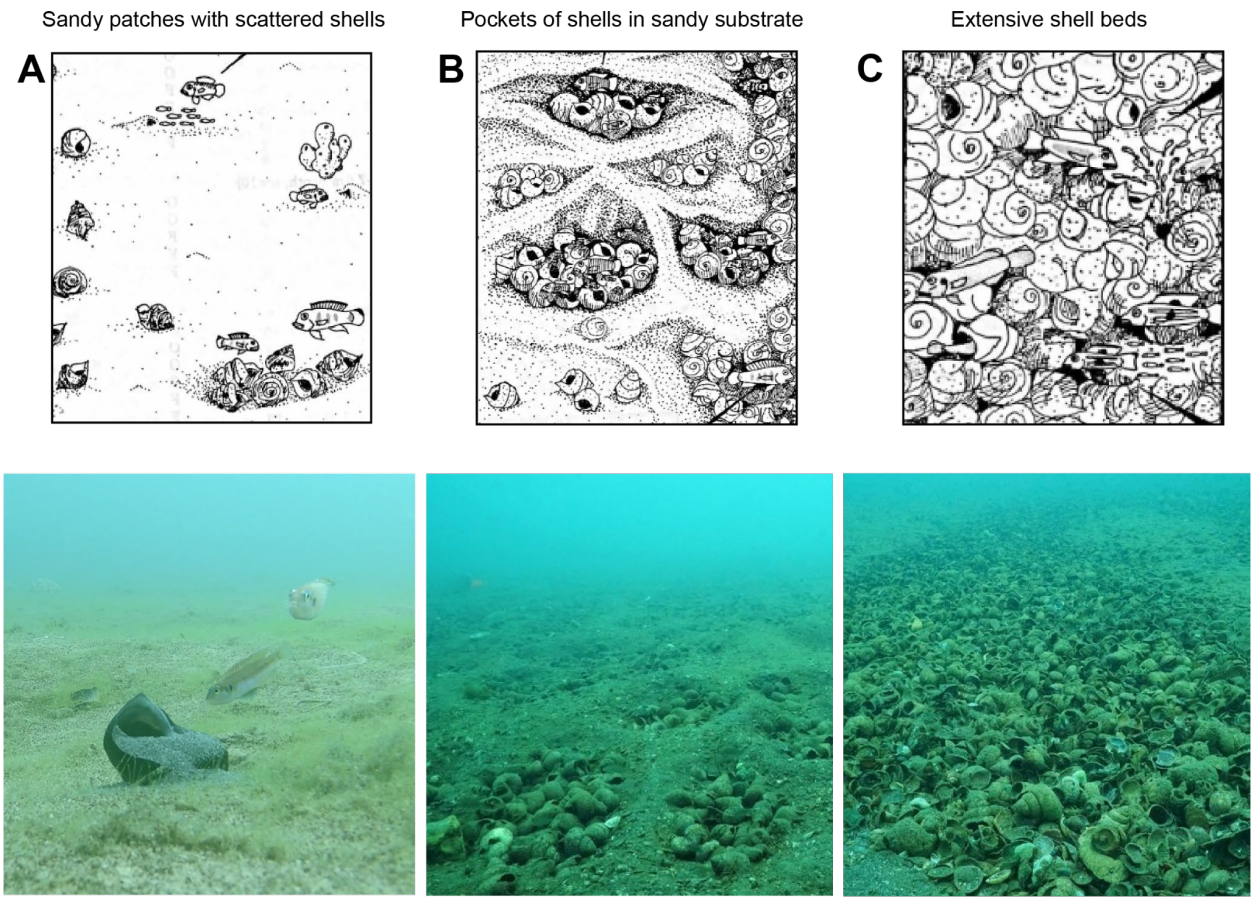


Figure 4: Distinctive shell niches found on the floor of Lake Tanganyika. A) Sandy patches with shells scattered, often half or fully buried into the substrate by the shell-dwelling cichlids, such as *Lamprologus ocellatus* (pictured) and *L. ornatipinnis*. **B)** Pockets of shells can be found interspersed with sandy substrate. This landscape is often engineered by the fish themselves, with *Neolamprologus multifasciatus* and *L. Callipterus* acting as the main culprits. **C)** Extensive homogeneous shell beds are created from water currents that constantly deposit empty shells into the same zone. The dense shell patches host the richest shell-dwelling species diversity. Illustrations taken from Sato & Gashagaza, (1997), with permission while corresponding photographs were taken by the Jordan Lab (**A**) and Aneesh Bose **(B** and **C)**.

1.2.2.2 Lamprologus ocellatus *as a model*

In this thesis, I investigate the reproductive strategies and parental care paradigm of *Lamprologus ocellatus*, one of the few obligate shell-dwellers (Fig. 6A). This species primarily resides in sand and mud substrates, where it strategically buries its shells to facilitate breeding and enhance protection of offspring (Bills, 1996; Konings, 1998; Sato & Gashagaza, 1997). This behaviour comprises an inspection phase, followed by manipulation of the shell and surrounding substrate to obtain an optimal positioning, and then a final covering-over phase to attain the desired nest (Fig. 6B; Haussknecht & Kuenzer, 1991). Male *L. ocellatus* typically reach 5 cm in

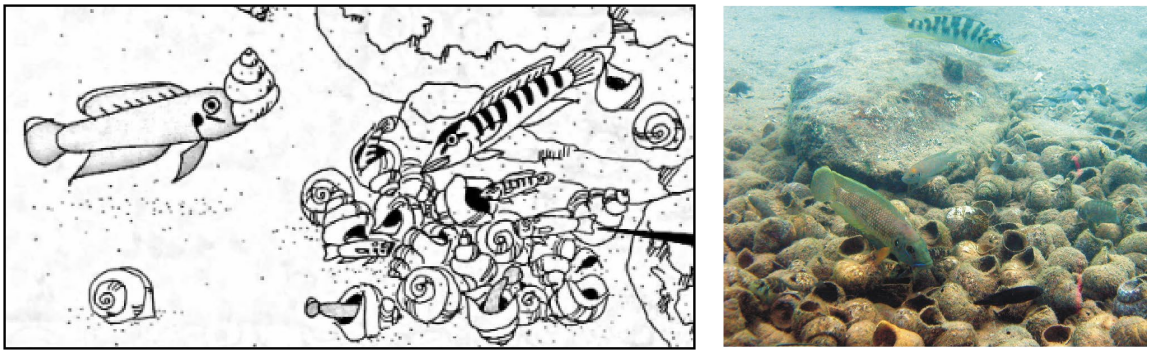


Figure 5: Nesting pile created by male *Lamprologus callipterus*. Male *L. callipterus* are large enough to carry empty snail shells by the mouth, accumulating them into a nesting pile (Schuetz & Taborsky, 2005). The size of these piles is a sexually-selected trait. Remarkably, the females are small enough to fit inside the shells, resulting in *L. callipterus* exhibiting the greatest sexual dimorphism among vertebrates. Illustrations taken from Sato & Gashagaza, (1997), with permission, while the corresponding photograph was taken from Koblmüller et al. (2008) (open access: CC BY 2.0 DEED).

length and control territories ranging from 1–3 m², within which they distribute and bury 1–10 shells (Fig. 6C; Bills, 1996). Females, slightly smaller at 4 cm, have their own shells within the male's territory. The male strategically places each female at shells to maximise distance between them, reducing the likelihood of aggressive encounters during brooding (Brandtmann et al., 1999; B. Walter & Trillmich, 1994).

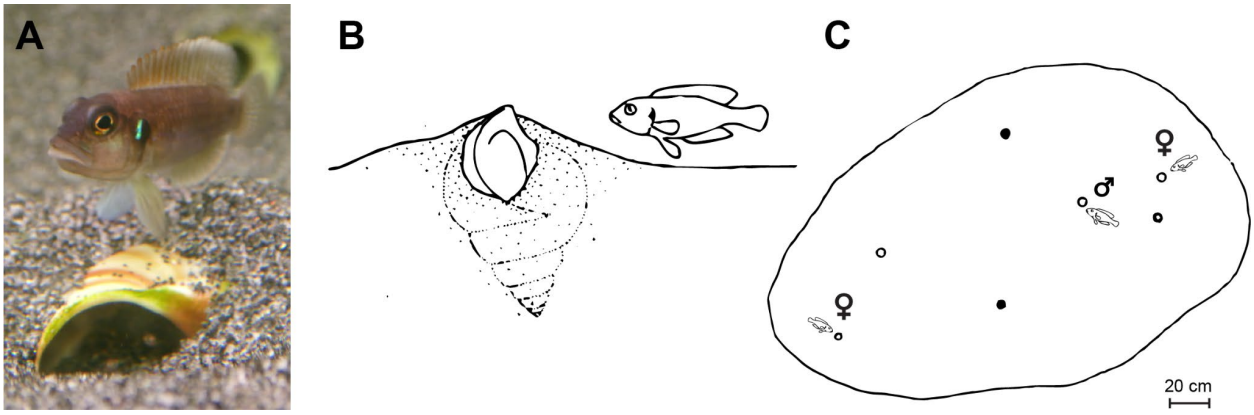


Figure 6: *Lamprologus ocellatus* and their shells. A) A female *L. ocellatus* in a defensive position over her shell entrance. **B)** An illustration of the final position of the shell after the nest building in *L. ocellatus*. **C)** A diagram of a typical territory held by a male *L. ocellatus*. Open circles represent buried but accessible shells, while closed circles represent shells completely hidden under the substrate to prevent other fish from using them. Illustrations adapted from (Bills, 1996) with permission.

The female will lay her eggs inside her own shell and provide all the direct care to her offspring before they become free-swimming (Fig. 7A–C). Once the larvae are first seen emerging from the

shell, the female continues to guard the free-swimming fry as they move in and out of her shell. After several weeks the juveniles move across to the father's shell where they benefit from his further protection (Fig. D; Bills, 1996; Haussknecht & Kuenzer, 1991; Sato & Gashagaza, 1997). Unsurprisingly, there is little known about larval development or the parental-offspring interplay in this species owing to the protective environment in which they raise their offspring.

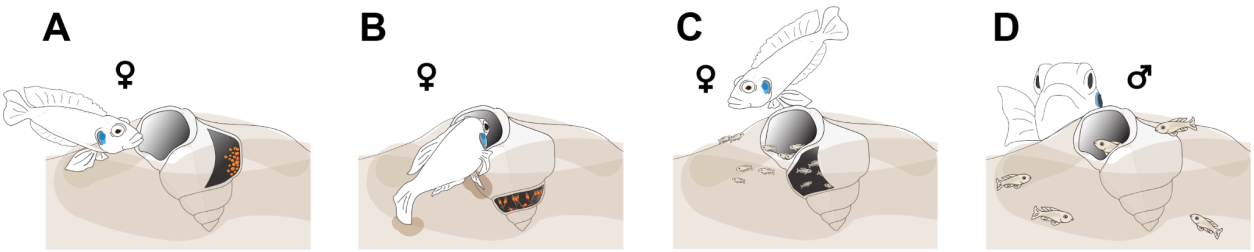


Figure 7: Brood care stages of *L. ocellatus*. A) Mother laying eggs inside the first whorl of the shell nest, **B)** hatched fry stay deep within the shell, **C)** larvae are seen emerging from the shell, **D)** juveniles move across to the father's shell. Illustrations by Swantje Grätsch and adapted from Parker et al. (2023; with permission)

Most previous studies on shell-dwellers are limited to observations within Lake Tanganyika (Bills, 1996; Bose et al., 2020; Ota et al., 2012; Sato & Gashagaza, 1997; Schuetz & Taborsky, 2005). While these studies provide great context for the natural behaviours and habitat conditions of shell-dwellers, they do not allow for detailed study or manipulations in a controlled environment. These fish are also conveniently eager to build shell nests and breed in captivity, presenting behaviours similar to what has been reported in the lake (Brandtmann et al., 1999; Haussknecht & Kuenzer, 1991; B. Walter & Trillmich, 1994). Successful breeding in captivity is crucial as it permits direct observation of behaviours that are typically hidden within the dark confines of snail shells, such as brood care behaviours, larval development and intricate parent-offspring interactions.

This study provides an opportunity to explore the unique adaptations of shell-dwelling cichlid parental care paradigms. While shell-dwellers are a form of substrate brooders, the nature of the shell offers a distinct environment, comparable in some ways to the confined space used by mouthbrooder larvae. Although considerable research has compared mouthbrooders and substrate brooders (Balshine & Abate, 2021; Kuwamura, 1986; Sefc, 2011), there is a lack of substantial studies examining how the reproductive strategies and larval development of shell-dwellers fit into this spectrum. Understanding these adaptations can shed light on the broader evolutionary context of cichlid reproductive strategies.

Lastly, studying the *L. ocellatus* parental care paradigm in a laboratory environment has allowed us to observe and dissect the mechanisms of adaptive behaviour in the shell-dwelling parental paradigm. The larvae spend the first days of their life deep within the shell, and at some point, they must emerge for the first time. This behaviour represents a significant transition as the larvae are exposed to a vast, new environment filled with light, predators, and prey. I sought to understand the factors contributing to this emergence behaviour, specifically determining whether the mother, the offspring, or both influence this transition. This process parallels the fledging behaviour observed in birds, where the timing of leaving the nest potentially involves a complex interplay between parental guidance and offspring readiness, highlighting the broader relevance of this study to understanding parental care strategies across taxa.

1.3 Methodological Innovations and Study Design

Since the foundational work of Nikolaas Tinbergen and Konrad Lorenz in the mid-20th century, the study of animal behaviour has undergone significant evolution (Font, 2023; P. K. Smith, 1990). Initially reliant on simple observational techniques, it now integrates sophisticated technological tools (Berman, 2018; Datta et al., 2019; M. W. Mathis & Mathis, 2020; Pereira, Shaevitz, et al., 2020). Early research on cichlids primarily took place in their natural habitats, with early ethologists using observational descriptions and ethograms to record behaviours (Baerends & Baerends-Van Roon, 1950; Bills, 1996; Gashagaza, 1991; Nakano & Nagoshi, 1990; Yanagisawa et al., 1996). Ethograms are comprehensive catalogues of all observable behaviours displayed by an individual or group across a given time, allowing researchers to systematically record and compare the actions of their subjects (Friard & Gamba, 2016).

While these studies serve as an essential foundation of the studies of cichlids, this early research faced notable limitations. For example, difficulties in continuous monitoring, especially during the night, the potential influence of researchers' presence on animal behaviour, and the challenge of studying behaviours inside structures like shells where direct observation is not possible. These limitations hindered the progress in our understanding of complex evolutionary mechanisms and called for more controlled environments and experimental manipulations.

The advent of video technology revolutionised ethological research, enabling non-invasive, slowed-down analyses of complex behaviours that are often missed in real-time observations (London et al., 1998). This advancement additionally allowed researchers to conduct longer experiments in both natural and laboratory settings where environmental variables can be manipulated to observe resulting behavioural responses.

The work in this thesis integrates both classical and modern methodologies to study the parental care paradigm of the shell-dweller, *L. ocellatus,* in a controlled laboratory setting designed to mimic their natural habitat. I was able to keep record of individuals using tattoo identifiers, create simplified and standardised environments, and record detailed behavioural observations over long periods of time.

One of the primary challenges was observing the interactions within the shells. To address this, I designed 3D-printable shells with windows, allowing near-infrared cameras to record day and night behaviours without disrupting the natural activities of the mother and offspring. With the assistance of colleagues, I made use of current data acquisition and large data storage solutions before employing recent computer vision technology.

The last ten years have seen significant advancements in animal tracking software, driven by the integration of computer vision and machine learning. Tools like DeepLabCut and SLEAP offer markerless pose estimation from video footage, while TRex can uniquely identify and track hundreds of individuals (A. Mathis et al., 2018; Pereira, Tabris, et al., 2020; T. Walter & Couzin, 2021). However, these systems have limitations, such as requiring a constant number of individuals in frame and optimal lighting conditions, which are not always feasible in complex environments like shell interiors.

To overcome these challenges, I employed object detection technologies, which have also benefited from the advancements in computer vision since their inception in the 1970s (Marr, 1982). Modern approaches, such as the YOLO (You Only Look Once) algorithm, use neural networks to predict bounding boxes and class probabilities directly from images, facilitating real-time object detection (Redmon et al., 2016).

In this study, ethograms were used to meticulously document the behaviours of mothers and the development of offspring behaviours within the shell. Additionally, object detection was employed to analyse larval distribution and maternal visitation patterns over the time larvae are restricted to the shell and their first few days exploring the area outside the shell. By comparing these behaviours across various experimental set-ups, I aimed to create a comprehensive description of the *L. ocellatus* parental care paradigm, shedding light on shell-dweller mating strategies and uncovering the evolutionary mechanisms facilitating larvae emergence from the shell.

1.4 Research Objectives and Hypotheses

To address the gap in understanding of reproductive strategies and parental care in shell-dwelling cichlids, this thesis observes and dissects the brood care adaptations in *L. ocellatus*. The study is structured by three main objectives:

1. **Descriptive analysis of behavioural development:** I first aim to describe the behavioural development of shell-dwelling cichlid larvae and maternal care within the protective confines of the shell. This involves observing and analysing the independent activities of the larvae during early stages of development as well as the interactions between mother and larvae inside the shell.
2. **Comparative adaptations:** Next, the study compares these behavioural adaptations and reproductive tendencies to those observed in other cichlid species within Lake Tanganyika, specifically substrate brooders and mouthbrooders. This comparison aims to highlight the evolutionary context of shell-dwellers within the broader spectrum of cichlid parental care strategies.
3. **Mechanisms of larval emergence:** The final objective is to investigate the drivers behind the initial emergence of larvae from the shell. This part of the research tests three hypotheses concerning the timing of larval emergence:

   Maternal control: The first scenario proposes that the mother controls the emergence timing of the larvae through her behaviours. In her absence, it is hypothesised that larvae emergence time would be affected.

   Larval development: The second scenario suggests that the larva's emergence is governed by intrinsic developmental factors, independent of maternal presence. Here, the absence of the mother is hypothesised to have no effect on the natural timing of emergence.

   Coordination: The third scenario hypothesises that the correct emergence timing requires synchronisation between maternal cues and larval development. A mismatch in these timelines, due to the absence of the mother or other disruptions, is expected to alter the natural emergence schedule.

Through these objectives, I was able to observe the parental care paradigm of shell-dwellers within their shells and describe the reproductive niche of shell-dwellers in the context of the substrate brooder–mouthbrooder spectrum within Lake Tanganyika. By observing larval and maternal behaviours inside the shell, I discovered for the first time that the mother transports her

newly-hatched offspring by mouth to the deeper chambers of the shell, where the larvae remain for another six days. The next significant transition for the larvae is their initial emergence from the shell. Through a series of manipulations to the parental care paradigm, I demonstrated that emergence is timed by two intrinsic behavioural mechanisms: one from the mother, who prevents the larvae from leaving the nest until they reach a specific stage, and the other from the larvae, whose phototactic behaviour switches from a preference for darkness to light in anticipation of emergence. These findings provide new insights into the complexity of parental care strategies and highlight the intricate interplay between maternal and offspring behaviours, setting the stage for a deeper understanding of the evolutionary adaptations in cichlid fishes.

Chapter 2: Methods

2.1 Animal Husbandry

L. ocellatus were bred in 51 litre tanks (600 x 270 mm, 320 mm deep) with one male and two females at the Max Planck for Biological Intelligence, Martinsried, Germany, in accordance with institutional guidelines established by the Max Planck Society and regulations from the regional government (Regierung von Oberbayern). The colonies, originating from wild-caught populations collected at Isanga Bay, Zambia, were provided to our lab by our collaborator Alex Jordan from the Max Planck Institute of Animal Behavior. The breeding tanks contained 4–5 cm of beach sand (0.4–1.4 mm grain-size), and each individual was given one of two types of 3D-printed Tanganyika shells: males typically occupied the larger, complete shells, while females generally chose one of the two window shells (models adapted from Bose et al., 2020). These window shells were pressed against the glass, with their backs shielded from room light by opaque tape, allowing for daily checks for new egg clutches. If male and female pairs did not breed within two weeks, individuals were swapped out until an established breeding pair was formed—defined as a pair that successfully raised at least one clutch of offspring together. Breeding fish, as well as those used in experiments, were fed live artemia twice daily and maintained under constant conditions (13:11 hour light-dark cycle, 27°C water, pH 8.2, conductivity ~550 µS). Between 7 am and 8 pm, the room lights were on, and at night, they were turned off, leaving only a faint light from aquarium computer monitors.

2.2 Staging Embryonic and Larval Development

Freshly fertilised eggs were collected by my colleague in a 100 mm Petri dish from the mother's shell in the breeding tanks. The larvae were then raised at a density of 20 fish per dish in a 27°C incubator with a 14:10 hour light-dark cycle, with fresh facility water changed daily. Eggs and larvae were visualised under a stereo microscope (Nikon SMZ25) equipped with a 1x SHR Plan Apo objective. During brightfield image acquisition, the exposure time and illumination intensity were adjusted using the NIS-Elements imaging software.

2.3 Testing if *L. ocellatus* can See in Near Infra-red Light

A 27 litre tank (190 x 190 mm, 300 mm deep) filled with 10 litres of water from the facility was housed in a light-impenetrable box (Thor Labs). The set-up included a string of white light LEDs and a single 850 nm near-infrared (NIR) light strip (12 Osram-Olson LEDs, 700 ma) were able to be turned on and off independently. A NIR camera (Ximea, MQ042RG-CM, Germany) was positioned above the tank, recording at 30 frames per second to capture detailed observations.

An experiment was performed where a female was placed in this tank and allowed to acclimate for 15 minutes in ambient room light. The box was then shut and only the NIR light source was turned on. After 2 minutes the white light was additionally turned on for a further 2 minutes, after which the white light was turned off, leaving only the NIR light illuminating the tank. The fish was observed over the computer and descriptions of her eye movements and swimming behaviours across the three light changes were noted.

2.4 Behavioural Observations Inside the Shell

2.4.1 Behavioural Arena Set-up

I designed and had a custom-built 100 litre experimental tank insert made (420 x 660 mm, 360 mm deep) which is described in the results section of this thesis (see 3.1.2.1. The filming arena). Briefly, the tank insert was made with light-absorbing white acrylic bottom and three sides into which two 3D-printed shells, 430 mm apart, were inserted. On the outside of the tank, the open backs of the window shells were protected from visible light with NIR-penetrable plastic covers (LUXACRYL-IR,1698, 0.8 mm thick, Germany). The tank was illuminated constantly with six 850 nm NIR light strips (NIR; 12 Osram-Olson LEDs, 700 mA) from above and two 850 nm NIR light strips directed from the side into each of the two filmed shells. As this tank was part of the housing facility, with constant water exchange and visible lighting, water conditions and feeding were as described above (See 2.1 Animal husbandry). Four NIR cameras (Ximea, MX042RG-CM-X2G2-FF, Germany), two filming inside the window shells using a 50 mm lens (Edmund Optics, Germany) and two filming from above with a 12.5 mm lens (Navitar, NMV-12M1, USA). Media acquisition and processing was programmed with custom-written python scripts written by a colleague and Ximea software to acquire images (2048x2048 pixels; JPEG) every 7 minutes and take 20 minute videos (30 frames per second, avi) at specific times of the day from fertilisation of

the eggs until and including 11 days post-fertilisation (dpf). The 7-minute interval between time-lapse images was chosen to balance the need for detailed behavioural data and practical data processing. This interval was sufficient to capture stable larval behaviours and frequent enough to monitor maternal visits to the shell accurately.

2.4.2 Analysing Behavioural Video Data

To assess the behavioural repertoire of the larvae and the mother inside the shell, I annotated behaviours using BORIS software (Friard & Gamba, 2016). For the larvae, 10 individuals were observed and scored for 5 minutes at noon on 1, 3, 5, 7, 9, and 11 dpf across two independent control experiments. The behaviours annotated included visible, head-attached, resting on the floor, wriggling, and swimming (Table 1). For the mothers, individuals were scored over 20-minute videos at noon on 1, 3, 5, 7, 9, and 11 dpf, as well as at 3 am on 7 dpf. The behaviours scored included visible, fanning, and mouthing (Table 1). Mothers across all experiments were scored when present; however, certain days were excluded if there was any suspected disruption to natural behaviours, such as the introduction of water flow or food into the shell, or after the clutch swaps.

Table 1: Larvae and mother behavioural repertoire scored in the BORIS software. The behaviour code, subject, type of event and the description of the behaviour used when scoring videos in BORIS

Behavior code	Subject	Type	Description
Head attached	Larvae, egg	State event	Larva head attached to shell wall
Resting on floor	Larvae, egg	State event	Head not attached but larva is weighed down by yolk, lying on shell floor
Wriggling	Larvae	State event	Larva performs high frequency tail beating
Swimming	Larvae	State event	When larva are actively or passively displaced within the shell
Mouthing	Mother	State event	Mother actively engages with eggs or larvae using her lips or picking up individuals and circulating them in her buccal cavity
Fanning	Lother	State event	Mother rapidly moves her anal and caudal fin to facilitate water exchange inside the shell

Using the BORIS software, we exported a CSV file of the aggregated events for the mother and the larvae separately for each dpf and experimental run. I then used Python to process the data and produce Figures 13A–D and 14E–F. Data for the mother and larvae were processed

separately. For both, I first concatenated all the results across experiments for each dpf, ensuring each individual was uniquely named. I then calculated the total duration each individual engaged in each behaviour and normalised this by dividing by the total duration the individual was visible during the observation period. The mean and standard deviation were calculated across all individuals for each dpf and behaviour.

2.4.3 Distribution of Mother and Larvae Inside the Shell

To identify the mother and eggs or larvae within the shell, I utilised YOLO v5 (Redmon et al., 2016), training the network over 300 epochs with a batch size of 4. This was facilitated by a custom Python GUI developed by a colleague. The network was trained on 1481 manually annotated images (using labelImg, Github) from 14 datasets, ensuring balanced coverage across the experimental timeframe. The labelled object categories included egg, larvae, mother's head, and mother's pectoral fins. The trained model (Fig. 8) was then applied separately to each dataset to predict the x and y coordinates of each identified object's centre. Since the shell's position remained constant throughout each experiment, an image of the shell was segmented into three regions of interest (ROI): entrance area, laying chamber, and deep chamber. This segmentation was performed using the software labelme (Github), with ROIs drawn manually (see Fig. 10F). Using custom Python scripts, developed in collaboration with a colleague, we processed the data to extract time information and image sequences. The data for the larvae were then processed separately from the data for the mothers.

2.4.3.1 Determining larvae distribution inside the shell

For the larvae, we filtered the preprocessed positional data to include only the information on eggs and larvae, setting a threshold for the predicted detection confidence level at 0.5 and above. The age of the larvae (dpf) was determined using the time information, and each coordinate was assigned to one of the three ROIs. A pivot table was used to evaluate the number of larvae in each ROI for each time frame..

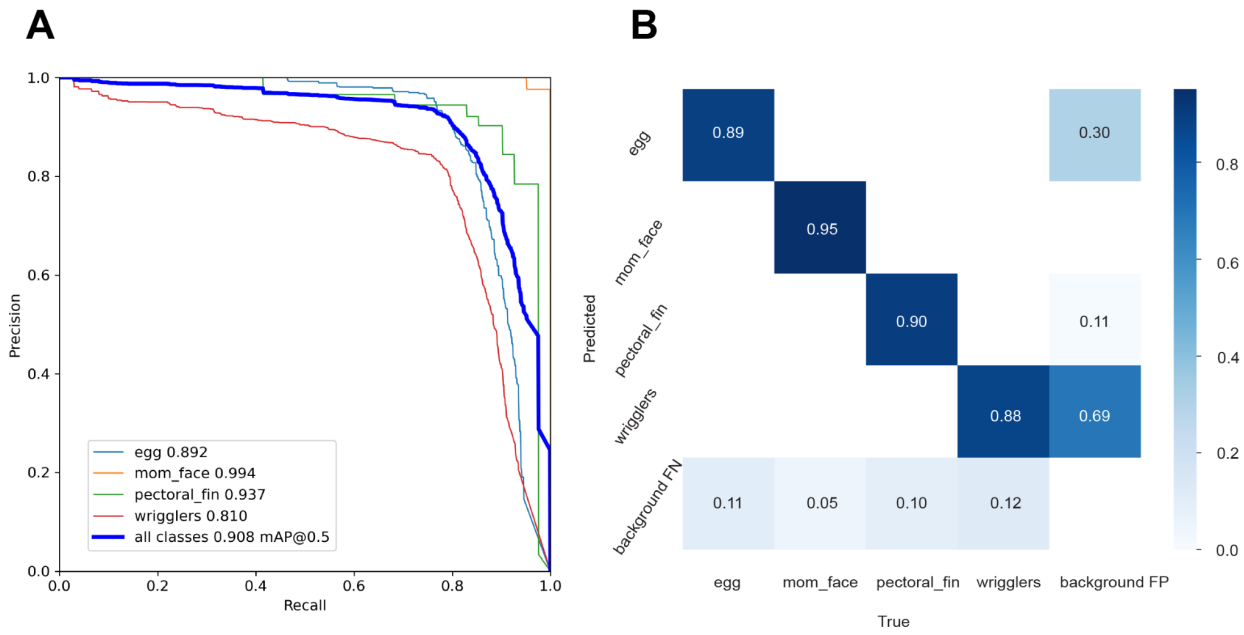


Figure 8: Evaluation metrics of the YOLO neural network for object detection within the shell. A) The precision-recall curves and average precision values for each object class (blue, mother; orange, mother's face; green, pectoral fin; red, wrigglers) and for all classes combined (navy), with a mean average precision (mAP) at 0.5. **B)** The confusion matrix for the four aforementioned classes and the background. Figure taken from Parker et al. (2023) with permission.

From the pivot table, we identified the maximum number of larvae detected in each ROI as well as in the entire shell at any given time during the experiment. We created Figures 14C, 17A–D, 18A–C, and 20A–B, which are tile plots, by plotting a vertical line for each frame, separately for each ROI. The colour of the line represented the ROI, while the alpha component (or transparency) of the colour corresponded to the number of larvae in that ROI for the given frame, divided by the maximum number of larvae found in that ROI throughout the experiment, with a higher fraction resulting in a higher alpha component. Additionally, key time points, manually inputted, were plotted onto the figure, including the time of parent removal, fresh water provision, food provision, and clutch swapping. The emergence time, also plotted on the figure, was automatically detected as the first frame in which two larvae were found in the entrance chamber in the same frame after 5 dpf.

The emergence times were also used to create Figures 17E and 18D. Experimental runs were grouped according to experimental manipulation, and the average emergence time was plotted as a bar graph. Additional transition time points were added to these figures. The first transition,

at hatching, where larvae move from the laying chamber to the deep chamber, was calculated as the last frame in which an egg was detected in the laying chamber. The second transition, from the deep chamber to the laying chamber, was calculated as the first frame where two larvae were detected in the laying chamber after 4 dpf. These transition points, along with the emergence times for each experiment, were plotted in Figures 17E and 18D according to their respective experimental manipulation categories.

2.4.3.2 Determining mother visitations and use of the shell

For the mother, the preprocessed positional data was filtered to include only those predictions with a confidence level above 0.59 for the mother's head and pectoral fins. To determine the mother's position within the three ROIs, we primarily used the position of the mother's head. If the head was not detected (due to obstruction by the shell whorl), we used the position of the mother's pectoral fin with the highest confidence level, assigning her position to one chamber deeper than the location of the pectoral fin, unless it was already in the deep chamber. Similarly to the larvae, we calculated the dpf and used a pivot table to determine the presence of the mother in each ROI for each time frame.

Using the pivot table, we created Figure 16B, a tile plot, as described for the larvae, showing the presence of the mother across the different ROIs for each frame, without the alpha component as the mother's presence was binary. No additional key time points were added to the plots. For each experiment, we also plotted the percentage of frames in which the mother visited the shell during the day and night for each dpf. I collated the visitation data across all experiments, except for the cross-fostering experiments, to create a bar graph showing the mean percentage of frames detecting the mother during the day (Fig. 16C) and night (Fig. 16D) for each dpf. Data for each dpf was only included when filming occurred and the mother was present in the set-up for the full 24 hours of that dpf.

2.4.4 Manipulations to the Parental Care Paradigm

For the control conditions the fish were left undisturbed for the duration of filming in the set-up described above (see 2.3.1 Behavioural arena set-up)

2.4.4.1 Parent removals

For experiments where the only manipulation was parent removal, the set-ups were treated as in the controls, but at 5 or 7 dpf both the mother and father were removed with nets from the tank and the time point noted. Shells with larvae were left untouched. I chose 5 and 7 dpf as parent removals earlier than 5 dpf lead to too many offspring fatalities, whereas at 5 dpf only two offspring died across 2 experiments and none died after parent removals at 7 dpf. For all of the experiments thereafter requiring a removal of parents, the removal occurred at 7 dpf to ensure a greater survival chance for the offspring.

2.4.4.2 Supplying fresh water to the shell

The shell was adapted by drilling a 2 mm hole from the outside of the shell into the deep chamber and inserting a tube connector (Harvard, No.72-1475, USA). The tube (0.5 mm inner diameter) attached was then connected to a syringe pump (Aladdin A-1000, Germany) outside the tank with a 50 ml syringe (BD Plastipak, 300865, Germany). Once water flow into the shell was started at 4–6 dpf (flow rate: 4.5 ml/hour) it was continuous until the end of the experiment, with the syringe refilled with facility water every 8–14 hours. Parents were removed at 7 dpf as described above (see 2.3.4.1 Parent removals).

2.4.4.3 Feeding inside the shell

For these experimental manipulations, the set-up was treated as in the controls, but the same adapted shell used for the freshwater experiment was used. Here, I infused 5 ml of freshwater containing 20–30 live artemia at 5 ml/min, once at 7 dpf and 8 dpf to test if the larvae would start eating, which they did not, and then multiple times at 9 dpf once the larvae started performing prey capture within the shell.

2.4.4.4 Cross fostering experiments

Two females were identified with clutches of eggs two days apart in the breeding facility. When larvae were at 4 and 6 dpf, respectively, they were removed from the mother's shell into separate 100 mm petri dishes and counted. The experimental tank was divided in two by an opaque barrier, with one filmed window shell in each. Larvae clutches were then put into one of the two window shells with a pipette, ensuring the number of larvae was equal to the size of the smaller of the two clutches. The mothers were added to experimental tanks, receiving the shell containing the clutch from the other mother. Both shells were filmed from the switch point as described for the control conditions.

2.5 Phototaxis Experiment

To test light preference in the larvae, 6 dpf larvae were removed from their mother's shell in the breeding facility, and 4–5 larvae were placed in a custom-made box (30 x 50 mm, 10 mm deep; see Fig. 21A) situated inside the experimental tank described previously (see 2.3.1 Behavioural arena set-up). This set-up exposed the larvae to the same water and light conditions as in all previous experiments. The box, constructed from clear acrylic, had side walls and half of the lid covered in NIR-penetrable plastic (LUXACRYL-IR, 1698, 0.8 mm thick, Germany). This design created a box where one half was exposed to visible light while the other half was shielded from it. After placement, the larvae were allowed to acclimate before initiating a time-lapse (5 images per minute) using the same software mentioned earlier (see 2.3.1 Behavioural arena set-up). The time-lapse ran from midnight at 7 dpf until midnight at 11 dpf, with a NIR camera (Ximea, MX042RG-CM-X2G2-FF, Germany) equipped with a 50 mm lens (Edmund Optics, Germany), filming from above. The box was not water-tight, but the larvae could not escape. The lid, held down by magnets, was flipped daily by the experimenter between 12–2 pm to change the dark side of the box, during which filming was temporarily paused.

To determine the light/dark preferences of the larvae between 7–10 dpf, I used a custom-written Python GUI (see 2.3.3 Distribution of mother and larvae inside the shell) and labelImg (Github) to manually annotate larvae in 506 images across 6 datasets, ensuring an even spread across the dpf of each experiment. Using our GUI, I trained a YOLO model (300 epochs, batch size=4; Fig. 9), which was then applied separately to the 6 complete datasets to predict the x and y coordinates of each larva's centre point. Using labelme (Github), the experimental box was divided into two

ROIs for each dpf: light (where the lid was clear) and dark (where the NIR-penetrable plastic covered the lid). The larvae could be detected equally well on both sides as the NIR camera filmed through the NIR-penetrable plastic.

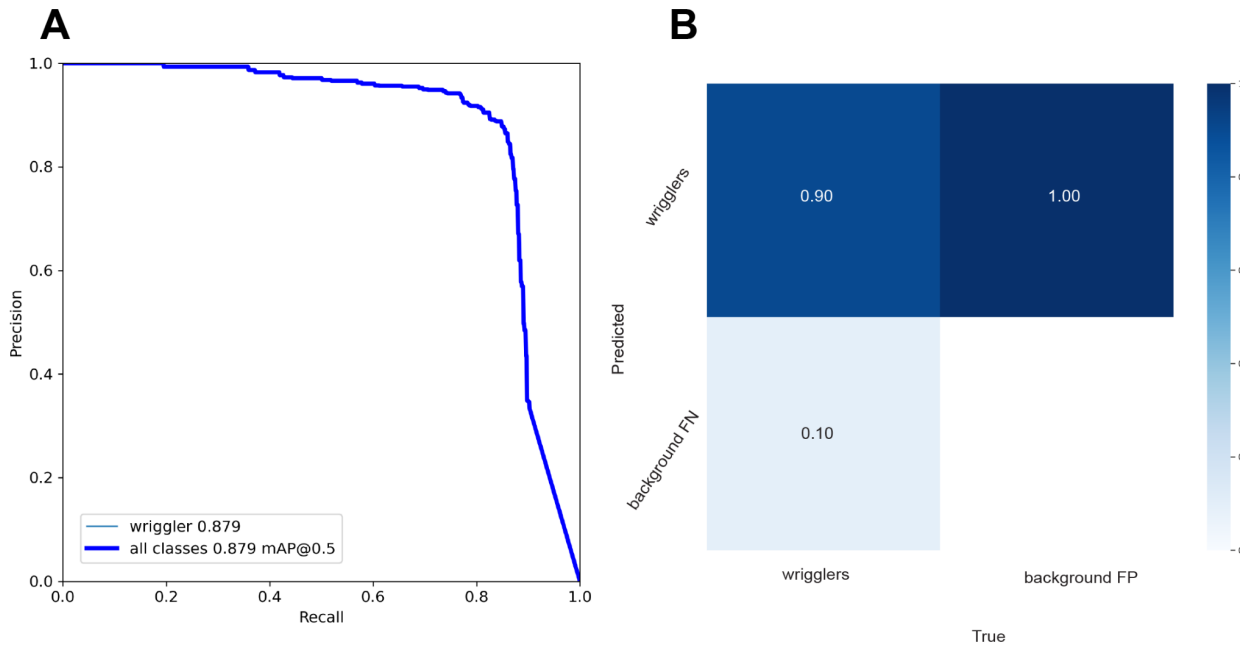


Figure 9: YOLO neural network evaluation metrics for object detection in phototaxis assay. A) The precision recall curves and average precision values for wrigglers, with an mean average precision (mAP) at 0.5. **B)** The confusion matrix for the wriggler detections and the background. Figure taken from Parker et al. (2023) with permission

The position data of all larvae was processed using a custom-made Python script for each dpf and all experimental runs. Predicted detections with a confidence level below 0.25 were filtered out. Time information was extracted from the images, assigning each larva detected to a specific dpf and hour of the day. The location of each larva was categorised into one of the two ROIs, and all dpf files from one experimental run were concatenated. Using a pivot table, I totalled the number of larvae in the dark versus the light for each hour of each dpf and calculated the percentage of larvae in the dark at those time points.

At this stage, all experimental runs were combined. To create Figure 21B, I plotted the average ± standard error percentage of larvae in the dark for each hour of each dpf. In Figure 21C, I compared the average percentage of larvae in the dark during the day pre-emergence (7 and 8

dpf) to that post-emergence (9 and 10 dpf) for each dataset and performed a two-sided Wilcoxon signed-rank test.

2.6 Comparison of Reproductive Strategies

I acquired a dataset from Niclas Kolm, first author of Kolm et al. (2006a, 2006b), which includes extensive information on the majority of African cichlid species, compiled from various publications cited in Kolm et al. (2006a). Using this dataset, I recreated Kolm's bivariate correlations for species data and incorporated data for *L. ocellatus,* comparing log values of egg diameter versus clutch size, care duration versus clutch size, and body length versus clutch size (see Fig. 22). Subsequently, I focused on species native to Lake Tanganyika, classifying them as mouthbrooding, substrate brooding, or shell-dwelling (see Fig. 22D–F). I performed the same comparisons, now segregating the data based on the care strategy. Additionally, I plotted bar graphs to compare clutch sizes and egg sizes among substrate brooders, mouthbrooders, and shell-dwellers within this Tanganyikan species subset (see Fig. 22G–H).

Chapter 3: Results

3.1 Establishing a Paradigm to Watch Cichlid Development and Parental Care

3.1.1 Designing a Shell with a Window

To investigate the development and behaviours of *L. ocellatus* larvae inside the shell, it was necessary to gain visual access to as much of the shell interior as possible, while maintaining the natural behaviours of the mother and larvae. To achieve this, I adapted a 3D model file, originally based on the shell morphology of the snail species *Neothauma tanganyicense* (Fig. 10A), provided by the lab of Alex Jordan (Bose et al., 2020; Fig. 10B). Using Blender 2.82 software (Blender Foundation, 2020) to alter the design, the back of the shell, opposite to the aperture and just behind the inner spiral, was removed (Fig. 10C). Following this, the cut walls were extruded outward (Fig. 10D) to restore the volume lost during the cutting process. This modified 3D printed design facilitated optimal visibility into the shell while ensuring the mother's ability to navigate through the deeper chambers and perform her natural behaviours (Fig. 10E).

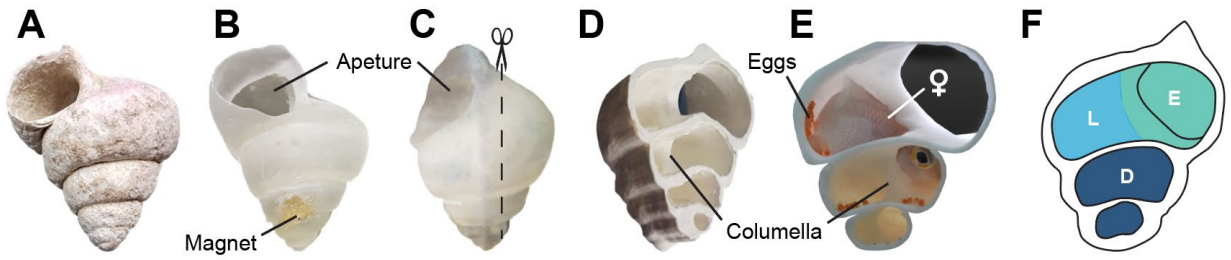


Figure 10: Construction of a functional 3D printable window shell that allows maximal visual access to the inner spaces. A) A natural shell from Lake Tanganyika, from the species *Neothauma tanganyicense*. **B)** A 3D printed version of the natural shell, consisting of two halves held together with a pin and magnet to facilitate easy access to the fish inside the shell. **C)** The two halves of the 3D printed shell were merged and an off-centre cut was made opposite of the shell's aperture (dotted line). **D)** The cut shell walls were extruded to recreate the space needed for the mother to swim around the inner spiral. **E)** The mother can use the shell as she would naturally, laying her eggs and reaching the deeper chambers of the shell. **F)** For analysis purposes, the space inside the shell was divided into the entrance area (E; teal), laying chamber (L; blue) and deep chamber (D; navy). Figure adapted from Parker et al. (2023) with permission

These adapted 'window' shells were placed against the inner glass of an aquarium, providing a viewpoint from which the total shell interior, excluding areas obscured by the columella, could be seen. From this view of the shell, the inside space was divided into three regions of interest: the entrance area, laying chamber and deep chamber (E, L, D respectively in Fig. 10F).

3.1.2 Building a Tank Set-up to Film Parental Care Inside the Window Shell

3.1.2.1 The filming arena

A 100-litre experimental arena was designed and constructed with versatility in mind (Fig. 11). The arena featured light-absorbing white acrylic bottoms and three walls. Typically *L. ocellatus* is housed with a sand substrate and will manipulate the sand and shells to produce a nest with a vertically-oriented shell submerged in the sand with only the aperture exposed. To replicate this in the absence of sand, two window shells were inserted into specially designed cut-outs in the acrylic, 430 mm apart, mimicking a prepared nest (see Fig. 6BC). The arena was submerged in the facility tanks, with the open side firmly pressed against the glass tank wall. The facility's water exchange mechanisms were integrated into the experimental set-up to allow for the indefinite housing of fish.

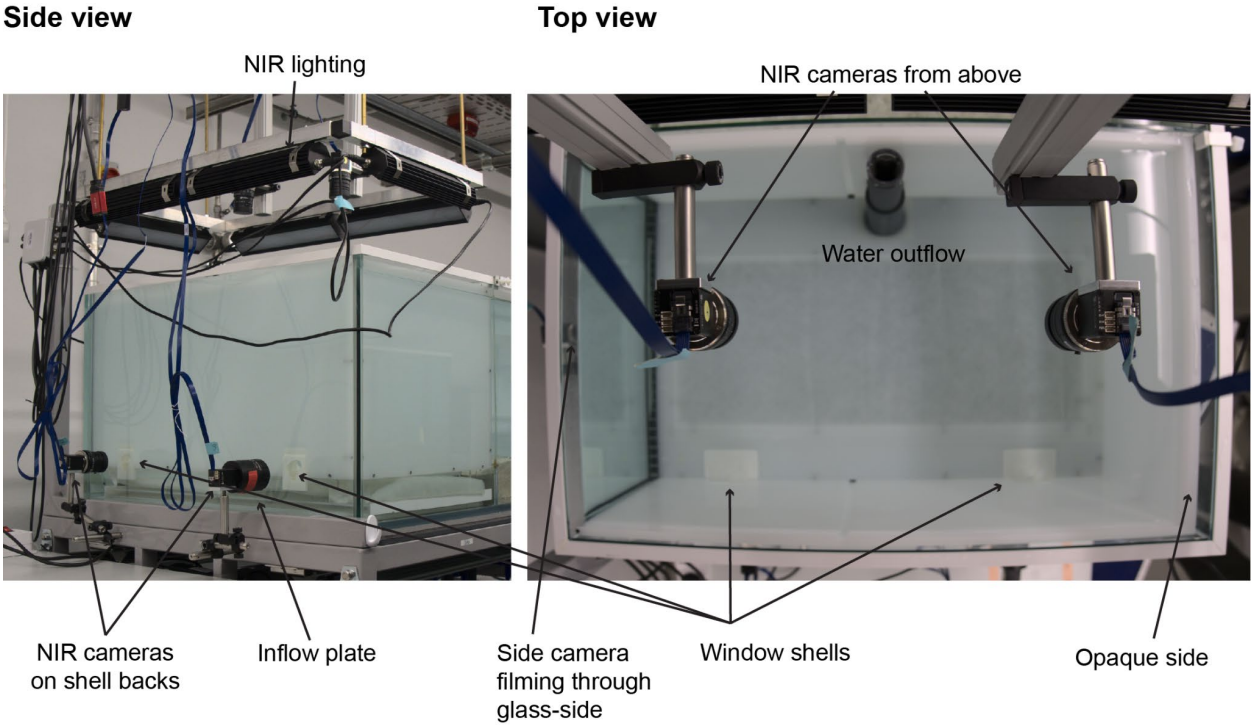


Figure 11: Self-constructed 100 litre observation tank used for all behaviour experiments. The design allowed for the insertion of two window shells into the light-absorbing acrylic tank insert. An inflow plate ensuring the even entry of water into the tank was located under the tank insert. An outflow tube was located near the wall opposite to that of the shells. The shell backs were imaged with NIR cameras for observing the larvae and mother inside the shell. The two top-view NIR cameras were used to film the full tank space, and a side camera was used to film through the open

side of the arena. The tank was lit by 6 NIR lights from above and two NIR lights (not imaged) were placed next to the camera filming the shell backs to illuminate the shell interior with NIR light. Figure adapted from Parker et al. (2023) with permission.

To capture data from multiple perspectives with high resolution, five cameras were installed: two positioned above, one mounted on the glass side, and two focused on each of the two window shells. All cameras were synchronised using PCIe aggregation hardware (xSwitch by Ximea), and rapid data transfer to a computer was facilitated through a FireFly copper ribbon cable. Custom-written software, developed in collaboration with a computer science student, was utilised to collect and compress incoming imaging data (Fig. 12A–D) to ensure that no frames were lost and to merge the two field of views from the top cameras into a single field of view (Fig. 12D).

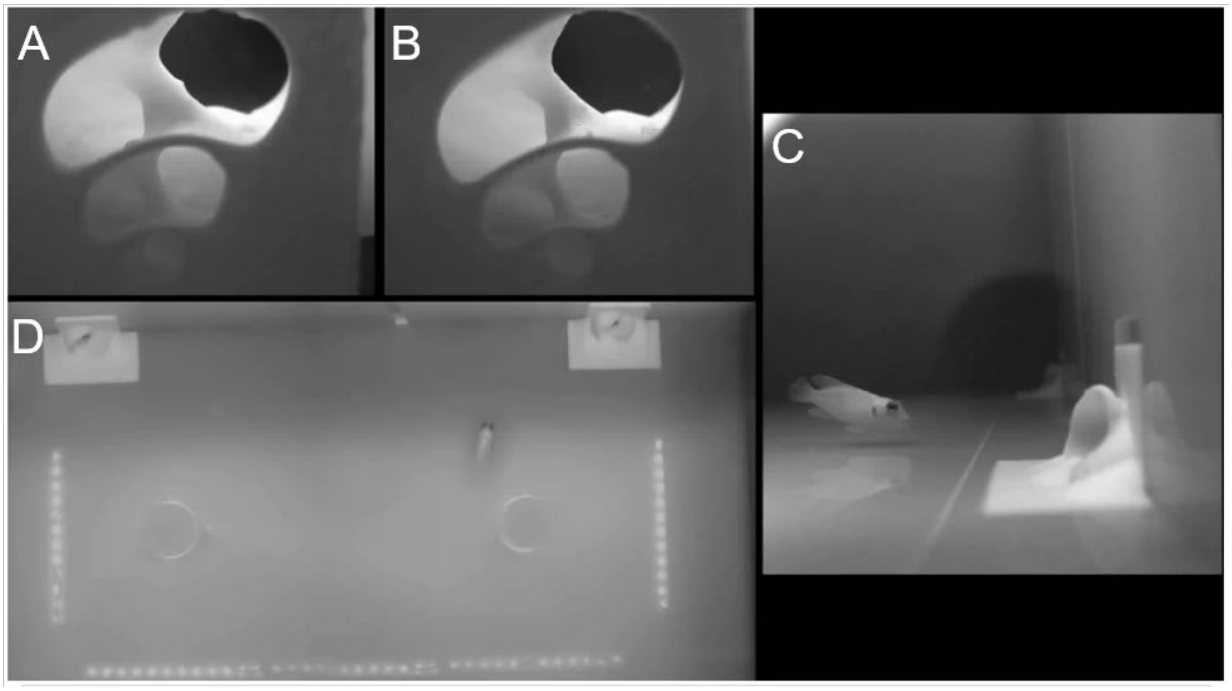


Figure 12: Synchronised imaging data acquired from five cameras connected to a xSwitch. A–C) Field of view captured by the cameras filming the shell interiors (**A** and **B**) and the side of the tank (**C**). **D**) The merged field of view from the two cameras filming from above the behaviour arena.

As shell interiors are dark in the natural environment, I faced the challenge of illuminating the shell interior sufficiently in order to capture high-quality images and videos, while still maintaining a relatively dark natural environment within the shell. Additionally, since I wanted to capture data across day and night, I needed a light source that would not adversely affect the behaviour of the

fish. Though near-infrared (NIR) light is typically used in these situations, there have been no studies to date showing that cichlids are incapable of seeing into the NIR spectrum.

3.1.2.2 Observing L. ocellatus *under NIR conditions does not disturb their natural behaviour*

To investigate whether cichlids could detect NIR light sources, a female was introduced into a set-up where she was exposed to three different light conditions within a dark enclosure. The first condition involved illumination from above with IR light only, the second condition involved illumination from both NIR and white light, and the third condition involved again only NIR light from above. The female froze for the entire duration of 2 minutes with only the NIR light illuminating the arena. When the white light was added, she exhibited normal swimming behaviour and eye movements when the white light was turned on. When the white light was turned off, leaving only the NIR light source, she again displayed a freezing response for over a minute. This suggests that when only NIR light was available, the fish perceived its surroundings as dark, and possibly exhibited freezing behaviour as they required additional light to obtain more visual information to perform swimming or exploratory behaviours. Thus NIR seems not to disturb the normal behaviour of the fish. Taking these results into account, the back of the window shell was covered with NIR-penetrable plastic to block the visible light and provide a dark environment inside the shell while allowing continuous illumination of NIR light for filming (Fig. 13A). A six-panel NIR lighting rig was built to illuminate the tanks day and night from above (Fig. 11), while two NIR panels were used to flood light into each shell through the NIR plastic.

3.1.2.3 The parental care and development paradigm

To capture the brood care behaviour, a couple was placed inside the arena and time-lapse images and video data were collected for 11 days from egg-laying (Fig. 13B). Importantly, *L. ocellatus* bred successfully in these shells, with females presenting behaviours similar to what19 has been reported in the lake, including egg-laying and fry-guarding (Bills, 1996; A. Jordan et al., 2021; Sato & Gashagaza, 1997).

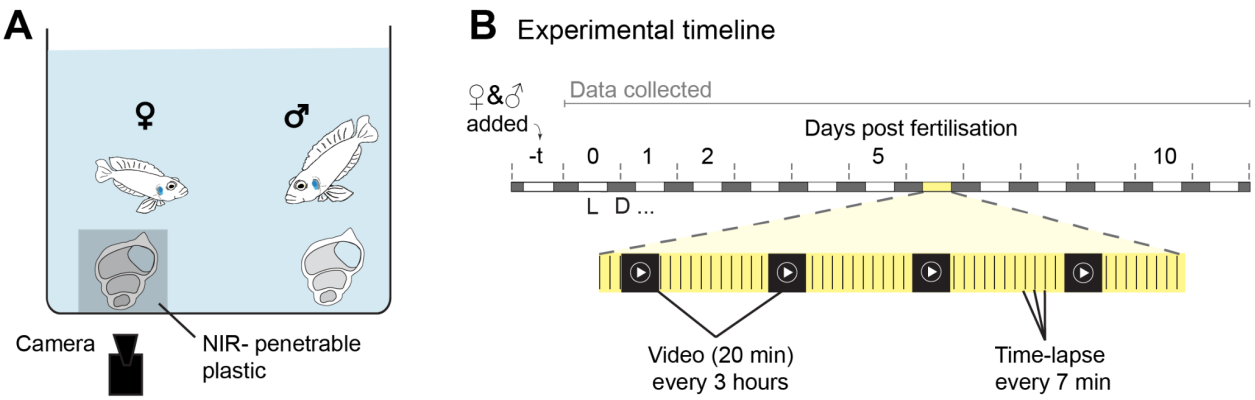


Figure 13: Filming the parental care paradigm inside the shell. A) The tank set-up includes a male and female breeding pair, each provided with a 3D printed shell attached to the glass and covered with NIR-penetrable plastic to prevent visible light entering into the shell. **B)** The experimental timeline shows the introduction of a breeding pair t days before fertilisation, and the filming paradigm over 11 days across daytime (L; white) and nighttime (D; grey). Figure adapted from Parker et al. (2023) with permission.

3.2 Development of Larvae Cichlids

I wanted to get a full understanding of the behavioural capabilities of the larvae within the shell, which included observing their morphological development during these early stages. With assistance from a colleague, larvae were reared in a petri dish at 27 ℃ and imaged daily under a light microscope until 9 days post-fertilisation (dpf) to assess morphological development (Fig. 14A) alongside the time-lapse images of the clutch inside the shell (Fig. 14B, video 1). A machine-learning object detector, YOLO (Redmon et al., 2016), was trained to detect all the eggs and larvae inside the shell across all frames over the 11 days of image acquisition. With this detection data, we could identify and sort the position of individual larvae over the observed period into each of the three pre-defined chambers: entrance area, laying chamber or deep chamber (Fig. 14C).

3.2.1 Morphological Development

3.2.1.1 Eggs to hatching

Females were recorded laying their eggs inside the shell, typically on the side walls of the first whorl of the shell, or laying chamber (Fig. 14Bi, C). In 10 of the 12 experiments where laying and fertilisation were recorded, the female laid her eggs in the morning between 5:30–11:30. The laying and fertilisation process took on average 1.99 ± 1.6 hours (mean ± standard deviation (sd),

n=12) and clutch size could vary from around 10 to 50 eggs with an average of 28.15 ± 10.15 (± sd; n=20). Each egg, on average 1.42 ± 0.08 mm (± sd; n=114) in diameter, contains a large orange yolk, leaving minimal space for the embryo to develop within the chorion (Fig. 14Ai).

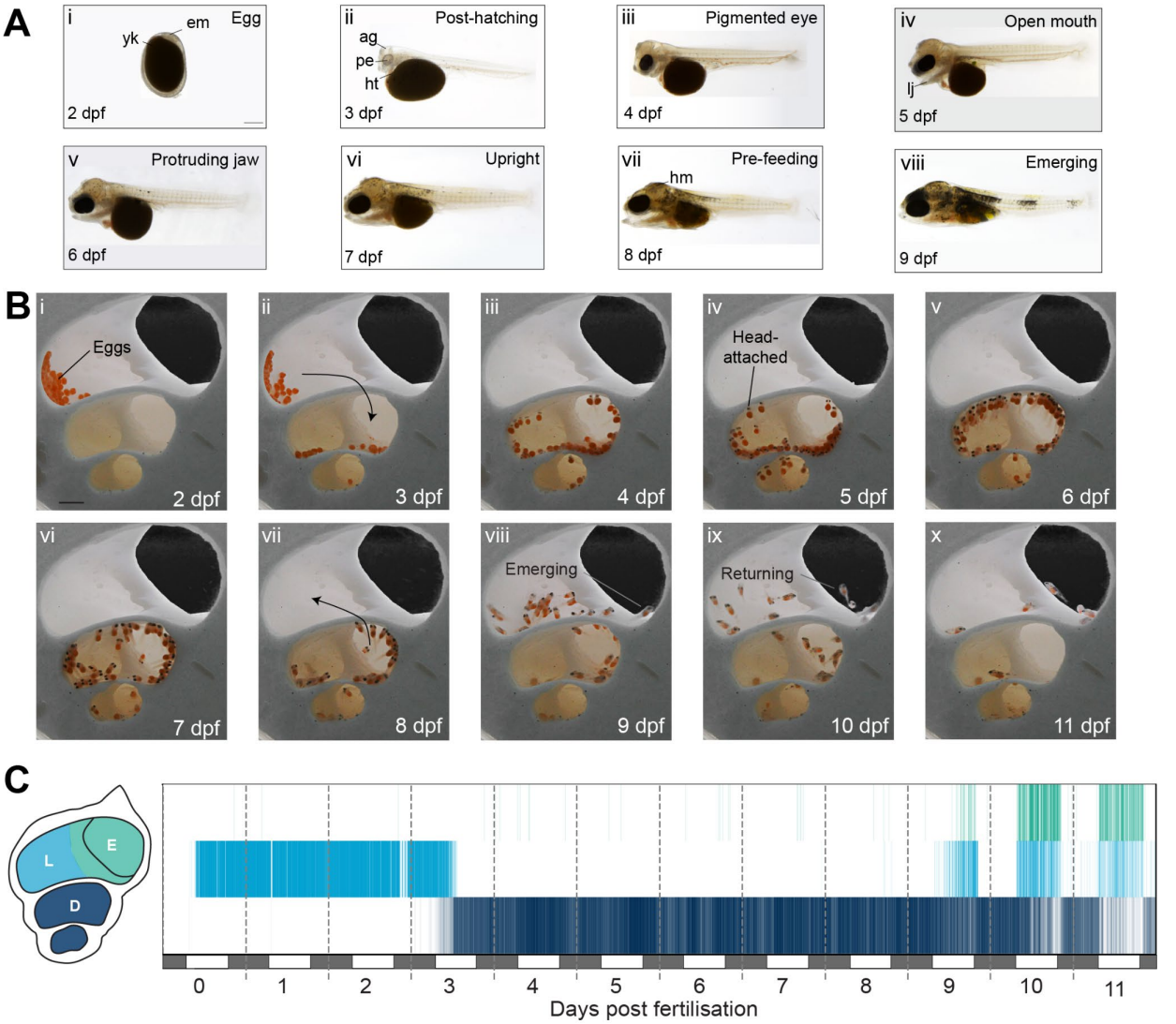


Figure 14: Larval morphological and behavioural development inside the shell. A) Morphological development: **Ai)** Egg stage and **aii–viii)** Larval stages from 3–9 dpf. Scale bar = 0.5 mm. Abbreviations: yk, yolk; em, embryo; ag, adhesive gland; ht, heart; pe, pigmented eye; lj, lower jaw; hm, head melanophores. **B)** Shell space utilisation of egg- and larval-stage fish: **Bi,ii)** Egg stages, **Bii–vii)** wriggler stages, and **Bvii–x)** emerging larvae. Curved arrows represent larvae migration through the shell chambers. Scale bar = 5 mm **C)** Typical distribution of eggs and larvae in each chamber from 0–11 dpf from one clutch. Each vertical line represents a single frame taken across the 11 days, with the alpha value of each vertical line in each chamber representing the normalised proportion of larvae at the time of the snapshot. White and grey alternating bars under the plot indicate day and night, while vertical dotted grey lines separate each dpf. Abbreviations: E, entrance area; L, laying chamber; D, deep chamber. Adapted from Parker et al. (2023) with permission.

The chorion of the egg is surrounded by a sticky mucus, glueing the eggs to one another and the shell wall (Kratochwil et al., 2015).

3.2.1.2 Hatching to emergence

The eggs hatched mostly between the late evening of 2 dpf, up to the early morning of 3 dpf. As each egg hatched, the mother picked it up and translocated the egg into the deep chamber of the shell where all the larvae then resided for the next 6 days (Fig. 14Bii, C). Hatched larvae carry an adhesive gland on the top of the their heads, a feature present in most substrate brooding cichlid larvae (Balshine & Abate, 2021; Kratochwil et al., 2015; Peters & Berns, 1983), which serves to anchor the larvae to the shell wall before they become free swimming (Fig. 14Aii, Bii–viii). At 3 dpf the cranium of the larvae was still transparent and the fore-, mid- and hind- brain were visible. The pigments of the eye were starting to appear and the first red blood cells began to circulate. By 4 dpf, the eyes were fully pigmented and the head and body had grown. The lower jaw was also now visible (Fig. 14Aiv) but only really became prominent at 5 dpf (Fig. 14Av). At 6 dpf, the lower jaw freed from the yolk, extending the head in a more anterior direction (Fig. 14Avi). From this stage, the larvae were constantly opening and closing their mouths. Pigmented cells also started appearing on the head and dorsal side of the larvae.

Notably, the larvae resting on the floor underwent a shift in posture between 7 and 8 dpf, from lying on their sides to ventral-side down (Fig. 14Avi, Bvi–vii) possibly as a result of swim bladder inflation (Kratochwil et al., 2015). At 8 dpf, the pigmented cells increased in density on the head and anterior spine. By 9 dpf, an additional two patches of pigments appeared along the spine, alternating with areas lacking pigment, giving the larvae a striped appearance (Fig. 14Aviii).

3.2.1.3 Emergence and post-emergence

The larvae underwent an important transition at 9 dpf. They started to move up to the laying chamber, into the entrance chamber and also began to explore outside the shell (Fig. 14Bviii, C). This was the first time the larvae were seen returning to the laying chamber post-hatching, coinciding with the day they first emerged from the shell through the entrance chamber. From 9 dpf onward, the nighttime behaviour became distinct from that of the daytime. In the days following emergence, the larvae utilised all chambers of the shell during the day, while at night they predominantly resided in the deep chamber for refuge (Fig. 14Bviii–x, C). The yolk of the

larvae continually depleted over the course of development as the fish grew but was still visible at 11 dpf (Fig. 14Bx).

3.2.2 Observation of Larvae Behavioural Development Inside the Shell

In addition to the time-lapse data collected, 5 minutes of video footage captured at noon every second day during larval development was scored using BORIS (Friard & Gamba, 2016) to ascertain any fine-grained behaviours inside the shell (Fig. 15A–D; n=10). From the video footage, four key behaviours performed by the larvae were identified: wriggling, resting, head-attached and swimming, examples of which can be viewed in Video 2. Additionally, feeding behaviour was monitored in the larvae raised in a petri dish.

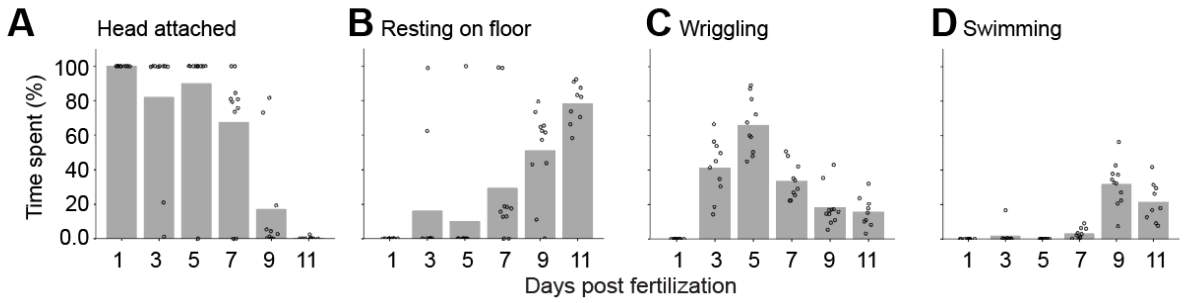


Figure 15: Behavioural changes of developing larvae inside the shell. Larvae (n=10) were observed on video for 5 minutes and scored using BORIS for the following behaviours: **A)** head-attached, **B)** resting on the floor, **C)** wriggling, and **D)** swimming. Figure adapted from Parker et al. (2023) with permission.

3.2.2.1 Head-attached and resting within the shell

Once hatched, the larvae spent most of their time attached to the inside of the shell: adhered to the inner walls by their adhesive glands (Fig. 15A), or in some instances, grounded due to the weight of their yolk (Fig. 15B). From 3 to 7 dpf, larvae were constantly head-attached to the inner shell wall or resting on the shell floor. However, these mutually exclusive behaviours change in proportion to one another with larval development. At 3 dpf, 80 % of the larvae scored were constantly attached via their head's adhesive gland, while 20 % were either only resting on the shell floor or spent time attached in both positions. Head-attachment remained the dominant position until 7 dpf, where 60 % of the larvae switched between the two attachment forms, while the remaining 40 % were equally split between being constantly head-attached and constantly

resting on the floor. By 9 dpf, larvae were only attached 70 % of the time, with resting on the floor now being the predominant position for most larvae. This switch from head-attachment to resting of the shell floor continued up to 10 dpf, after which larvae were no longer recorded being head-attached to the shell walls.

3.2.2.2 Wriggling behaviour

Head-attached and larvae resting on the floor performed intervals of high-frequency tail beating, termed “wriggling” (Courtenay & Keenleyside, 1983). Wriggling bouts lasted from 0.03–50 seconds. The amount of time spent wriggling varied as the larvae aged (Fig. 15C). Freshly-hatched larvae at 3 dpf spent an average of 41 % of their time wriggling. The average time increased to a peak of 66 % at 5 dpf before dropping down at 7 dpf, to an average of 34 %. There was a further decrease in wriggling duration to 20 % at 9 and 11 dpf, which coincided with an increase in swimming periods (Fig. 15D).

3.2.2.3 Swimming development

Here, swimming refers to periods where larvae were recorded detaching from the shell wall or floor. In the earlier larval stages, these periods were seldom and short-lived, lasting a maximum of 6.7 seconds before larvae reattached (Fig. 15D). At 9 dpf, there was a significant increase in time spent swimming which was maintained up to 11 dpf, the end of recording. In these later stages, the swimming appeared more deliberate and was used by the larvae to navigate around shell interior and to exit the shell. While making these movements, the larvae swam continuously rather than in bouts, until the larvae rested on the shell floor.

3.2.2.4 Feeding onset

I tested the larvae’s ability to catch prey by transferring larvae as young as 7 dpf to petri dishes and adding live brine shrimp (*Artemia salina*). Larvae only begin hunting this prey from 9 dpf onward, at which point we deem them independently feeding.

To summarise, these results indicate that once hatched, the larvae predominantly spend their time attached to the shell wall, adhering via their sticky mucus glands on their heads while periodically wriggling in place. As they develop, the larvae become increasingly mobile, detaching

and reattaching more frequently. Gradually, they spend less time adhered to the shell wall by their heads and more time resting on the shell floor or swimming purposefully around the deeper chambers. By 9 dpf, they make their way through the laying chamber and emerge from the entrance chamber for the first time.

3.3 Observations of maternal brood care activities

The mothers of these shell-dwelling larvae were also observed over the 11 days of data acquisition. I obtained each mother's head position inside the shell by analysing time-lapse images captured at 7-minute intervals. I used the YOLO object detection tool, trained with a model specifically for the mother (see methods) and reported her location within the shell in a similar way to the larvae (example: Fig. 16A, B). From this data, I could compare across clutches and different mothers to pull out trends in visitation frequency over the 11 days and also compare her day (Fig. 16C) and night time (Fig. 16D) presence in the shell. Additionally, BORIS was used to analyse the mother's behaviours inside the shell during the 20 minute videos taken at noon on 1, 3, 5, 7, 9 and 11 dpf (Fig. 16E and F; white bars) and at 7 dpf for nighttime (Fig. 16E and F; grey bars) behaviour, see Video 3 for behaviour examples.

3.3.1 Visiting Patterns during Brood care

The results show that of the three chambers, the mother mostly visited the entrance of the shell (Fig. 7B). Notably there were recurring periods where the mother was almost constantly present in the entrance. These periods corresponded with nighttime data (Fig. 7B, D; n=3–5) and I observed that across all mothers, they were present in the shell almost throughout the night. Looking at the daytime data alone, I saw all three chambers being used more evenly (Fig. 7B). There was an increase in visitation during 2 and 3 dpf (Fig. 7B, C; n=4–12). The mother played an active role in moving freshly hatched larvae with her mouth from the laying chamber to the deep chamber.

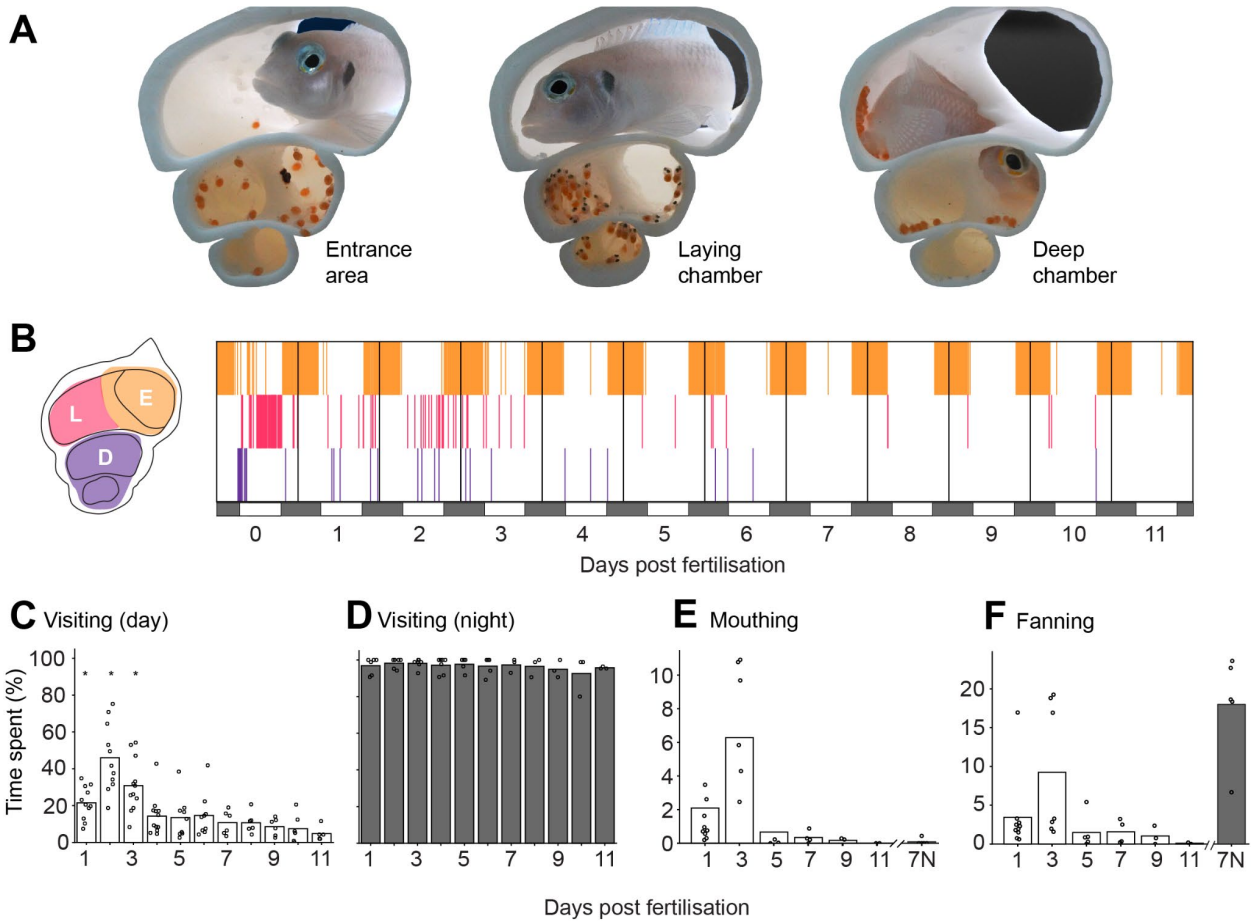


Figure 16: Mother's behavioural repertoire inside the shell during brood care. A) Images demonstrating the classification of mother's positions within the shell. **B)** An example of the mother's location inside the shell during the first 11 days of brood care. Automated mother detection (YOLO) and positional classification were used. Each vertical coloured line indicates the presence of the mother's head in one of the chambers (E, entrance; L, laying; D, deep) for each snapshot taken every 7 minutes. White and grey alternating bars under the plot indicate day and night, while vertical dotted grey lines separate each dpf. **C–D)** The mean percentage of time mothers spent in the shell during the day (n=4–12) **(C)** and night (n=3–5) **(D)** up to 11 dpf. **E–F)** The percentage time mothers (n=3–8) spent mouthing **(E)** or fanning **(F)** the eggs or larvae during 20 minute video recordings at noon, daily (white bars) or 3 am at 7 dpf (7N; grey bars). Circles on bar graphs represent the individual values for each mother during a 20 minute recording. Figure adapted from Parker et al. (2023) with permission.

3.3.2 Mouthing Behaviours Change with Offspring Age

Transporting the larvae was just one of the ways in which the mother used her mouth to interact with her offspring. The mother was also frequently seen touching the eggs with her lips and removing dead eggs. Once hatched, she would also pick up the larvae and swirl them around in her buccal cavity before depositing them into the deep chamber. These mouthing behaviours occurred predominantly at the egg stage and at 3 dpf when transporting the freshly hatched larvae was required (Fig. 16E; n=3–8). Mouthing occurrences seemed to drop dramatically for the later

stages and were not recorded to take place at night. However, if a larva strayed from the deep chamber or out of the shell, the mother would rapidly pick it up and deposit it back into the deep chamber.

3.3.3 Fanning the Offspring Changes with Age and Time of Day

Another behaviour performed by the mother was fanning, characterised by rapid movements of the anal and caudal fins to facilitate water exchange within the shell. The behaviour was predominantly observed within the entrance chamber, with occasional occurrences in the laying chamber. Across the initial 9 dpf, mothers consistently allocated 2–3.5 % of their time to fanning, executing this behaviour approximately 15–20 times per hour. An exception lies at 3 dpf, an increase in fanning activity was observed coinciding with egg hatching, where mothers exhibited an average of 55 fanning events per hour, corresponding to a heightened fanning duration of approximately 9.5 % of their time (Fig. 16F; n=3–8).Interindividual variability was notable, particularly evident at 1 and 3 dpf, although fanning activity remained consistently minimal by 11 dpf. During the nocturnal period, measured at 3 am on 7 dpf, maternal investment in fanning peaked, with mothers dedicating up to 24 % of their time to this behaviour, initiating an average of 96 fanning events per hour.

Taken together, the results show the mother is frequently attending to her offspring, performing mouthing and fanning behaviours. This is especially true in the early dpfs, before and during hatching. While mouthing behaviours almost totally stop after hatching, fanning continues, but less frequently. However at night the mother is constantly in the shell and fans on average more than during the day.

3.4 Determining the Mechanism Behind Emergence at 9 dpf

From the repeated observations of larval development in the shell, what stood out was the consistent timing of the larvae's initial emergence from their shell at 9 dpf (Fig. 17E). Before this, the larvae were confined exclusively to the deep chamber of the shell once they had hatched, not even using the laying chamber before the day of emergence (Fig. 17A). This naturally led to the question of what was driving this sudden change in behaviour. Investigations hereafter focused on whether this change was influenced by extrinsic factors, such as changes in the mother’s behaviour, or intrinsic factors, such as a shift in larval development.

3.4.1 Investigating Extrinsic Factors Involved in Emergence

In order to determine the extrinsic factors affecting the emergence time of the larvae from the shell, manipulations were made to the natural in-shell-raising paradigm, henceforth referred to as the control (Fig. 17A).

3.4.1.1 Maternal absence accelerates larval emergence

The first objective was to remove the parents and assess how this would affect the emergence time of the offspring compared to the control. In pilot experiments, the parents were first removed pre-hatching but none of these eggs survived. Instead, removal was delayed to either 5 or 7 dpf and the behaviour of the orphaned larvae was observed. Removal of the parents at both 5 and 7 dpf led to the larvae immediately moving up into the laying chamber (Fig. 17B, C, E; n=5). The larvae then began to use the entrance chamber and emerged within 24 hours of parent removal, premature in comparison to the control conditions. After emergence, day-night behavioural differences were less pronounced compared to the control, where larvae resided exclusively in the deep chamber at night but explored all three chambers during the day. In three of the five experiments, none of the orphaned larvae returned to the shell in the day or night by 11 dpf. This suggests in the absence of the mother the larvae leave prematurely, however, it was important to determine the driving reason behind this behavioural change. For future experiments involving parent removal, the mother and father were removed at 7 dpf as I experience no larval fatalities in these experiments, whereas one or two larvae died after the parents were removed at 5 dpf.

3.4.1.2 Artificial supply of freshwater corrects emergence time in absence of mother

I hypothesised that deteriorating water quality inside the shell could be a factor driving out orphaned larvae prematurely and thus tested larvae behaviour when fresh water was provided to the shell. In this experiment the parents were removed at 7 dpf but the larvae were provided with a continuous source of fresh water directly into the deep chamber of the shell before parent-removal until the end of the experiment. Similar to the previous parent-removal experiments, the larvae moved into the laying chamber earlier than control clutches, but they were only observed in the entrance and emerged at 9 dpf, as in control experiments (Fig. 15D, E; n=2).

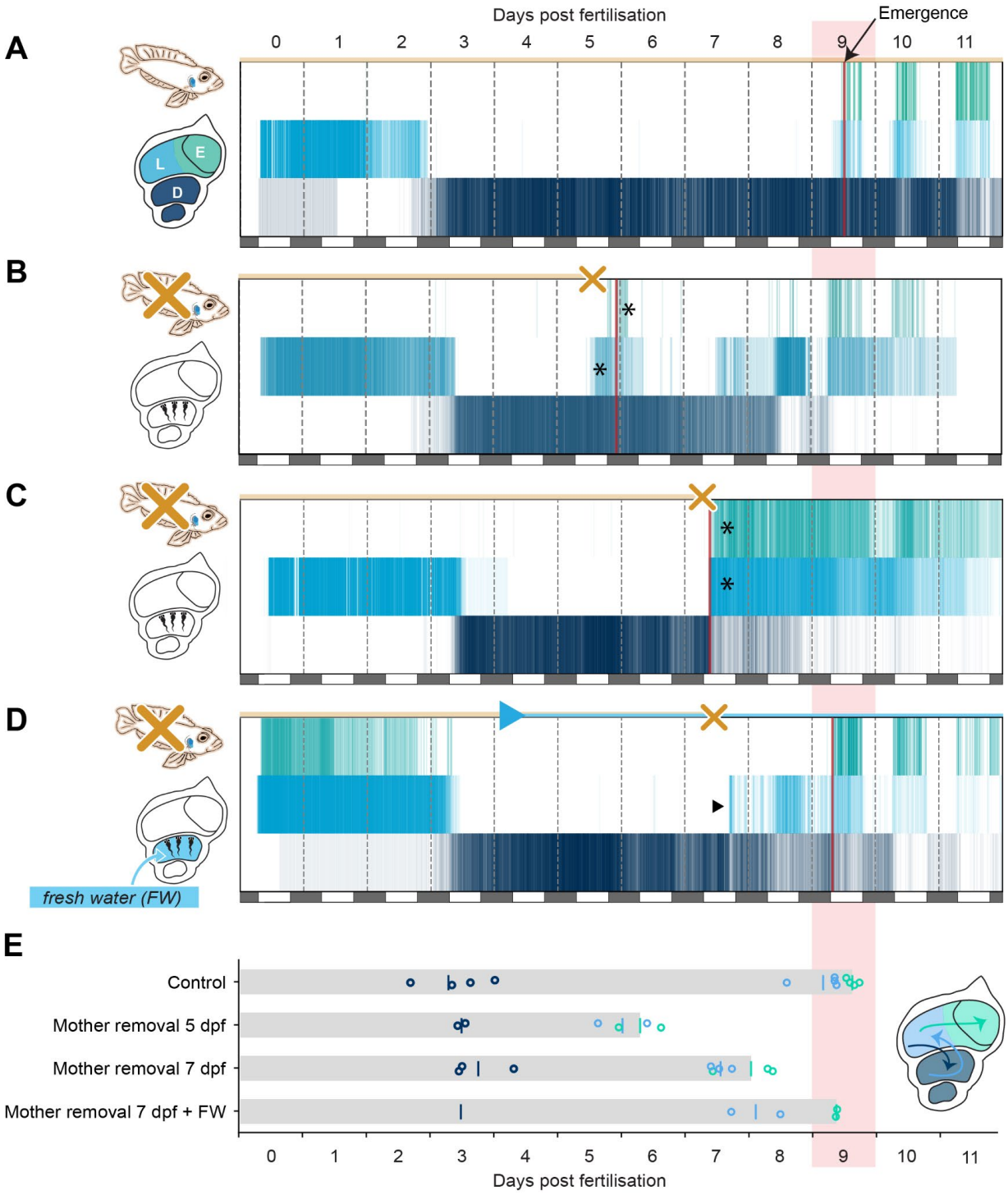


Figure 17: Larvae emerge prematurely from the shell when orphaned unless provided with fresh water. A–D) Larvae distribution across three chambers (E, teal, entrance; L, blue, laying; D, navy, deep) over 11 dpf with presence of the mother (orange bar) and emergence time indicated (red line), asterisk and the black arrowhead point out deviations in the chamber transition timing between experiments and controls. Each figure is an example of the **A)** control condition, **B)** removal of the parents at 5 dpf (orange X), **C)** removal of the parents at 7 dpf (orange X),

D) removal of the parents at 7 dpf while supplying larvae with continuous fresh water from 4 dpf (blue triangle and blue bar). White and grey alternating bars under the plots indicate day and night, while vertical dotted grey lines separate each day. Expected emergence on 9 dpf is indicated by a transparent red bar behind the plots. **E)** Average transition time to the deep chamber after hatching (navy), then to the laying chamber (blue) and average emergence time (teal) of larvae across different manipulations. Individual circular points correspond to each experiment. Figure adapted from Parker et al. (2023) with permission.

Furthermore, the larvae initially exhibited a similar day-night behavioural pattern as the controls after emerging, as they returned to the deep chamber at night until 10 dpf. However, they subsequently continued to at least return to the entrance of the shell at night thereafter. By providing fresh water in the mother's absence, I could restore the natural emergence time of the offspring, thus suggesting the larvae emergence time must have an intrinsic component.

3.4.1.3 Forcing asynchrony between maternal and offspring timelines disrupts emergence

Having established that the larvae possess intrinsic mechanisms that govern emergence, it is still unclear if the mother additionally alters her behaviours to facilitate emergence. The mother's brood care behaviour may, too, be governed by an independent timer, initiated perhaps by the significant act of egg laying. Alternatively, she might continuously adjust her care and provisioning depending on the signals and status of her offspring. To distinguish between these scenarios, the clutches of mothers were exchanged with offspring either 2 days younger or 2 days older than her original clutch, and maternal and offspring behaviours were then both analysed (Fig. 18).

In five out of six experiments, the foster mothers readily accepted the foreign offspring. When mothers fostered offspring 2 days younger than her original clutch (Fig. 18B, D; n=2), the larvae transitioned to the laying chamber earlier than sibling controls that remained with the original mother (Fig. 18A, D; n=4). Moreover, fostered larvae emerged at their biological age of 9 dpf (expected age of 11 dpf to the foster mother).

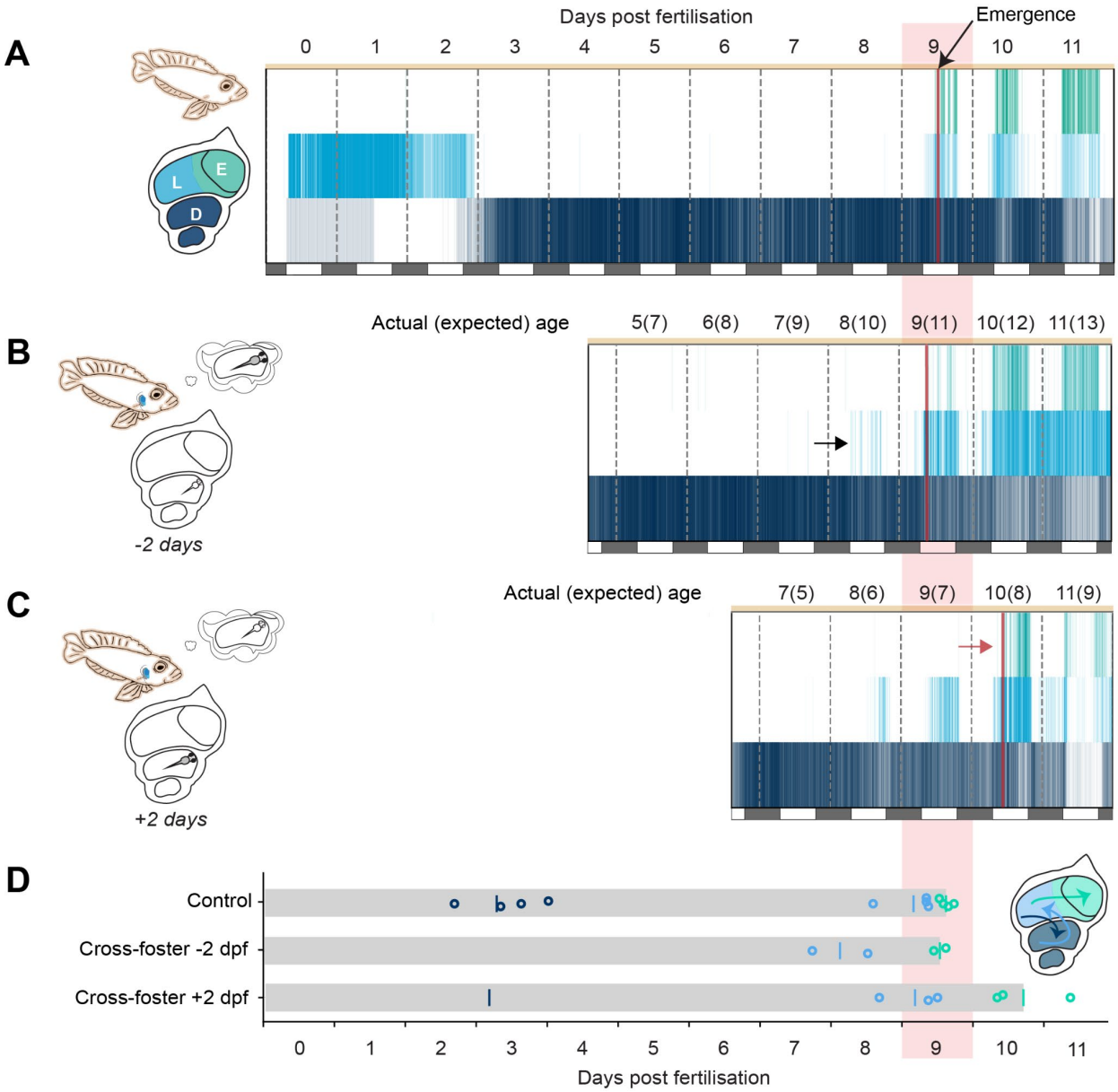


Figure 18: Larvae emergence is delayed when the foster mother perceives offspring as premature. A–C) Larval distribution across three chambers (E, teal, entrance; L, blue, laying; D, navy, deep) over 11 (dpf) with presence of the mother (orange bar) and emergence time indicated (red line). Each figure is an example of a cross-foster experiment where larvae are **B)** 2 days younger than the mother's original clutch and **C)** 2 days older than the mother's original clutch. **D)** Average transition time to the deep chamber after hatching (navy), then to the laying chamber (blue) and average emergence time (teal) of larvae across different manipulations. Individual circular points correspond to each experiment. Figure adapted from Parker et al. (2023) with permission.

Conversely, when mothers fostered offspring 2 days older than expected, emergence was delayed by at least one day to 10 or 11 dpf (Fig. 18C, D; n=3). Observations of the mother revealed increased mouthing activity compared to controls on this day (Fig. 19A; n=3). Although

the time spent fanning was not in all cases higher in foster mothers compared to controls (Fig. 19B; n=3), video recordings depicted these foster mothers vigorously fanning larvae, generating a strong inward current to push the clutch deeper into the shell, a deliberate behaviour not seen in control mothers (see Video 4). The foster mothers were also seen retrieving stray larvae from the laying chamber and depositing them back into the deep chamber. Collectively, these experiments suggest that the mother's brood care also follows an independent intrinsic timing schedule.

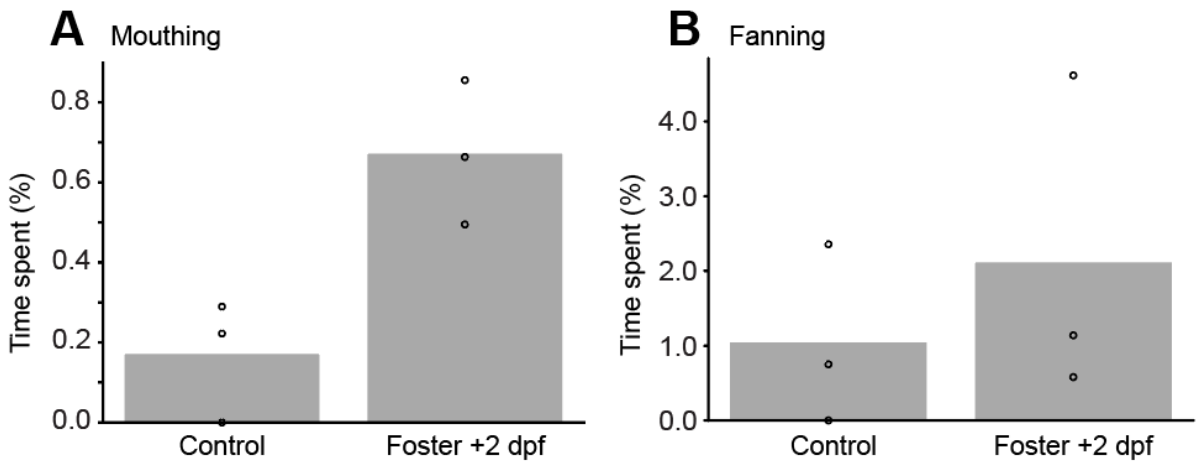


Figure 19: Foster mothers increase care behaviours towards adopted offspring older than her original clutch at 9 dpf. The percentage of time control mothers (with biological offspring) compared to mothers fostering larvae 2 days older than her original clutch, spent **A)** mouthing (n=3) or **B)** fanning (n=3) the 9 dpf larvae during 20 minute video recordings at noon. Bars graph represents the mean percentage across mothers recorded and circles are the values for the individual mothers. Figure adapted from Parker et al. (2023) with permission.

3.4.2. Investigating Larval Intrinsic Drive for Emergence

In discovering that orphaned larvae that were provisioned with fresh water still maintained their natural schedule to emerge at 9 dpf, I wanted to further explore the intrinsic factors that govern this precise emergence time. It was clear that mobility or physical development of the larvae was not hindering an earlier emergence, as seen in the parent-removal experiments where larvae were able to emerge as early as 5 dpf. Therefore, I investigated whether change in internal state (e.g. hunger, dark-light preference switch) could drive the emergence at 9 dpf.

3.4.2.1 Hunger does not drive emergence

To assess the potential influence of hunger on emergence, small amounts of live brine shrimp (*Artemia salina*) were provided to the larvae before and on the expected emergence day in the control paradigm. For this, food was provided at 7 and 8 dpf to test if larvae would eat and then from early 9 dpf using a thin tube inserted directly into the deep chamber. Video recordings revealed larvae engaging in prey capture within the shell and consuming brine shrimp from 9 dpf, but leaving the shell again shortly afterwards. This feeding behaviour did not postpone emergence from the shell (Fig. 20B; n=1). This finding implies that the drive to seek food plays a minimal, if any, role in the decision to leave the nest.

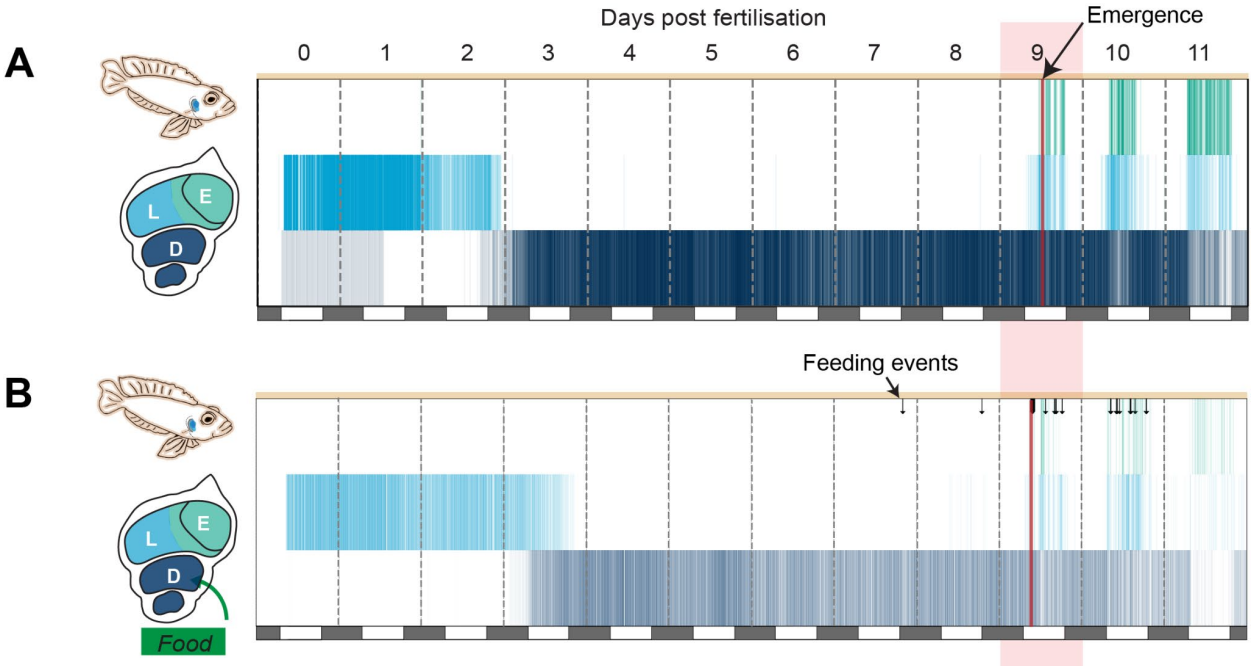


Figure 20: Food provision inside the shell does not affect emergence time of larvae. Larvae distribution across three chambers (E, teal, entrance; L, blue, laying; D, navy, deep) over 11 dpf with presence of the mother (orange bar) and emergence time indicated (red line). White and grey alternating bars under the plots indicate day and night, while vertical dotted grey lines separate each day. **A)** An example of a control experiment and **B)** an experiment where larvae were fed multiple times inside the shell, indicated by the black vertical arrows. Expected emergence on 9 dpf is indicated by a transparent red bar behind the plots. Figure adapted from Parker et al. (2023) with permission.

3.4.2.2 Light preference switch correlates with emergence timing

Next, I hypothesised that the emergence of larvae might be influenced by an intrinsic preference for light. Pre-emergent larvae could be drawn to dark environments, thus remaining within the darker interior of the nest. This preference may switch at 9 dpf, thereby prompting them to swim towards light and migrate out of the shell. To see if there is a correlation between phototactic switch and emergence time, I developed a new assay: Larvae were collected from shells at 6 dpf and placed in a testing arena in small groups of 4 to 5 animals (Fig. 21A). The arena was divided into two halves, with one half kept dark and the other exposed to the same ambient light cycle as in my previous experiments (see Methods for details). I found across 6 experimental groups that at 7 dpf, nearly all larvae preferred the dark environment over both day and night (Fig. 21B). By 8 dpf, approximately 72 % of the larvae still favoured the dark chamber on average, but this preference waned as the day progressed, dropping to an average of 65 % larvae preferring to remain in the dark. By 9 dpf, the balance shifted towards a light preference where larvae began to migrate to the light: Only 40 % of the larvae remained in the dark during the day. Similar trends were observed at 10 dpf.

During nighttime, when ambient light levels significantly decreased, a persistent preference for darkness was observed across all ages, with averages ranging from 56–100 % of larvae on the dark side of the arena. Overall, there was a significant decrease (p=0.03, two-sided Wilcoxon signed-rank test) in daytime preference for darkness during pre-emergent stages (7 and 8 dpf), with an average of 76 % in the dark compared to post-emergence stages (9 and 10 dpf) where an average of 38 % remained in the dark (Fig. 21C).

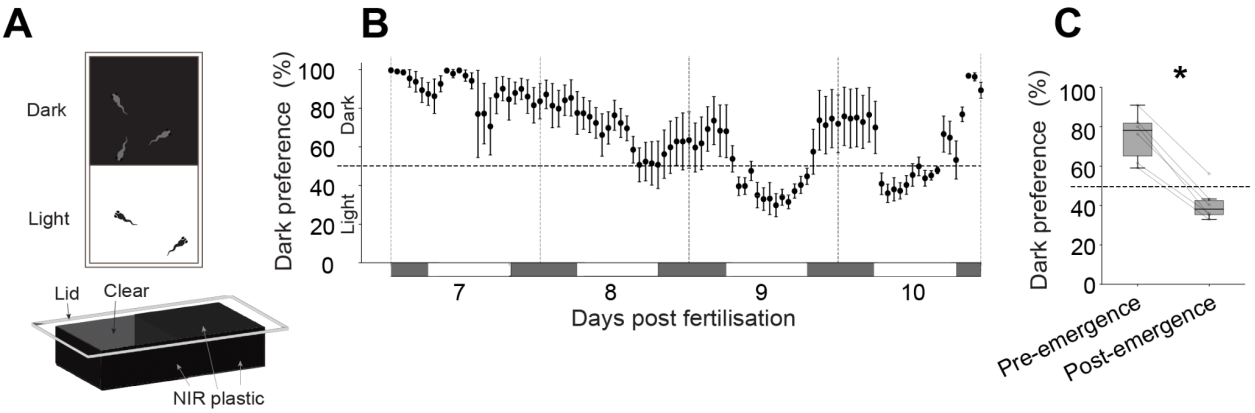


Figure 21: Larvae show a switch in light preference coinciding with their emergence time. A) The experimental box used to test light preference with one side of the lid blocking visible light (black rectangle, NIR plastic) and the other allowing visible light through (white rectangle, clear). **B)** The mean percentage and standard error of larvae (n=28) that preferred the dark side of the box in each hour across 7–10 dpf. White and grey alternating bars under the plot indicate day and night, while vertical dotted grey lines separate each dpf **C)** A box plot of the percentage of larvae in the dark during the daytime before and after emergence. Each data point indicates the mean preference in each experiment before and after emergence. Overall difference is significant (asterisk) (p=0.03, Wilcoxon signed-rank test). Figure adapted from Parker et al. (2023) with permission.

These results indicate that there is indeed a switch from dark preference to light preference and the timing of this switch coincides with the day the *L. ocellatus* larvae emerge from the shell. There is also a consistent preference for the dark at night, regardless of age.

Chapter 4: Discussion

4.1 Summary of Findings

This doctoral thesis makes two key advancements within the field. Firstly, it introduces *Lamprologus ocellatus*, a shell-dwelling cichlid, as a novel model organism, demonstrating its suitability for laboratory investigations and its utility in studying fundamental concepts such as development, evolutionary adaptations, social dynamics, and brood care behaviour. Filming data was collected on the embryonic and larval development of *L. ocellatus* inside the protective confines of their nest, providing the first comprehensive delineation of the brood care paradigm of shell-dwelling cichlids. Using artificial neural networks, the spatial distribution of fry and mother inside the shell were tracked, and the parent-offspring interactions were recorded continuously over an 11-day period from the time of fertilisation to a late-larval stage. This sheds light on the adaptations necessary for the morphological and behavioural development of larvae within a shell and reveals the central role of maternal care during the initial stages of their development.

Secondly, this thesis dissects a pivotal behavioural change occurring at 9 dpf, namely the first emergence of larvae from the shell. Subsequent experiments reveal the interplay between two key factors: the intrinsic phototactic switch of the larvae that dictates the timing of their emergence, and the mother's independent timeline of care, which acts as a regulating mechanism to prevent premature emergence. It is essential for these factors to align harmoniously to ensure that natural emergence takes place at 9 dpf.

4.2 Adaptations to Maternal Care and Larval Development Inside a Snail Shell

This is the first study of shell-dwelling cichlid larvae development and parental care observed from inside the shell. Comprehensive studies exist on early morphological development in substrate brooding larvae from South America (Kratochwil et al., 2015; Meijide & Guerrero, 2000) and mouthbrooding species from Africa (Fujimura & Okada, 2007; Woltering et al., 2018). And earlier research in the 1980s provided basic insights into larval morphological and behavioural development in other closely related Lamprologian substrate brooding species (Nagoshi, 1983; Nagoshi & Gashagaza, 1988; Yanagisawa, 1987). However, due to the lack of accessibility, the larval and parental activities within the shell remained elusive.

4.2.1 Shell-dwelling Brood Care in the Context of Substrate- and Mouthbrooding Cichlids

The two major parental care systems in cichlids are mouthbrooding and substrate brooding. These two strategies demonstrate distinctive reproductive characteristics, which include caregiver roles, egg and clutch sizes, and required investments. Such patterns can be attributed to evolutionary forces and divergent reproductive pressures experienced by these two groups, which I further discuss. Shell-dwellers represent a specialised form of substrate brooders, yet the reproductive behaviour of *L. ocellatus* exhibits intriguing parallels with both substrate brooders and mouthbrooders.

4.2.1.1 Mating systems and parental care

In cichlids, monogamy is often accompanied by biparental care, and mostly observed in substrate brooding species (Fryer & Iles, 1972; Keenleyside, 1979; Kuwamura, 1986). This adaptation is believed to have emerged in response to heightened predation risks faced by exposed fry anchored to the substrate, necessitating the joint efforts of both parents in defending progeny (Balshine & Abate, 2021; Keenleyside, 1979; Kuwamura, 1986; McKaye & Kocher, 1983). On the other hand, in polygynous mating systems usually females bear sole responsibility for offspring care as seen in mouthbrooders. Females carry offspring in their buccal cavity, unrestricted by territorial ties (Fryer & Iles, 1972; M. H. A. Keenleyside, 1979; Kuwamura, 1986). The safety of the buccal cavity diminishes predatory threats on offspring, rendering additional paternal protection redundant, freeing males to invest in additional mating opportunities over offspring.

Interestingly, a comprehensive study on the mating systems in shell-dwellers, showed that these substrate brooders mostly display a polygynous mating system, with a few exceptions (Sato and Gashagaza, 1997) In *L. ocellatus*, it has been reported that a single male will hold a large territory with a harem of 1–3 females, with the composition of the harem remaining stable over time. (Bills, 1996; Sato & Gashagaza, 1997). Females are the primary caregivers who tend to and guard their offspring at their own shell over the first few weeks post-fertilisation. Perhaps due to the added protection of the shell that lowers predation pressure, these species have been able to adopt a polygynous mating strategy that only requires a single primary carer at any given time. The male takes over caregiving at later stages of offspring development, providing month old juveniles with shelter within his shell (Haussknecht & Kuenzer, 1991). This division of labour between females and males is reminiscent of a small group of mouthbrooding species, *Eretmodus cyanostictus*,

Tanganicodus irsacae, *Xenotilapia boulengeri*, and *X. longispinnis*, where males take over the duty of mouthbrooding during later stages of brood care (M. H. A. Keenleyside, 1991; Kuwamura, 1986).

4.2.1.2 Brood and eggs size

There is a direct trade-off in nature between egg size and clutch size (Roff, 2002). In cichlids, clutch size correlates positively with care duration and body size, but negatively with egg size (Fig. 22A; Kolm et al., 2006a). Kolm et al. (2006a) identify two primary cichlid reproductive strategies: small-bodied species produce large eggs in small clutches with an extended pre-hatching period but reduced post-hatching care, while larger-bodied species produce small eggs in large numbers with a brief pre-hatching period but prolonged post-hatching care. Large eggs are costly because the growing embryo is provided with large volumes of yolk, resulting in fewer eggs being produced by females. These embryos take longer to hatch but are more developed and independent once the yolk is absorbed, reducing the need for parental care. Conversely, smaller eggs hatch quickly and remain vulnerable even after yolk absorption. Females can produce many more of these smaller eggs, distributing their resources among a larger number of offspring.

A deeper dive into the literature showed that this trend separates out mouthbrooders and substrate brooders (Fig. 22D–F). Mouthbrooders are, on average, smaller than substrate brooders (Fig. 22F; Kolm et al., 2006b) and tend to produce larger eggs, ranging from 1.7–7.1 mm (Fig. 22D, H; Santos et al., 2023; Yanagisawa, 1987; Yanagisawa et al., 1996). The clutches are small, <50–80 eggs, and parents provide less care after hatching (Sefc, 2011; Yanagisawa & Sato, 1990). Substrate brooders tend to produce large clutches of small eggs and provide extended post-hatching care (Fig. 22D, G–H). Shell-dwellers, unlike other Lamprologini substrate brooding cichlids, have small clutch sizes similar in number to mouthbrooding species (Gashagaza, 1991; Sefc, 2011; Yanagisawa et al., 1996; Fig. 22G). However, I found slightly larger clutch sizes in *L. ocellatus*, ranging from 10–50 eggs, compared to the 10–20 eggs for shell-dwellers reported in the wild (Bills, 1996; Sefc, 2011). Individual egg size, averaging at 1.45 mm, falls within the typical range for Lamprologian substrate brooding species (1.44–2.1 mm; Fig. 22H; Gashagaza, 1991; Yanagisawa et al., 1996).

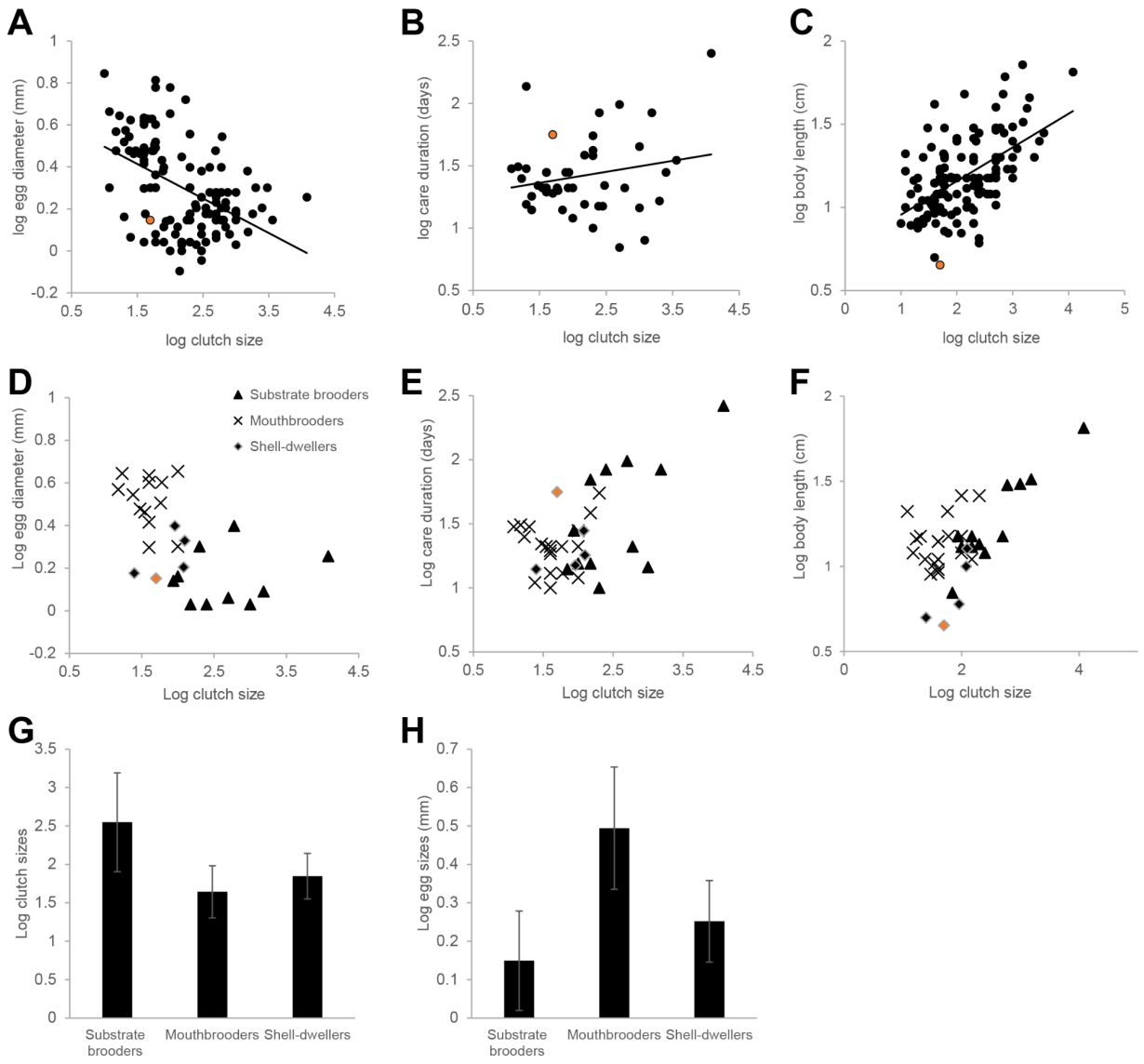


Figure 22: Reproductive trends according to data collected from Kolm et al. 2006a across cichlid species. A–B) Bivariate correlations for raw cichlid species data on A) egg diameter versus clutch size (124 species), **B)** care duration versus clutch size (46 species), **C)** Body length versus clutch size (143 species), adapted from Kolm et al. (2006a; with permission) with added information for *L. ocellatus* (orange). **D–F)** Bivariate correlations for a subset of Kolm et al. (2006a) Lake Tanganyika cichlid data (with permission) with additional information on whether species are substrate brooders (triangle), mouthbrooders (X) or shell-dwellers (diamond) and data points for *L. ocellatus* in orange. **G)** Average maximum clutch sizes for 12 substrate brooder versus 19 mouthbrooding versus 5 shell-dwelling species all inhabiting Lake Tanganyika. Lines represent standard error. **H)** average recorded egg diameter for 12 substrate brooder versus 15 mouthbrooding versus 5 shell-dwelling species—all inhabiting Lake Tanganyika. Lines represent standard error.

An analysis by (Kolm et al., 2006b)) predicts a 99 % likelihood that the ancestral state had large clutch sizes and small egg sizes, and a 77 % probability of large body sizes. The authors suggest

that the evolution towards small-bodied cichlids with small clutches of large eggs likely involved an initial increase in egg size followed by reductions in body and clutch size. However, this pattern does not hold for *L. ocellatus*. These shell-dwellers produce small eggs in modest clutches with brief pre-hatch periods but extended care durations (Fig. 22A–F). As *L. ocellatus* has adapted to in-shell living by reducing its body size, clutch size has correspondingly decreased compared to substrate brooders. Nevertheless, the ancestral traits of small eggs and extended care durations typical of substrate brooders remain intact in *L. ocellatus*.

4.2.1.3 Larvae development

The hatching time of *L. ocellatus* eggs aligns with what has been reported for other Lamprologian species (Nagoshi, 1983; Nakai et al., 2006; Rossiter, 1991; Yanagisawa, 1987) and substrate brooders (Baerends & Baerends-Van Roon, 1950), which is typically shorter than that of mouthbrooding species (Watanabe, 2000).

In many substrate brooding species, it is common for parents to relocate larvae after hatching (Baerends & Baerends-Van Roon, 1950; Noble & Curtis, 1939; Sefc, 2011), and this is also the case for *L. ocellatus*. In this species, the mother transfers newly hatched fry from the laying chamber to a deeper chamber within the shell at the onset of the so-called “wriggler stage”. During this stage, larvae perform high-frequency tail beats while attached to a fixed surface by sticky head glands—anatomical structures unique to substrate brooders (Yanagisawa, 1987; Yoshida et al., 1996). While (Baerends & Baerends-Van Roon, 1950) described this behaviour as constant tail beating at 3–5 beats per second, I observed that *L. ocellatus* takes breaks between wriggling sessions. This wriggling behaviour is believed to create a current, aiding in waste removal and water exchange (Baerends & Baerends-Van Roon, 1950).

Within the shell's confines, larvae reside in the deepest chamber, where they absorb their yolk sacs in preparation for the free-swimming stage—when larvae detach from the substrate and reorientate ventrally. Other studies on substrate brooding larvae report that the free swimming phase is the point at which the larvae typically leave the nest for the first time (Baerends & Baerends-Van Roon, 1950; Balshine & Abate, 2021). In *L. ocellatus*, I observed that larvae start to detach from the shell wall and switch to an upright position at 7 dpf, two days prior to emergence from the shell. However, consistent with Balshine & Abate (2021), the time that larvae spent head attached significantly decreases at 9 dpf, coinciding with their initial emergence from the shell. The onset of prey-capture behaviour also coincides with emergence time: I observed petri dish-

raised larvae displaying hunting behaviours and actively consuming prey at 9 dpf, despite the remaining yolk. It has been reported in both mouth- and substrate brooders that fry begin eating before the complete depletion of the yolk sac (Yanagisawa, 1987; Yanagisawa et al., 1996). Increased mobility and hunting drive may both play roles in determining the timing of emergence and will further be discussed in later sections. However, another key player who may influence emergence time is the mother, who frequently visits the shell and interacts with her offspring.

4.2.2 Maternal Adaptations to the Shell

The first caregiving behaviour the mother performs is to lay her eggs inside the confines of the shell in order to protect eggs from predators (Mousseau & Fox, 1998). The exact location of oviposition is likely determined by how deep the female is able to enter into the whorl, in order to protect eggs from predators (Bills, 1996; Takahashi et al., 2012; Winkelmann et al., 2014). Female shell-dwellers have evolved to be small enough to enter the shell to cater to the needs of their fry (Schuetz & Taborsky, 2005; Sefc, 2011). The maternal behaviours observed within the shell, while not entirely unique among substrate brooding species, appear to be adapted to the shell's architecture.

4.2.2.1 Fanning to refresh water within the shell

One such necessary behaviour is fanning, which serves multiple purposes such as supplying oxygen, removing waste and reducing fungal infection on eggs and larvae, all of which greatly increase the probability of offspring survival (Baerends & Baerends-Van Roon, 1950; Hale et al., 2003; A. Jordan et al., 2021; Reebs & Colgan, 1991, 1992; St Mary et al., 2004). Given the closed environment and limited circulation within a shell, fanning is particularly vital for water exchange. Notably, the fanning behaviour of *L. ocellatus* differs from the descriptions of other substrate brooders, where fanning is performed by both parents in tandem, ensuring fresh water to flow almost constantly over the developing embryos. Typically, these substrate brooders hover approximately 2 cm above their eggs and sweep their pectoral fins alternatingly at 1 beat a second, while the dorsal and caudal fins undulate laterally at a slower tempo (Baerends & Baerends-Van Roon, 1950; Balshine & Abate, 2021; Reebs & Colgan, 1991; Smith-Grayton & Keenleyside, 1978). In contrast, when *L. ocellatus* females are in the shell, they are not constantly fanning, but rather spend a maximum of 20 % of their time fanning during the day. The mother predominantly fans from the entrance chamber, vigorously beating her caudal and anal fins,

sometimes arching her body to follow the shell's spiral. Pectoral fin movements are observed but are likely countering the forward movements created by fanning, thereby stabilising the mother's position within the shell.

Over the course of 11 days observed, mothers spent significantly more time fanning their offspring at night compared to the daytime. Similar findings have been reported in substrate-brooding cichlid species such as *Abudefduf saxatilis* (Albrecht 1969*), *Cichlasoma nigrofasciatum* and *Herotilapia multispinosa* (Reebs & Colgan, 1991), which all show increased nighttime fanning. The authors suggest this behaviour is influenced by both darkness and circadian rhythms. For shell-dwelling species, the increased nighttime fanning could be due to the mother's continuous presence in the shell throughout the night, leading to faster oxygen depletion and necessitating more fanning to maintain adequate oxygen levels.

4.2.2.2 Mouthing offspring is important for transport and cleaning

Another common behaviour observed in substrate brooding cichlids is mouthing, often referred to as cleaning (Baerends & Baerends-Van Roon, 1950; Balshine & Abate, 2021; D. H. Brown & Marshall, 1978; Smith-Grayton & Keenleyside, 1978). During the egg stages, mothers visit the shell and move their lips over or nip at the eggs. This behaviour intensifies as the eggs approach hatching (Baerends & Baerends-Van Roon, 1950). Research indicates that this action serves as a way for the mother to taste the eggs and to eliminate diseased or dead eggs, thus preventing the spread of contamination in the shell (Balshine & Abate, 2021; D. H. Brown & Marshall, 1978; Smith-Grayton & Keenleyside, 1978). Once eggs are hatched, mouthing takes on a different form. I observed mothers picking up the hatchlings in their mouth and performing buccal cleaning or gargling with the offspring before depositing them down into the deep chamber of the shell. In other substrate brooders, parents often do this when transfering the larvae from the spawning site to the dugout pits or other surface where the wrigglers will be guarded (Smith-Grayton & Keenleyside, 1978). Mouthing is seen less often after 4 dpf in *L. ocellatus,* but if a larva strays out of the deep chamber during the wriggling phase, the mother will retrieve her offspring and return it to the lower whorls of the shell.

4.2.2.3 Raising offspring in the dark

One significant adaptation for shell-dwelling mothers is raising their brood in constant darkness within the shell. While substrate brooders often rely on visual cues, such as the eggs or small larvae, to interact with their offspring (Baerends & Baerends-Van Roon, 1950; Noble & Curtis, 1939), we can gain insight into the cues shell-dwelling mothers might employ from studies on nighttime parental care behaviours by (Reebs & Colgan, 1992). Here the authors showed that in the absence of light, chemical cues from eggs sufficed in eliciting egg-oriented behaviours from the mother, whereas tactile cues did not. It is thus likely that the shell-dwelling mothers are using chemical cues inside the shell to guide the care behaviours required of her at the different development stages of the larvae.

4.2.3 Concluding Remarks on the Evolutionary Context of Brood Care in Shell-dwelling Cichlids

The examination of the brood care paradigm in shell-dwelling cichlids reveals a potential interesting interplay of factors influencing the evolution of this reproductive strategy. While shell-dwellers exhibit parallels with both substrate and mouthbrooders, their unique niche within this spectrum is shaped by the specific challenges imposed by their habitat. The similarities shared with mouthbrooders, such as mating strategies and clutch size constraints, exemplify the convergence of these strategies, perhaps driven by the offspring growing up in a confined space. Conversely, traits reminiscent of substrate brooders, such as smaller egg sizes, larval anatomy and behaviours, as well as maternal interactions with offspring highlight the lingering influence of evolutionary history. These observations suggest multiple factors, including ecological context, evolutionary history, and mating systems are at play in the evolution of parental care in shell-dwellers. As we delve deeper into the intricate world of shell-dwelling cichlids, it becomes increasingly evident that their unique brood care strategies serve as a testament to the remarkable adaptability and ingenuity of the parental care paradigm.

4.3 Dissecting the Mechanisms Behind Larval Emergence

In observing the brood care paradigm of *L. ocellatus*, the timing of emergence across different clutches and mothers was strikingly consistent. While the duration of hatching could occur anywhere from late 2 dpf to 3 dpf, emergence reliably transpired at 9 dpf between 12 pm and

3:30 pm. This abrupt transition, from a larva spending its early life within the deepest whorls of a dark shell to suddenly exploring the expansive, bright world above, piqued my curiosity and prompted an investigation into the factors governing this emergence behaviour. I postulated that these changes might be influenced either by extrinsic forces, such as the mother's active influence, or by intrinsic developmental changes within the larvae themselves, which could prompt such a precisely timed shift in behaviour.

These two scenarios are in line with the two debated hypotheses of the optimal fledging age of songbirds, as mentioned in the introduction: the parent manipulation hypothesis, where parents and offspring are in conflict over the optimal fledging age (Jones et al., 2020; Martin et al., 2018), and the nestling choice hypothesis, suggesting fledging occurs when offspring reach a developmental threshold (Johnson et al., 2004, 2017; Nilsson & Svensson, 1993).

4.3.1 Extrinsic Influences on Emergence

4.3.1.1 Removing the mother causes premature emergence from the shell

To investigate the maternal influence on larval emergence, I removed the mother at various stages and observed the effects on larval behaviour and timing of emergence. Mother removals right after egg laying led to high offspring mortality from infections, but removals at 5 dpf and later showed notable behavioural changes. The orphaned larvae immediately expanded their activity to the laying chamber and emerged prematurely within 24 hours, completely abandoning the shell by 11 dpf, whereas control larvae, with the mother present, continued to utilise the shell throughout the observation period. Additionally, orphaned larvae showed altered nocturnal behaviour, failing to cluster in the deep chamber of the shell at night like the control groups. This behaviour parallels behaviours observed in orphaned jewel cichlids (*Hemichromis bimaculatus*), where larvae failed to form their usual compact groups at night in the absence of parents (Noble & Curtis, 1939).

The distinct movements of the larvae at different stages, first to the laying chamber and later to the entrance, suggest deliberate transitions rather than random dispersal into available space. Despite almost constant adhesion to the shell wall, wrigglers are able to direct their general movements and exit the shell from as early as 5 dpf, ruling out mobility as a restriction of shell

emergence. These results also indicate that the mother plays a critical role in larval behaviour and affinity to the shell.

4.3.1.2 Fresh water supply restores natural emergence time in the absence of the mother

To further investigate the maternal behaviours that promote larvae retention within the shell, I explored replacing the basic care provided by the mother, specifically maintaining frequent water exchange inside the shell. The results showed that an artificial supply of fresh water restored normal emergence times for orphaned larvae. Day and night behaviours normalised, mirroring control conditions, and larvae continued using the shell until the end of the observation period. The behavioural changes seen in previous mother-removal experiments likely stem from the accumulation of toxic conditions within the shell as larvae consume oxygen and release waste products (Baerends & Baerends-Van Roon, 1950). Without the mother's fanning behaviour, the crucial exchange of fresh water within the shell ceases and larvae are driven out in search of a better environment.

Interestingly, a consistent behaviour was observed across all experiments following the removal of the mother, even with fresh water provision: the larvae quickly ascended into the laying chamber. This suggests that the mother at least plays an active role in keeping the larvae in the deep chamber until the day of emergence. While it appears that larvae have an intrinsic cue for emerging around 9 dpf, it is also plausible that maternal behaviours, such as fanning, may be adjusted to encourage this timing.

4.3.1.3 Maternal behaviours are governed by an independent intrinsic timeline

Building on this observation, I sought to determine whether maternal behaviour within the shell follows a fixed schedule that is initiated by egg-laying, or if it dynamically adjusts in response to the needs and behavioural shifts of the offspring. To this end, I exchanged clutches between two females at 4 and 6 dpf. Previous studies on various cichlid species during the brood care phase have demonstrated that many parents accept conspecific offspring at different stages, provided they are within 3–4 days of their original clutch (Nelson & Elwood, 1997). I hypothesised that if the foster mother follows a fixed behavioural timeline during brood care and adjusts her behaviour at her 9 dpf to facilitate emergence, this would disrupt the intrinsic emergence time of the adopted larvae due to asynchrony between foster mother and larvae.

We found that larvae who were 2 days younger than expected by the mother, did not emerge from the shell until they reached their biological age of 9 dpf (i.e. expected age of 11 dpf). This suggests that the mother did not cause premature emergence, as would be expected if she stopped fanning or altered her behaviours to induce emergence. However, it was noted that the larvae ascended into the laying chamber at their biological 8 dpf, a day earlier than observed in the control clutches. This behaviour aligns with observations from experiments where the mother was removed, but fresh water was provided, and reinforces the notion that the mother is responsible for maintaining the larvae in the deep chamber until her 9 dpf. It is possible that she reduces her oppressive behaviours that keep the larvae in the deep chamber when she anticipates emergence, but according to her own schedule rather than in response to larval signals.

Further evidence supporting the idea that the mother operates on a predetermined schedule was observed in an alternative experiment where mothers received larvae 2 days older than their own clutch, resulting in delayed emergence from the typical 9 dpf to 10 dpf (i.e. expected age 8 dpf). Video footage showed mothers engaging in vigorous fanning and actively retrieving larvae to return them to the deep chamber, indicating an attempt to synchronise emergence with their biological timing, regardless of the larvae's older age. Similarly, experiments by (Baerends & Baerends-Van Roon, 1950) involved switching *Cichlasoma meeki* eggs for *C. bimaculatum* eggs, which hatched earlier than expected. This resulted in parents delaying the transfer to the pit, also pointing towards an intrinsic parental timeline. Contrarily, (Noble & Curtis, 1939) observed that jewel cichlid parents adapted immediately to premature detachment of eggs, resuming the next parenting phase promptly. These observations suggest that while some cichlid species show flexibility in parenting behaviours or respond to cues, others adhere strictly to their internal schedules.

Following my observations on maternal behaviours within *L. ocellatus*, existing literature provides insights that could help determine the regulatory mechanisms behind this intrinsic maternal timeline. For instance, fluctuations in prolactin levels have been shown to significantly influence parental behaviours during critical postnatal periods in species such as mallard hens (*Anas platyrhynchos*) and zebra finches (*Taeniopygia castanotis*), where prolactin adjustments correspond with changes in care intensity post-hatching (Boos et al., 2007; Smiley & Adkins-Regan, 2016). Given these parallels, hormonal priming from egg-laying could be an important mechanism for setting a specific maternal care timeline in *L. ocellatus*. Additionally, nonapeptides,

such as oxytocin and vasopressin analogues, have been shown to be important in regulating paternal care in fish species, including anemonefish (*Amphiprion ocellaris*; DeAngelis et al., 2017) and convict cichlids (*Amatitlania nigrofasciata*; O'Connell et al., 2012), making them strong candidates for investigating similar mechanisms of hormonal regulation in *L. ocellatus*.

4.3.2 Intrinsic Influences on Emergence

While maternal hormonal regulation may provide one layer of understanding, it is also crucial to consider the developmental changes occurring within the larvae themselves. I hypothesise that a developmental change underlies the behavioural switch in the young *L. ocellatus*. Minot (1988) posed two key questions when observing a sudden change in animal behaviour during development: How can a gradual process like development provoke a sudden change, and what is the function of such a change in the animal's life? Addressing the latter, *L. ocellatus* needs to emerge from the shell to feed and continue growing, thus reducing competition for space inside the shell.

To examine the intrinsic factors that may influence the emergence time of the larvae, I manipulated both feeding and phototactic behaviours. Although larvae were observed performing prey capture at 9 dpf in petri dishes, coinciding well with emergence time, I found that supplying the larvae with food inside the deep chamber from 8 dpf did not influence emergence time. Larvae quickly accepted the live prey inside the shell but returned to exploring the outside shortly after consuming their meals.

Phototactic behaviour was evaluated in a new set-up designed to eliminate spatial and gravitational influences within the shell. My results clearly demonstrate a transition from a preference for darkness at 7–8 dpf to a preference for daylight at 9 dpf, aligning with the larvae's emergence time. This suggests that the shift in light preference could be a key mechanism driving the larvae to explore outside the shell. Interestingly, even though the larvae exhibit photophilia during the day, they consistently retreat to the darker side of the arena at night. This behaviour mirrors their natural tendency to retreat into the deep chamber at night, in agreement with observations made by (Noble & Curtis, 1939)) of Jewel cichlid larvae seeking safety in darkened environments. This adaptation is biologically significant, as it likely provides the larvae with protection from nocturnal predators, which are more challenging to detect in their natural habitat at night (Bills, 1996).

A switch between phototactic preferences during development is observed in a diverse array of organisms, including species of beetles (Meng et al., 2019), frogs (Muntz, 1963) and birds (Minot, 1988). Starling nestlings (*Sturnus vulgaris*), for example, also transition from light avoidance to attraction, crucial for fledging the nest (Minot, 1988). In *Xenopus laevis* tadpoles, early exposure to light is essential for developing photopositive behaviour; young tadpoles opt for a white background while metamorphic ones prefer black, but this can be influenced by their rearing conditions (Adebogun et al., 2023; Copp & McKenzie, 1984; Moriya et al., 1996). Zebrafish larvae (*Danio rerio*) also initially have a preference for light, which promotes foraging in shallower waters away from predators (Lau et al., 2011). This behaviour is mediated by an asymmetrical pathway in the left dorsal habenula, which receives illumination information from arborization field 4 via the eminentia thalamus (Zhang et al., 2017). However, at about 3 weeks post-fertilisation, they shift to avoiding light (Lau et al., 2011), though the mechanisms underlying this transition remain unclear.

From this section, we see that the observed shift in phototactic preferences in *L. ocellatus* aligns with emergence, suggesting that certain innate mechanisms, potentially involving neural development, trigger these transitions to optimise survival. Similar patterns of phototactic shifts are evident across various species, indicating a common evolutionary strategy to enhance adaptability during development. Additionally, even simple single-cell organisms can efficiently perform phototaxis. This fundamental, computationally-accessible behaviour thus does not require complex neural structures, making it a viable trait for evolution to exploit early in developmental processes (Hill & Häder, 1997; Hill & Vincent, 1993). These findings contribute to a broader understanding of how environmental and biological cues are integrated during early development to regulate behaviour in a manner that maximises ecological success.

4.3.3 Aligning Multiple Factors for Natural Emergence in *L. ocellatus*

In this section, I sought to unravel the mechanisms underpinning the initial emergence of *L. ocellatus* larvae from the shell through experimental manipulations within the brood care paradigm. My findings indicate that the larvae remain intrinsically avoidant of the lighter environment outside the shell until a phototactic switch to light preference occurs at 9 dpf. Prior to emergence, these larvae demonstrate goal-directed movement and prefer to distribute themselves throughout all shell chambers except the entrance, which is exposed to light.

However, under control conditions, larvae predominantly reside in the deep chamber, a behaviour likely conducted in response to maternal actions. The precise methods by which the mother retains her offspring deep in the shell remain unclear, but frequent visits and fanning could provoke a retreat into deeper chambers via detection of the larvae's lateral line. Other potential cues might include visual, chemosensory, or auditory signals, though further experimentation is required to clarify these mechanisms.

The mother operates on her own intrinsic timeline of care behaviours, which I have explored by bringing the independent timelines of mother and offspring into conflict through my experiments. These showed that the mother's efforts to retain the larvae in the shell persist until 9 dpf, coinciding with the larvae's phototactic shift that prompts their emergence. Post-9 dpf, maternal efforts wane, allowing the larvae to exit the shell freely. Interestingly, artificially introduced premature larvae remain within the shell past the foster mother's 9 dpf, driven by their aversion to light, while older adopted larvae actively conflict with the foster mother's timeline, moving towards the entrance before the mother's 9 dpf and eliciting vigorous maternal responses to keep them in the deeper chambers of the shell.

This research has demonstrated the possibility of creating competing interests regarding emergence timing, where offspring are ready to leave but the mother prevents, what she deems, premature emergence. In *L. ocellatus*, the larvae are driven to emerge by a developmental switch, potentially preparing them for independent feeding once their yolk has depleted. From the maternal perspective, it could be that retaining larvae within the shell until they are free-swimming minimises the effort required to secure all larvae in the face of danger, especially considering larvae's limited mobility due to a heavy yolk sac and underdeveloped musculature at early stages.

A similar parent-offspring conflict is observed in three-spined sticklebacks (*Gasterosteus aculeatus*), where larvae attempt to propel themselves out of their father's nest to gulp air at the water surface, necessary for inflating their swim bladder (Feuth-De Bruijn & Sevenster, 1983). However, the father catches and returns them to the nest, preventing them from gaining the air needed for increased mobility. This restriction likely protects the larvae from predation by neighbouring males, to which they would be more vulnerable once they roam further from the nest. Only larvae that manage to escape the father are likely fast enough to survive outside the nest, illustrating an evolutionary strategy where the father's behaviour ensures that only the most mature offspring gain early mobility.

A conflict between parents and offspring is also one of the debated hypotheses for the mechanisms behind avian fledging. However, in birds this conflict is flipped: parents encourage early fledging to commence another brood, while offspring delay fledging to enhance growth and survival chances (Jones et al., 2020; Martin et al., 2018). My findings in *L. ocellatus* align more closely with the nestling choice hypothesis (Johnson et al., 2004, 2017; Nilsson & Svensson, 1993), suggesting that offspring depart upon reaching a developmental threshold—here, the shift from dark to light preference that encourages exploration outside the shell.

Notably, when the mother is removed, larvae leave prematurely without additional fresh water, indicating that their inherent aversion to light can be overridden by inadequate conditions. Moreover, the observation that larvae return to the shell at night after becoming photophilic is interesting as it suggests the larvae are drawn to the darker environment of the shell as external light levels decrease. These points highlight the modulation of phototactic responses by environmental conditions and suggest further investigation into how these influences might alter larval behaviour.

4.4 Conclusions and Outlook

This doctoral thesis has described the behavioural developments and adaptations of a shell-dwelling cichlid brood care paradigm. I have been able to describe the reproductive niche of *L. ocellatus* in the context of the substrate brooder–mouthbrooder spectrum within Lake Tanganyika, showing the convergent characteristics with mouthbrooders, while retaining some substrate-brooding traits due to a shared evolutionary history. Observing for the first time the development of the larvae within the shell, I show that the larvae undergo two major transitions within the shell. Initially, from eggs attached to the wall of the first whorl, they hatch into wrigglers, who are moved by the mother into the deeper chambers. In the second transition, driven by a combination of intrinsic and extrinsic factors, the free-swimming larvae emerge through the shell entrance at 9 dpf. Experiments suggest that the intrinsic factor driving this emergence is the larvae's shift from dark to light preference at 9 dpf. For this transition to occur successfully, the larvae must be maintained in an optimal environment, notably with constant water exchange facilitated by the mother. Additionally, the synchronisation of the mother's care phase with her offspring's development is essential for her to facilitate and accept the free-swimming fry's transition to the external environment.

Looking ahead, this study paves the way for several promising areas of future research. Conducting field studies on the brood care characteristics of *L. ocellatus* in its native environment of Lake Tanganyika could yield insights into the dynamics of this brood care in the context of ecological pressures such as competition and predation. Investigations might include comparing survival rates of shell-dwelling larvae to those of mouthbrooders and substrate brooders, thereby deepening our understanding of how environmental factors shape brood care strategies.

Furthermore, expanding comparative studies to include other shell-dwelling cichlid species would allow for a more comprehensive evaluation of brood care strategies across this diverse group. By examining a range of shell-dwellers, researchers could identify commonalities and differences in these strategies, providing deeper insights into their evolutionary context relative to mouthbrooders and substrate brooders. For example, (Lein & Jordan, 2021) describe shell-dwelling species as representing "Darwin's dream pond," noting significant variation in social organisation despite these species sharing the same ecological niche and life history. Such comparative studies are particularly useful when they involve species with minimal differences between them, as this allows for a clearer understanding of evolutionary mechanisms. Species of

particular interest include *Lamprologus ornatipinnis*, which shares a similar lifestyle with *L. ocellatus* but differs in shell orientation (Bills, 1996); *Neolamprologus multifasciatus*, known for their group-living and unburied shells in dugout pits (L. A. Jordan et al., 2016; Sato & Gashagaza, 1997); and *N. brevis*, where males and females cohabit in the shell but with the mother residing almost constantly inside with the offspring, and providing minimal post-emergence care (Ota et al., 2012; Sato & Gashagaza, 1997). By comparing these specific traits across species, we can discern which characteristics of *L. ocellatus* are common adaptations among shell-dwellers and which are unique to this species.

To further investigate the mechanisms behind the emergence of *L. ocellatus* larvae, one can follow up on the underlying changes in neural circuitry associated with the phototactic switch in larvae. Determining the neural mechanisms for such a behavioural switch could provide breakthroughs in our understanding of how new behaviours arise during development. Supported by advances in technology such as closed-loop behaviour assays, researchers can employ sophisticated experiments that can dynamically track and force choices in light preference in real-time. Following that, researchers can then assess the neural activity associated with these behaviours by using methods like mapping immediate early gene expression in pre- and post-emergent larvae.

We are currently working on creating stable transgenic *L. ocellatus* lines, which would further help answer neural circuit questions on light preference. I propose creating a calcium indicator line and performing phototaxis assays on larvae embedded in agarose, with their tails freed. This set-up would allow us to correlate tail movements, indicative of preferred light environments, with areas of high activity in the brain. The immediate early gene expression assay, mentioned previously, will help identify candidate brain regions. Performing these studies in pre- and post-emergent larvae and comparing the results could inform us of how the switch in preference occurs in the brain, for example if the same circuit is sequestered.

Following Zhang et al. (2017)'s work on zebrafish larvae phototaxis, the habenula would be a good first target region. By using the *gng8* promoter to drive Channelrhodopsin—a light-gated ion channel—in the habenula, we aim to employ optogenetics to determine if we can elicit a light preference behavioural response in blind larvae. Here, we could shine the light that activates the ion channels on one side of the phototaxis behavioural arena and allow the fish to swim freely. If the mechanisms in larvae zebrafish are conserved in cichlids, I would expect pre-emergent larvae

to avoid the side of the arena that activates channelrhodopsin, whereas I would expect post-emergent larvae to be attracted to this side.

Finally, to further unravel what drives the mother's behavioural timeline across the brood care period, additional experiments focusing on hormonal influences could be insightful. Investigating the roles of hormones such as prolactin, oxytocin, or vasopressin in modulating maternal behaviours could provide key insights. However, this approach presents significant challenges due to the invasive nature of hormone level assessments, which typically require blood samples that could be detrimental to the fish's health. Consequently, these hormone levels might need to be inferred from alternative, less invasive methods or studied post-mortem, which would preclude longitudinal studies within individual fish. Additionally, manipulating the circadian cycle of the mother could offer insights into whether she possesses an internal mechanism for counting days post-fertilization. This would help determine if the timing of brood care behaviours is influenced by an innate sense of time rather than hormonal changes alone. Such studies could explore whether adjustments to light cycles affect maternal care patterns, potentially revealing a circadian or time-keeping component in her brood care strategy.

In conclusion, the comprehensive studies conducted in this thesis not only deepen our understanding of *L. ocellatus* but also underscore the vast potential of this species as a model organism in neuroethology. The unique ecological and behavioural traits I have observed in these shell-dwelling cichlids offer great opportunities to explore complex topics such as nest-building behaviours, extended phenotypes, intricate social dynamics, behavioural development including play in juveniles, spatial navigation, and shell-caching. It is my hope that the groundwork laid by this research will propel *L. ocellatus* into the forefront of neuroethological model systems. By unlocking the secrets held within their ecological adaptations and social structures, we can gain insights into fundamental biological processes that are broadly applicable across taxa. As this species gains traction in the scientific community, I am optimistic that the findings from *L. ocellatus* will enrich our understanding of animal behaviour in natural contexts, providing a window into the evolutionary pressures that shape complex living systems. I am eager to see how future research will build on these findings, exploring the depths of behavioural ecology and the neural mechanisms that underpin it, and I am proud to have contributed to setting the stage for a new era of discovery in neuroethology.

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Affidavit

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation **Adaptations to the Brood Care Paradigm in the Shell-dwelling Cichlid *Lamprologus ocellatus*** selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe. Ich habe das AI-Sprachmodell ChatGPT-4 verwendet, um die Grammatik und den Fluss zu verbessern.

I hereby confirm that the dissertation **Adaptations to the Brood Care Paradigm in the Shell-dwelling Cichlid *Lamprologus ocellatus*** is the result of my own work and that I have only used sources or materials listed and specified in the dissertation. I have made use of the AI language model ChatGPT-4o to improve grammar and flow.

Ash Parker

München, den 24.03.2025

Declaration

Hiermit erkläre ich, dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist, und dass ich mich anderweitig einer Doktorprüfung ohne Erfolg nicht unterzogen habe.

Ash Parker

München, den 24.03.2025

Curriculum Vitae

Personal information

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Academic Education

2018-2025	Doctoral studies at the Max Planck Institute of Biological Intelligence, Thesis: Adaptations to the Brood Care Paradigm in the Shell-dwelling Cichlid *Lamprologus ocellatus*. LMU, Germany
2016-2018	M.Sc. in Molecular and Cell Biology working of the role of Wnt/B-catenin in bat wing development, University of Cape Town
2015	B.Sc. Honours in Molecular and Cell Biology, Univ. of Cape Town
2012-2014	B.Sc. in Ecology and Evolution, and Genetics, Univ. of Cape Town

Publications

Parker, A. V., Stemmer, M., Grätsch, S., Dorigo, A., Ramirez, O. R., Adel, A., ... & Baier, H. (2025). Intrinsic timing of brood care in shell-dwelling cichlids. Current Biology, 35(3), 672-680.

Mearns, D. S., Hunt, S. A., Schneider, M. W., **Parker, A. V.**, Stemmer, M., & Baier, H. (2023). Diverse prey capture strategies in teleost larvae (p. 2023.10.03.560453). bioRxiv.

Eckalbar, W. L., Schlebusch, S. A., Mason, M. K., Gill, Z., **Parker, A. V**., Booker, B. M., ... & Illing, N. (2016). Transcriptomic and epigenomic characterization of the developing bat wing. *Nature genetics*.

Academic Prizes

2021	Poster prize at Cichlid Science, Cambridge, United Kingdom
2021	1st place for oral presentation at the 29th IMPRS seminar, IMPRS-LS, Martinsried, Germany
2016	2nd place for oral presentation at the Molecular and Cell Biology Research Day, University of Cape Town, South Africa
2016	2nd place for oral presentation at the Univ. of Cape Town Science Colloquium, Cape Town, South Africa

2016	Royal Society of South Africa poster and research prize, 25th South African Society of Biochemistry and Molecular Biology Congress, East London, South Africa
2016	Conviron 3rd place poster prize in Protein Biochemistry, 25th South African Society of Biochemistry and Molecular Biology Congress, East London, South Africa
2014	Class medal in Genetics (Univ. of Cape Town)
2012-2014	Dean's Merit List (Univ. of Cape Town)

Courses Attended

Jun 2022	CAJAL Quantitative Approaches to Behavior (Champalimaud Center for the Unknown)
Dec 2021	Awareness & Intercultural communication with Maria Prahl (Max Planck Gesellshaft)
Dec 2021	Dealing with ethnic diversity & racism in academic organizations with Dr. des Lorenz Narku Laing (Max Planck Gesellshaft)
Nov 2020	LeTS GEPs Training Course on Gender Equality, Gender Equality Plans and Gender Budgeting (Modena, Italy)
Dec 2018	Fast Forward Implementation Program with Nadine Sinclaire
Dec 2018	DeepLabCut Markerless Animal Tracking Workshop with Dr. Mathis (Harvard) (Ludwig-Maximilian University, Munich)
June 2018	Max Planck Institute Python Class with Nick del Grosso (Munich)
Sept 2018	IMPRS-LS Retreat: Workshop in Communication in Science (Obergurgl, Austria)
Apr 2018	International Zebrafish and Medaka Course (Karlsruhe Institute of Technology)
Mar 2017	Medical Science Workshop in Image J (University of Cape Town and Janelia Research campus)
Nov 2016	Science Faculty Animal Ethics Course (Univ. of Cape Town)
June 2015	Software Carpentry Workshop (Univ. of Cape Town)
June 2015	Basic Histology Techniques Course (Univ. of Cape Town)

Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

München, den 10.06.2024 Ash Parker

(Unterschrift)

Erklärung

Hiermit erkläre ich, *

☑ dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.

☑ dass ich mich anderweitig einer Doktorprüfung ohne Erfolg **nicht** unterzogen habe.

☐ dass ich mich mit Erfolg der Doktorprüfung im Hauptfach .....

und in den Nebenfächern .....

bei der Fakultät für ..... der .....

(Hochschule/Universität)

unterzogen habe.

☐ dass ich ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.

München, den 10.06.2024 Ash Parker

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*) Nichtzutreffendes streichen