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Early and late developmental arrest as complementary embryonic bet-hedging strategies in African killifish

TOM PINCEEL[1]*, BRAM VANSCHOENWINKEL[2], PIETER DECKERS[1], ARNOUT GRÉGOIR[1], TOM VER EECKE[3] and LUC BRENDONCK[1]

[1]*Laboratory of Aquatic Ecology, Evolution and Conservation, KU Leuven, Charles Deberiotstraat 32, 3000 Leuven, Belgium*
[2]*Department of Biology, VUB (Vrije Universiteit Brussel), Pleinlaan 2, 1050 Brussels, Belgium*
[3]*External expert*

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The production of dormant eggs is a crucial adaptation for African killifish of the genus *Nothobranchius* to survive in temporary waters. These habitats are often characterized by unpredictable variation in the suitability of growing seasons as a result of variable lengths of inundations and temporary colonization by piscivorous fish. Incomplete hatching could enable killifish to buffer against reproductive failure during unsuitable inundations. Although this phenomenon has been tentatively linked to variation in dormancy states, it has never been investigated under controlled conditions and its viability as a bet hedging strategy to distribute offspring over several inundations remains unclear. In the present study, we used common garden experiments to assess the contribution of environmental modulation and bet hedging to delayed hatching in *Nothobranchius* killifish by testing the feasibility of arrested development in the presence and absence of environmental cues. Overall, the results confirmed that the presence of cues signalling a threat (predator kairomones) inhibited hatching. However, delayed development also occurred independent of cues and was regulated at two stages. Developmental arrest in energy-efficient dormancy stages could present a means for long-term bet hedging over years, whereas arrest in the energy-consuming final stage may serve a similar purpose over shorter time scales.

ADDITIONAL KEYWORDS: annual fish – diapause – dormant eggs – kairomone – *Nothobranchius* – risk spreading – temporary waters.

INTRODUCTION

Annual killifish of the African genus *Nothobranchius* inhabit temporary ponds and wetlands that typically only hold water for a few months each year during the rainy season (Wildekamp, 2004; Williams, 2006). The production of drought-resistant dormant eggs enables populations to bridge dry periods *in situ*, whereas rapid growth and a short life cycle may increase the probability for reproduction before the habitat desiccates (Valdesalici & Cellerino, 2003; Terzibasi *et al.*, 2008; Blažek, Polačik & Reichard, 2013). After deposition, *Nothobranchius* eggs pass through three dormancy stages (D1, D2, and D3), which can be distinguished morphologically using an optical microscope. Embryos in the D1 stage lack visible morphological structures (Wourms, 1972a) and D2 embryos display paired blocks of mesodermic somites (Wourms, 1972b), whereas D3 embryos are fully developed with clearly visible eyes and a beating heart (Levels, Gubbels & Denuce, 1986; Podrabsky & Hand, 1999). Overall, breeding experiments and field observations suggest that embryos generally only reside in D1 for a number of days. However, D2 embryos are able to bridge dry periods of several months, and sometimes years, and D2 represents the stage of greatest resistance to environmental stress (Markofsky *et al.*, 1979; Podrabsky & Hand, 1999; Watters, 2009). By contrast, D3 embryos may only remain viable for up to approximately 4 months (Wourms, 1972a, b, c), presumably because a

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*Corresponding author. E-mail: tom.pinceel@bio.kuleuven.be developed embryo exhausts the energy supply in the yolk more rapidly. Generally, only embryos that have fully completed their development and reached the D3 stage are able to hatch after the onset of an inundation (Polačik, Donner & Reichard, 2011). Entering D3 therefore has an advantage in that the embryo can hatch when suitable conditions present themselves but at the cost of a reduced long-term viability of the dormant stage compared to embryos in more energy efficient 'pre-D3' stages.

Earlier tests indicate that hatching of *Nothobranchius* depends on environmental conditions such as water temperature, oxygen, and light (Markofsky *et al*., 1979; Genade *et al*., 2005), which may reflect the suitability of an inundation for growth and reproduction (Williams, 2006). The importance of abiotic environmental cues as reliable signals for the quality of growing seasons was also demonstrated for the hatching of freshwater crustaceans (Brendonck, 1996; Vandekerkhove *et al*., 2005; Vanschoenwinkel, Seaman & Brendonck, 2010; Pinceel *et al*., 2013). Next to this, hatching of freshwater crustaceans was shown to be influenced by biotic cues, such as the presence of a predation threat (but seeAngeler, 2005). For example, delayed hatching was demonstrated in *Daphnia magna* in the presence of golden orfe fish (*Leuciscus idus*) kairomones (Lass *et al*., 2005). In addition, Blaustein (1997) found indications for delayed hatching of dormant crustacean zooplankton eggs from temporary pools in the presence of predatory salamander larvae (*Salamandra infraimmaculata*), a pattern that was subsequently confirmed by Spencer & Blaustein (2001). Although predators, such as catfish (*Clarias* spp.), lack the adaptations to permanently inhabit killifish habitat, they can temporarily invade them during floods and prey on juvenile and adult *Nothobranchius* (Wildekamp, 1983). It is unknown, however, whether dormant killifish eggs adjust their hatching characteristics in the presence of such predators.

Besides being modulated by environmental cues, hatching of dormant stages can also be inhibited by internal mechanisms (Cohen, 1966; Evans & Dennehy, 2005; Gremer & Venable, 2014). Environmental cues are often unreliable and cannot be used to predict additional rains that are often essential to extend inundations (Tuytens *et al*., 2014). Therefore, partial hatching during each inundation can be a suitable strategy for ensuring population persistence in the face of environmental stochasticity (Seger & Brockmann, 1987; Venable, 2007). The potential importance of delayed hatching or germination of resting stages as a risk spreading (bet hedging) strategy has been acknowledged in various organisms, including plants and invertebrates (Evans & Dennehy, 2005; Childs, Metcalf & Rees, 2010; Simons, 2014). Theoretically, delayed hatching could arise from genetic polymorphism or from nongenetic variation passed on by the parents (Evans & Dennehy, 2005). Although bet hedging strategies are well supported by theoretical models (Cohen, 1966; Starrfelt & Kokko, 2012; Gremer & Venable, 2014), the underlying mechanisms that generate variation in dormancy strategies in nature are still poorly understood. This is also the case for dormancy in annual fishes. Despite anecdotal information and informally published reports belonging to the so called 'grey literature', experimental accounts of delayed hatching in *Nothobranchius* are rare (but see Wourms, 1972c) and the phenomenon has never been investigated over consecutive inundations and under standardized conditions.

In the present study, we conducted a standardized empirical test of delayed hatching in annual killifish both in the presence and absence of external cues signalling unfavourable conditions. First, we investigated whether dormant *Nothobranchius* eggs in the final (D3) dormancy stage adjust their hatching behaviour in the presence of predators by exposing them to fish kairomones during an inundation. We hypothesized that hatching would be lower in the presence of kairomones because these signal a potential predation risk that can be largely avoided by remaining dormant. Additionally, in a second experiment, we assessed variation in the hatching patterns and the transition through dormancy stages of dormant eggs of three *Nothobranchius* species during four subsequent identical inundations. Consistent with predictions from theoretical bet hedging models (Cohen, 1966; Evans & Dennehy, 2005), we hypothesized that a fraction of eggs will refrain from hatching, even though conditions are suitable, but will hatch under identical conditions during another inundation in the future. Here, we specifically assessed the relative importance of developmental arrest in the 'pre-D3' and D3 dormancy stages, which could reflect bet hedging over long and shorter time scales, respectively.

MATERIAL AND METHODS

NOTHOBRANCHIUS KILLIFISH

The genus *Nothobranchius* comprises approximately 62 described species (Froese & Pauly, 2013; http://www.fishbase.org), most of which are endemic to subtropical regions of eastern Africa, where they inhabit a wide variety of pools and streams that dry out seasonally (Watters, 2009) (Fig. 1). Although *Nothobranchius* is characterized by extensive intra- and interspecific morphological variation in traits

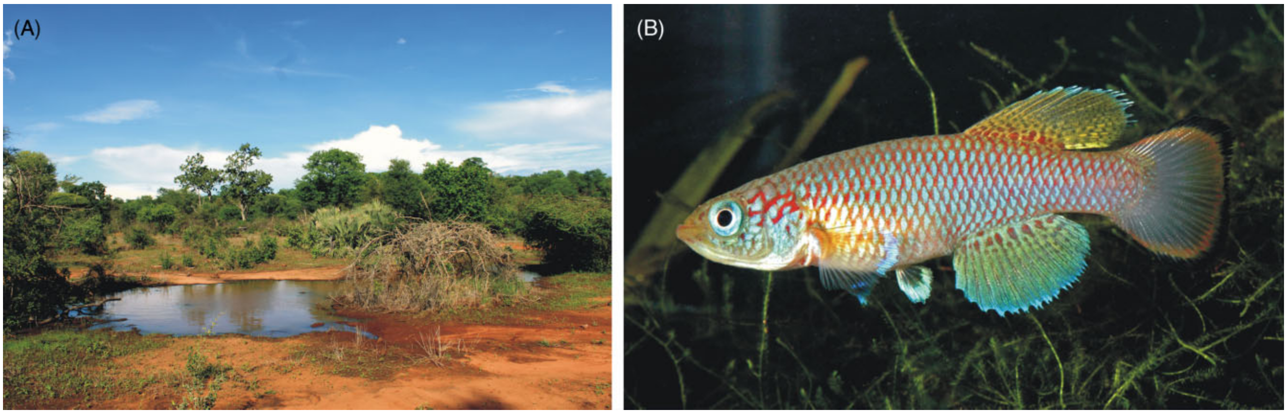

Figure 1. A, temporary savannah pools in South East Zimbabwe are typical *Nothobranchius* habitats (photograph by Karen Tuytens). B, *Nothobranchius lucius*, one of the killifish species from eastern Tanzania (photograph by Mike Jacobs).

such as body size (4–15 cm) and coloration, all species share a similar body plan and have an upward-pointed (superior) mouth. Additionally, all species display clear sexual dichromatism with brown–grey females and brightly coloured males (Wildekamp, 2004) and a large number of species are characterized by genetic colour polymorphism, sometimes even within populations (Reichard, Polačik & Sedlacek, 2009).

STUDIED POPULATIONS

All of the dormant eggs used in the hatching experiments originated from natural populations that were reared for at least three generations in captivity under common breeding conditions (22 °C, 12 : 12 h light/dark cycle) to establish laboratory lines prior to experimentation. Dormant eggs were stored in closed plastic zip-lock bags embedded in moist peat (Jiffy-7, Jiffy Products International BV) at 18 °C and under continuous darkness before the start of the hatching experiments.

The laboratory line of *Nothobranchius steinforti* (Kimamba, TZN 76-04) used in the first experiment originates from eastern Tanzania. The laboratory lines of *Nothobranchius kafuensis* (Lukanga swamp, ZAM 08-02), *Nothobranchius lucius* (Ruhoi River, TZN 09-10), and *Nothobranchius pienaari* (Panda, MZCS 08-81) used in the second experiment were originally collected in central Zambia, eastern Tanzania, and south-eastern Mozambique, respectively.

HATCHING PROCEDURE AND EXPERIMENTAL SET-UP

In a first hatching experiment, *N. steinforti* eggs were incubated in the presence (50 replicates) and absence (50 replicates, control group) of fish kairomones, aiming to determine whether dormant eggs adjust their hatching in the presence of potential predators. Eggs were all in the D3 stage (cf. stage 43, late pre-hatching embryo *sensu* Wourms, 1972a) and were randomly selected from 200 eggs of the same age (i.e. fertilized within a span of 48 h) after which each egg was placed individually in a transparent polypropylene container (10 × 7.5 × 4 cm) on 20 mL of peat. Each container was either filled with 200 mL of aged tap water (conductivity: 830 µs cm^{-1}, pH 8, KH 10) or with kairomone medium, both at a temperature of 10 °C. The kairomone medium was prepared using aged tap water in which predatory pumpkinseed sunfish *Lepomis gibbosus* (with a mean body length of 10 cm) were kept for 24 h at a density of one fish per litre^2. Pumkinseed sunfish kairomones were chosen as a substitute for African catfish kairomones because no catfish from *Nothobranchius* habitat could be obtained and previous studies indicated that the effect of kairomones on the hatching of resting stages is generally not specific to the fish species used (Lass *et al*., 2005). Yet, recent research has shown that some predatory fish may successfully adopt chemical camouflage to avoid detection through excreted kairomones (Resetarits & Binckley, 2013). Both the control and the kairomone medium were filtered over a 3-µm filter and subsequently a 0.45-µm filter (Jensen, Jakobsen & Kleiven, 1998; Lass *et al*., 2005), aerated, and cooled to 10 °C. After inundation, the plastic containers were positioned randomly in a temperature controlled incubator at 22 °C under a 12 : 12 h light/dark cycle, which converges to optimal *Nothobranchius* hatching conditions (Genade *et al*., 2005). The position of the containers in the incubator was randomized after each check. Hatching was evaluated at 12-h intervals until no additional hatching was observed for 48 h.

In a second hatching experiment, hatching or, in case of not hatching, the dormancy stage (D1, D2, D3) of *N. lucius*, *N. kafuensis*, and *N. pienaari* dormant eggs was assessed under standardized conditions during four consecutive inundations in the laboratory. Each inundation was alternated by a dry period of 7 days. The hatching experiment contained 56 replicates for *N. lucius* and 50 replicates for both *N. kafuensis* and *N. pienaari*. All dormant eggs were randomly selected from 200 eggs of the same age (i.e. fertilized within a span of 48 h) that had been stored for 7 months under common conditions (18 °C, total darkness). Each egg was placed individually in a plastic container (10 × 7.5 × 4 cm) on 20 mL of peat. The dormancy stage of each egg was determined both at the start and at the end of every inundation using a light microscope. Eggs were incubated under the same conditions and in the same medium as the control group from the first experiment. Hatching was evaluated at 12-h intervals until no additional hatching was observed for 48 h and the position of the containers was randomized after each check. After each inundation, each egg was stored individually, embedded in 20 mL of moist peat in a closed zip-lock bag, and kept at 18 °C for a 7-day period until the next inundation.

All statistical analyses were performed in R, version 3.1.1 (R Core Development Team) using the lme4 and car packages. Differences in the binary hatching response (0, 1) between the kairomone and control treatment in Experiment 1 were assessed using a generalized linear model (GLM) and specifying a binomial distribution and a logit link function. Pairwise contrasts were calculated to investigate variation in hatching fractions between treatments through time. To assess whether investment in long-term bet hedging [quantified as D3/(D1 + D2 + D3)] differed between species at the onset of the first inundation of Experiment 2, a GLM was constructed including species identity as a categorical predictor. In addition, separate GLMs were constructed to investigate the impact of species identity on cumulative hatching fractions of D3 eggs (measured as H/D3) during the first and second inundations. Pairwise contrasts were calculated to investigate intra- and interspecific variation in hatching fractions, within and among inundations. Pairwise contrasts were corrected for multiple testing using a sequential Bonferroni correction (Holm, 1979).

RESULTS

During all experimental inundations, killifish larvae only hatched from eggs that had reached the D3 stage by the end of the preceding dry phase. Hatching fractions could therefore be calculated as the proportion of D3 eggs that hatched during each inundation. During the first experiment, cumulative hatching of *N. steinforti* dormant eggs was significantly higher in the control than in the kairomone treatment ($F_{2,98} = 21.81$, $P < 0.001$), regardless of the time interval considered (Fig. 2, Table 1). Maximum cumulative hatching was reached after 36 h, both in the control (68%) and the kairomone (22%) treatments.

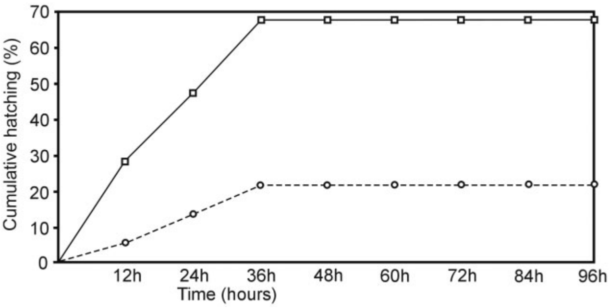

Figure 2. The impact of predator fish kairomones on the hatching of *Nothobranchius steinforti*. Cumulative hatching of dormant *N. steinforti* D3 eggs in the presence (dashed line) and absence (solid line) of predator fish kairomones.

Table 1. Generalized linear model output for the comparison of the hatching fraction of *Nothobranchius steinforti* resting eggs in the control group and the Kairomone treatment (K)

Time	Estimate	*z*-value	*P*-value
12 h (K)	−1.904	−2.839	0.005
36 h (K)	−1.735	−3.497	< 0.001
60 h (K)	−2.019	−4.423	< 0.001
84 h (K)	−2.019	−4.423	< 0.001
96 h (K)	−2.019	−4.423	< 0.001

All $P < 0.001$ (except at 12 h after inundation) after sequential Bonferroni correction.

At the start of the second experiment, not all of the randomly selected *N. kafuensis* eggs (D2: 2 eggs; D3: 48 eggs), *N. lucius* (D1: 14 eggs; D2: 24 eggs; D3: 18 eggs) and *N. pienaari* (D1: 7 eggs; D2: 15 eggs; D3: 28 eggs) had reached the D3 stage (Fig. 3). The GLM indicated that the fraction of D3/(D1 + D2 + D3) eggs at the start of the first inundation differed among species ($F_{2,153} = 26.45$, $P < 0.001$). At the onset of the second inundation, all remaining *N. kafuensis* and *N. lucius* eggs had reached D3, although there was still variation in the dormancy stage of the *N. pienaari* eggs (D1: 2 eggs; D2: 12 eggs; D3: 13 eggs). Although the hatching characteristics of *N. lucius* eggs could be evaluated during all four experimental inundations, hatching of the *N. kafuensis* and *N. pienaari* eggs could only be reliably analyzed during the first two inundations because only eight and eleven viable unhatched eggs of these species remained at the start

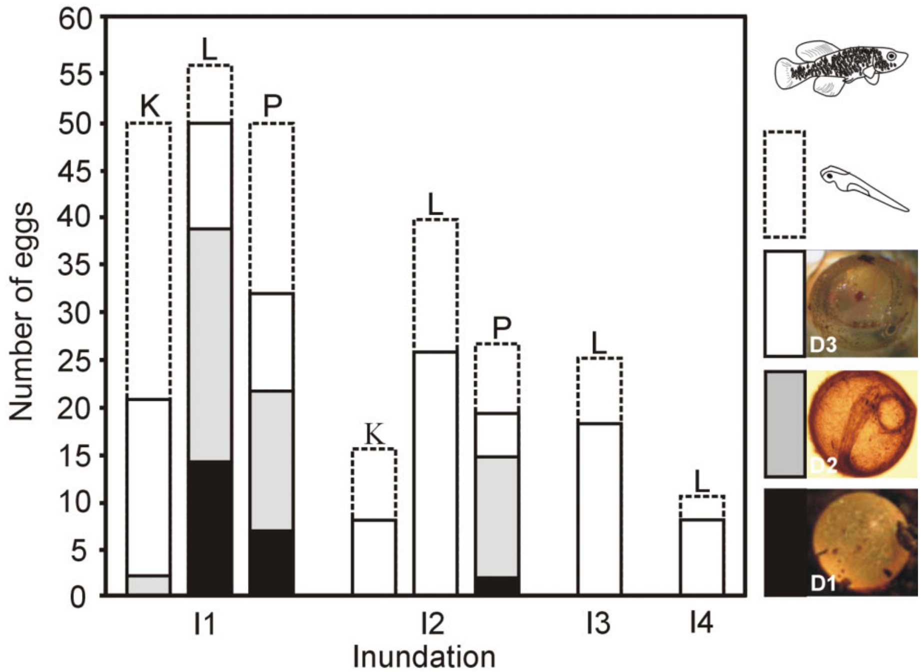

Figure 3. Hatching patterns of dormant eggs of *Nothobranchius kafuensis* (K), *Nothobranchius lucius* (L), and *Nothobranchius pienaari* (P) over four subsequent, identical, inundations. The proportion of D1, D2, and D3 eggs at the onset of each inundation is shown in black, grey, and white, respectively. Broken lines show the number of D3 eggs that hatched during each inundation. Eggs that died in dormancy are not shown.

of the third inundation. The GLMs did not demonstrate a significant overall species effect on hatching during the first inundation ($F_{2,91} = 2.24$, $P = 0.11$) or the second inundation ($F_{2,64} = 1.56$, $P = 0.22$). Within species, pairwise comparisons indicate that the cumulative hatching fraction was more or less constant over inundations (I) for all species considered; *N. kafuensis* (I1: 60%; I2: 50%), *N. pienaari* (I1: 64%; I2: 63%), and *N. lucius* (I1: 33%; I2: 35%) (Fig. 3, Table 2). In addition, the hatching fractions of *N. lucius* eggs (I3: 28%; I4: 27%) during inundations three and four were not significantly different from those measured during inundations one and two (Table 2). During inundations one and two, *N. pienaari* was characterized by the highest cumulative hatching fraction, followed by *N. kafuensis* and finally *N. lucius*; however, the GLM model did not demonstrate significant differences after sequential Bonferroni correction (Table 2).

DISCUSSION

The temporary habitat of *Nothobranchius* killifish is characterized by strong and often unpredictable variation in the timing and suitability of inundations for growth and reproduction (Seegers, 1997; Wildekamp, 2004). For example, variable rains may cause some inundations to be too short to allow inhabitants to

Table 2. A generalized linear model was constructed to compare hatching fractions between the three investigated *Nothobranchius* species (K: *Nothobranchius kafuensis*, L: *Nothobranchius Lucius*, P: *Nothobranchius pienaari*) during each inundation and within each species over the different inundations

Species (inundation)	Estimate	*Z*-value	*P*-value
K(1)–L(1)	−1.030	−1.777	0.076
K(1)–P(1)	0.251	0.512	0.609
L(1)–P(1)	1.281	2.011	0.044
K(1)–K(2)	−0.337	−0.581	0.561
L(1)–L(2)	0.074	0.124	0.902
P(1)–P(2)	−0.028	−0.038	0.970
K(2)–L(2)	−6.190	−1.032	0.302
K(2)–P(2)	0.560	0.698	0.485
L(2)–P(2)	1.179	1.662	0.097
L(1)–L(3)	−0.251	−0.375	0.707
L(1)–L(4)	−0.288	−0.342	0.732
L(2)–L(3)	−0.325	−0.586	0.558
L(3)–L(4)	−0.036	−0.045	0.964

Numbers in parentheses indicate the respective (1–4) experimental inundation. No *P*-values were significant after sequential Bonferroni correction.

grow and reproduce (Watters, 2009) or predatory fish to occasionally infiltrate *Nothobranchius* habitats during erratic river flooding events that may temporarily connect them with temporary ponds and wetlands in the floodplain (Wildekamp, 1983). These predators, however, are unable to establish persistent populations because they lack the adaptations to survive desiccation and die when the habitat dries out. If all *Nothobranchius* eggs in the sediment of a temporary pool were to hatch at the same time, a single unsuitable inundation would eradicate the complete fish-stock resulting in population extinction. In reality, however, this situation does not appear to occur because staggered hatching of *Nothobranchius* dormant eggs within and between inundations has frequently been observed, both in captivity and in natural habitats (Watters, 2009; Polačik *et al*., 2011). Although little is known about the underlying mechanisms in annual fish, this process of dormancy and delayed emergence of propagule or seed banks has been studied more extensively in annual plants (Cohen, 1966; Childs *et al*., 2010) and crustaceans (Brendonck, 1996; Philippi *et al*., 2001; Caceres & Tessier, 2003). What is more, the literature on dormancy in these other organisms highlights a number of potential mechanisms that could also contribute to delayed hatching in *Nothobranchius*. These include both environmental modulation and so-called intrinsic factors (i.e. genetically or nongenetically inherited hatching cues or inhibitors) (Evans & Dennehy, 2005; Starrfelt & Kokko, 2012).

Previous research showed that hatching of *Nothobranchius* dormant eggs is modulated by environmental cues that are indicative of suitable inundations. These cues include a low temperature and a high oxygen concentration indicative of copious rain (Wildekamp, 2004) and a sufficient light dose (Markofsky *et al*., 1979; Levels & Denucé, 1988), which could indicate that eggs are not covered by substrate (Pinceel *et al*., 2013). In addition to these cues, our results show that dormant eggs also adjust their hatching characteristics in the presence of predator cues. We observed reduced hatching of *N. steinforti* D3 eggs in the presence of fish kairomones, which suggests that they adequately respond to the presence of these infochemicals that signal a potential predation threat (Kats & Dill, 1998). Given that dormant *Nothobranchius* eggs in the sediment most likely face a much lower risk of predation by fish than free-swimming larvae and that the presence of fish predators is expected to vary within the potential lifespan of dormant *Nothobranchius* eggs (weeks/months) (Levels *et al*., 1986), delayed hatching in the presence of predator kairomones could be an adaptive strategy to at least reduce predation. Previous findings of a similar reduction in the germination of dinoflagellates (Rengefors, Karlsson & Hansson, 1998) and the hatching of *D. magna* (Lass *et al*., 2005) in the presence of fish kairomones and *Cyzicus* sp. in the presence of salamander kairomones (Spencer & Blaustein, 2001) further suggest that prolonging dormancy of resting stages can indeed be an efficient strategy for evading predation. It remains to be shown, however, whether the ability of *Nothobranchius* killifish to react to a potential predation threat is a conserved strategy or whether it is genotype dependent, as was, for example, previously demonstrated in *D. magna* (Lass *et al*., 2005).

Although environmental modulation of hatching can help to avoid reproductive failure, environmental cues may be unreliable (Vanschoenwinkel *et al*., 2010; Tuytens *et al*., 2014). The results of our standardized hatching experiments indicate that, even when eggs were exposed to suitable cues, a fraction of viable eggs shows delayed hatching. In the mean time, they contribute to the formation of a dormant reservoir of genetic information in the sediment: a dormant egg bank (Hairston, 1996; Brendonck & De Meester, 2003) from which individuals can be recruited during inundations over subsequent months or even years in the case of annual killifish (Levels *et al*., 1986; Podrabsky & Hand, 1999). These observations suggest an intrinsic mechanism controlling the dormancy of *Nothobranchius* dormant eggs that allows for the redistribution of offspring over different future inundations as a bet hedging strategy that may reduce the risk of population extinction (Seger & Brockmann, 1987; Starrfelt & Kokko, 2012). Similar explanations have been formulated for the partial hatching of dormant zooplankton eggs and seeds of annual plants (Evans & Dennehy, 2005; Gremer & Venable, 2014). Because the developmental stage of *Nothobranchius* killifish eggs can be assessed in a nondestructive way, we were able to demonstrate that the emergence of larvae during different growing seasons can be regulated at two different points (Fig. 4). First of all, development of *Nothobranchius* embryos can be halted in energy-efficient 'pre-D3' stages (mainly D2), which may remain viable up to several years (Levels *et al*., 1986; Watters, 2003). Embryos in the more developed and hence more energy-consuming D3 stage, in turn, only remain viable for several months (Wourms, 1972a, b, c; Wildekamp, 2004). The differences in timing of the transition of embryos to D3, which we observed in all studied species, may therefore constitute a means to repartition offspring over a number of inundations and as a bet hedging strategy over the long term. Although the investigated species differed significantly regarding their investment in 'long-term bet hedging' at the onset of the first inundation, we were unable to accurately link this to

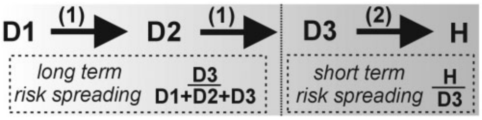

Figure 4. Conceptual diagram illustrating the potential control of bet hedging strategies in *Nothobranchius* killifish at two levels. (1) Variation in the timing of the transition of embryos from 'pre-D3' to D3 may constitute a means to ensure survival over several years as a long term bet hedging strategy, whereas (2) observed variation in emergence of D3 eggs could constitute a way to spread larval emergence over shorter time scales. Both environmental and intrinsic factors appear to influence the timing of these transitions.

temporal variation in habitat suitability and the timing of inundations as a result of a lack of long-term environmental information on the habitats of origin. In addition, repeated inundation and drying of resting eggs rapidly stimulated the transition of all eggs to the D3 stage, cancelling out further long-term bet hedging. Second, we also observed variation in the emergence of D3 eggs that are often assumed to be in a state of immediate response to suitable hatching cues. The observed delayed emergence of even part of the D3 eggs could represent a complementary mechanism for redistributing larval emergence, albeit most likely over a shorter time scale of only months. It is plausible that short-term fluctuations in habitat suitability could select for observed delayed development at the D3 stage whereas fluctuations over longer time scales could select for developmental arrest in the earlier, more energy efficient, stages. The relative contribution of both transitions as part of an integrated adaptive bet hedging strategy is therefore likely to depend on the temporal scale of uncertainty in environmental conditions and could be species, region, and even population specific, although this remains to be investigated.

Overall, our results confirmed environmental modulation of hatching in *Nothobranchius* killifish with predator kairomones reducing hatching fractions compared to controls. However, delayed development also occurred independent of environmental cues and appears to be regulated at two stages. Developmental arrest in the energy-efficient dormancy stages (mainly D2) could present a means to achieve long-term bet hedging over years, whereas arrest in the energy-consuming final (D3) stage may serve a similar purpose over months.

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