Local variation in embryo development rate in annual fish

M. Polačik*, M. Reichard and M. Vrtílek

Institute of Vertebrate Biology, Czech Academy of Sciences, Květná 8, 603, 65 Brno, Czech Republic

Extreme asynchrony in embryo development, a typical feature of annual killifish living in temporary pools, represents a bet-hedging strategy to cope with unpredictable rainfall. African annual killifish are distributed across a large precipitation gradient, raising the potential for local adaptation in the degree of developmental asynchrony (*e.g.* higher in arid areas, lower in humid areas). Eight populations of two sister species, *Nothobranchius furzeri* and *Nothobranchius kadleci*, from sites along the rainfall gradient were tested and compared for asynchrony and duration of embryo development. Degree of asynchrony and mean duration of embryo development did not differ across the examined range. Despite generally high developmental variability, fish from more arid regions (where rain is more erratic) produced a significantly higher proportion of short-developing embryos. Comparable developmental asynchrony, regardless of precipitation level, suggests that all populations tested need to cope with some level of rainfall stochasticity. By producing more short-developing embryos, however, fish in more arid areas with relatively more erratic rains are better adapted to very short pool durations and are more likely to produce multiple offspring generations within a single rainy season.

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Key words: diapause; erratic development; escape embryo; killifish; Mozambique; secondary pool.

INTRODUCTION

Temporary wetlands host a high diversity of organisms and there is growing interest in their protection and management (Kelly-Quinn *et al.,* 2017), especially in the light of ongoing climate change (Fay *et al.,* 2016). Temporary wetlands are especially sensitive to any shift in temperature and precipitation patterns due to their high surface area to volume ratio. In general, temperature is expected to increase resulting in a higher evaporation while precipitation events may become more extreme in terms of their intensity (extremely high or extremely low) and timing. For the temporary wetlands the consequences mainly include extended droughts and even more stochastic hydrological regime. (Junk *et al.,* 2013; Calhoun *et al.,* 2016). The climatic changes may promote establishment of alien animal and plant species potentially disturbing the ecological balance in the native community (Zedler, 2004; Carreira *et al.,* 2014). The ecosystem, which already imposes severe limitations to the existence of water organisms, coping with the cyclic shifts between aquatic and terrestrial phases, is going to become even harsher.

*Author to whom correspondence should be addressed. Tel.: +420 543 422 557; email: polacik@ivb.cz

Animals inhabiting temporary pools have evolved two main types of adaptation towards dry phases and environmental stochasticity. First, those taxa with life cycle involving a terrestrial form (*e.g.* amphibians, insects with aquatic larvae) escape the dry phase through metamorphosis. In addition, many have also adapted behaviourally by adjusting time of reproduction (Yasumiba *et al.,* 2016) or by actively searching for a suitable spawning site (Winandy *et al.,* 2017). Second, those taxa with no specific terrestrial stage survive through the dry season in a state of dormancy. While the dormant stages can be adult, they occur more often as drought-resistant eggs. Adult stages can react to local environmental conditions instantly through an adjustment of their behaviour (*e.g.* molluscs: Chapuis & Ferdy, 2012; lungfish: Polačik & Podrabsky, 2015) whereas producers of passive resistant embryos (*e.g.* rotifers, planktonic crustaceans) often display a form of diversified developmental bet-hedging. In such cases, embryonic development is highly asynchronous, ensuring that at least a proportion of the egg pool is always ready to hatch and match contemporary conditions (Waterkeyn *et al.,* 2013; Gilbert, 2016).

Embryonic dormancy as a means of surviving dry conditions has evolved in just a single group of vertebrates. Annual fish inhabiting temporary pools in South America and Africa hatch soon after the pool is inundated; they then mature rapidly and produce diapausing eggs before the pool desiccates once again. Annual-fish embryonic development is extremely variable as it comprises three facultative diapauses (dormancy stages), each of which comes at a well-defined developmental stage and can be entered or skipped (Wourms, 1972). Some embryos develop into the pre-hatching stage in less than 3 weeks, while others may take several years (Cellerino *et al.,* 2016). Such asynchrony commonly occurs not only at the population level but also within a single clutch incubated under the same conditions (Polačik *et al.,* 2017). Variation in embryo development allows the fish to cope with a false onset of the rainy season (Wourms, 1972) when light initial rains are followed by a further period of drought. It also enables appearance of several generations within the same rainy season, increasing an overall population abundance (Podrabsky *et al.,* 2010; Polačik *et al.,* 2014*a*; Reichard *et al.,* 2017*a*) and provides insurance against a complete absence of rainfall in an extremely dry rainy season by maintaining some unhatched eggs to the next rainy season (Watters, 2009).

Developmental bet-hedging maximizes geometric mean fitness and increases the chance that at least a proportion of progeny will hatch into a favourable environment (Simons, 2011). At the same time, investment into progeny that may mismatch actual conditions (by developing too early or too late) is wasted (Krug, 2009). Specifically, annual fish can halt their embryonic development at any of the three diapause stages, each of which differs markedly in energy consumption. For example, an embryo of turquoise killifish *Nothobranchius furzeri* Jubb 1971 that becomes dormant late in the advanced, pre-hatching stage (diapause III *sensu* Wourms, 1972) has a 15 fold higher metabolic rate than an embryo halting development in the relatively less complex diapause II (Furness, 2016), which occurs about midway through development prior to the major organogenesis phases (Wourms, 1972). Owing to the much higher energetic demands, the consumption of yolk reserves is faster in diapause III embryos and, therefore, the time window of embryo viability is restricted (Wourms, 1972; Furness, 2016). Consequently, natural selection should tune the timing of development of annual killifish to local conditions, matching the level of environmental predictability (Khatchikian *et al.,* 2009).
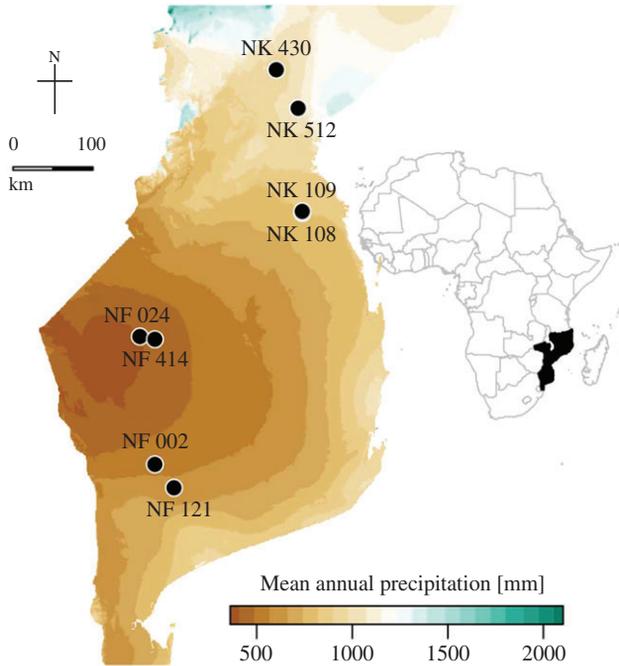
Fig. 1. Map of Africa showing Mozambique (▬) with enlargement of southern and central Mozambique showing mean annual precipitation isoclines and the collection sites of the eight experimental *Nothobranchius furzeri* (NF) and *N. kadleci* (NK) populations.

In this study, local adaptations for asynchrony and duration of embryo development using four *N. furzeri* and four *Nothobranchius kadleci* Reichard, 2010 populations originating from pools located along a precipitation cline (Fig. 1) were investigated. The precipitation cline coincides with substantial population genetic structuring (Bartáková *et al.,* 2013, 2015), providing an additional rationale for the potential existence of local adaptations. The two species are reciprocally paraphyletic in their evolutionary origin (Bartáková *et al.,* 2015), substantiating their treatment as a single evolutionary unit for the purposes of this study. Eggs from the eight populations were incubated individually in a common-garden environment and data on synchrony and duration of embryo development were recorded. A higher degree of developmental asynchrony in more arid regions (Khatchikian *et al.,* 2009; Watters, 2009) was predicted as an adaptation to the erratic character of rainfall (Geurts & Van den Berg, 1998).

MATERIALS AND METHODS

STUDY SYSTEM

Nothobranchius furzeri and *N. kadleci* represent a pair of sister species with allopatric distribution (Reichard *et al.,* 2017*b*) and are ecological vicariants with the same food niche (Polačik *et al.,* 2014*b*). While distinct in colouration (Reichard, 2010), DNA sequence data render them as paraphyletic evolutionary lineages (Bartáková *et al.,* 2015), distributed across southern and

TABLE I. Details of the eight *Nothobranchius furzeri* and *N. kadleci* populations tested, their site of origin and associated climatic conditions

Species	Population code	GPS coordinates	Mean annual rainfall (mm)	Mean annual temperature (°C)	Rainfall variability*	Genetic clade†
N. furzeri	MZCS 024	22° 30·498 S; 32° 33·046 E	443	24·2	80·4	Chefu
N. furzeri	MZCS 414	22° 33·278 S; 32° 43·635 E	463	24·5	78·9	Chefu
N. furzeri	MZCS 002	24° 03·808 S; 32° 43·932 E	576	24·3	73·9	Limpopo north
N. furzeri	MZCS 121	24° 21·471 S; 32° 58·446 E	621	24·6	68·7	Limpopo North
N. kadleci	MZCS 108	21° 00·728 S; 34° 32·219 E	840	23·5	57·9	Kadleci South
N. kadleci	MZCS 109	21° 00·439 S; 34° 32·366 E	847	23·5	57·8	Kadleci South
N. kadleci	MZCS 430	19° 16·838 S; 34° 13·230 E	942	24·9	65·8	Kadleci North
N. kadleci	MZCS 512	19° 44·698 S; 34° 28·898 E	1059	24·8	58·1	Kadleci North

*Measured as C.V. across 1957 to 2016 data, calculated for each month separately and averaged across months.
†Bartáková *et al.,* 2013; Bartáková *et al.,* 2015.

central Mozambique. The geographic range of *N. furzeri* is centred in the more arid southern region (Reichard *et al.,* 2009) while that of *N. kadleci* is centred in the more humid north (Bartáková *et al.,* 2015; Reichard *et al.,* 2017b). Four *N. furzeri* and four *N. kadleci* populations were used in the experiment and treated as a single evolutionary unit (Fig. 1).

Across the range of both species, there is a cline in mean annual rainfall (Table I). In Mozambique, a strong positive relationship exists between the mean annual rainfall and rainfall reliability (Geurts & Van den Berg, 1998). In concordance with the cline in the amount and predictability of rainfall, there is also strong geographic genetic structuring between populations. Populations of *N. furzeri* from semi-arid regions belong to a different genetic clade than those from more humid regions nearer the coast (Bartáková *et al.,* 2015; Fig. 1 and Table I). Similarly, nuclear markers indicate genetic separation between *N. kadleci* populations from the north and south, with northerly regions receiving more rainfall than those in the south (Bartáková *et al.,* 2015; Fig. 1 and Table I).

EXPERIMENTAL DESIGN

In order to evaluate developmental adaptation to local climatic conditions, synchrony and duration of embryo development were compared through regular visual inspections of eggs from each of the eight study populations incubated under identical conditions.

Eggs for the experiment were obtained through single day-pair spawning of 10–12 pairs per population of 8 week-old parental fish (the strain codes represent original field coding to allow for unambiguous identification of the wild-derived strains; Table I and Fig. 1). The parental fish were F2 generation of the wild-caught strain founders except *N. furzeri* MZCS 24 (F4), *N. kadleci* MZCS 108 (F5) and *N. kadleci* MZCS 109 (F3). Each pair was introduced into a 2 l plastic container with a 0·5 cm layer of laboratory glass beads and allowed to spawn for 2 h. After 12 h, all fertilized eggs from each experimental population were placed into Petri dishes and treated for 3 days with methylene blue to prevent the emergence of fungus (Polačik *et al.,* 2016). The eggs from each experimental population were pooled, with each female contributing at least five fertilized eggs to the pool.

After the anti-fungal treatment, 788 eggs were randomly chosen from the pool of fertilized eggs (95–100 per population) and individually incubated in 8 cm^3 compartments of plastic Petri dishes under a 1·5 cm layer of damp peat (Agro CS; www.agrocs.cz) (Polačik *et al.,* 2017). The position of eggs was randomized across different populations. This particular incubation mode was chosen as it is closer to natural conditions where the embryos are buried in the substratum (Polačik *et al.,* 2017) while incubation of killifish eggs on top of the substratum makes them develop relatively faster and more synchronously (Genade, 2005; Polačik *et al.,* 2016; Vrtílek *et al.,* 2017). Use of a substratum from a common source prepared prior to the study ensured a standard level of moisture and peat quality in all Petri dishes. Eggs were then incubated in a laboratory incubator (Q-Cell; www.pollab.pl) at 25° C.

Individual embryo development was checked visually during four inspections at 30 day intervals (30, 60, 90 and 120 days-post-fertilization; dpf). A focused light source was used to inspect each egg through the bottom of the transparent Petri dish (Polačik *et al.,* 2017). Where direct observation was compromised by the position of a particular egg (*c.* 2% of eggs), the substratum was disturbed briefly and the egg inspected directly. Two embryo development stages were scored based on degree of developmental progress: (1) fully developed embryos that had reached the pre-hatching stage, recognized based on full body formation, conspicuous golden eye colouration and an opaque appearance to the egg (Wourms, 1972; Podrabsky *et al.,* 2010; Furness *et al.,* 2015; Pinceel *et al.,* 2015), and (2) undeveloped embryos (diapausing or not) that had not attained the pre-hatching state at a given inspection. The presence of all dead embryos (white and opaque) was also recorded. Three embryo development rate categories were scored based on when the fully developed embryo was first recorded: (1) escape embryos (*sensu* Wourms, 1972; Podrabsky *et al.,* 2010; rapid developmental rate) scored as fully developed at the time of first inspection, *i.e*. 30 dpf, (2) intermediate embryos (intermediate developmental rate) scored as fully developed at 60, 90 or 120 dpf, and (3) slow developing embryos (slow developmental rate), scored as undeveloped at the last inspection (120 dpf).

DATA ANALYSIS

Data on mean annual rainfall, its variability (calculated as C.V. for each month averaged across 12 months for the period 1957–2016), mean annual temperature for the period 1957–2016 (source www.fetchclimate.org) and aridity index were collected for each study site. Aridity Index (I_A) was calculated as the ratio between the mean annual precipitation and mean annual potential evapotranspiration based on 1950–2000 averaged data (Trabucco & Zomer, 2009; Vrtílek & Reichard, 2016). The variable was strongly positively correlated with precipitation (Spearman rank correlation, $\rho = 0.929$, $n = 8$, $P < 0.01$) and therefore dropped as a redundant explanatory variable from further analysis.

Using Spearman's rank correlation, these variables were correlated with developmental asynchrony, mean embryo development rank duration (based on developmental rate categories 1–3, see experimental design), the proportion of *escape* embryos and the proportion of *slow* embryos. Developmental asynchrony was quantified using the index of qualitative variation (I_{QV}): $I_{QV} = [K(100^2 - \sum \% C^2)][\,100^2(K - 1)]^{-1}$, where K is the number of categories in the distribution and $\%C$ is the percentage of a respective category. The index ranges from 0 to 1, where 0 implies uniformity and presence of only a single category and 1 implies the highest possible diversity with all categories represented evenly.

RESULTS

Embryo survival during the experiment was 56·7%, with 35·4% of all embryos reaching the pre-hatching stage and 21·3% remaining undeveloped at the last inspection. The course of development appeared to follow a bimodal pattern, with 24·6% of surviving embryos developed by 30 dpf; 20·1% developed by 60 dpf; 3·6% by 90 dpf and 14·1% by 120 dpf (37·6% remaining undeveloped).
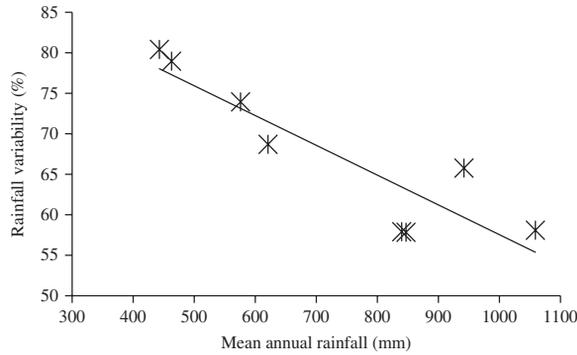
FIG. 2. Relationship between mean annual rainfall and rainfall variability (calculated as C.V. for each month averaged across 12 months for the period 1957–2016). Spearman's $\rho = -0.81$, $P < 0.05$.

Mean annual rainfall was negatively correlated with rainfall variability (Fig. 2). Mean annual temperature was not correlated to mean annual rainfall ($\rho = 0.191$, $P > 0.05$) and rainfall variability ($\rho = 0.381$, $P > 0.05$). No correlation was observed between the degree of developmental asynchrony (I_{QV}) and any climatic factor [Table II and Fig. 3(a)]. The experimental populations comprised a mix of relatively synchronous development (low I_{QV} values: *N. furzeri* 414, *N. furzeri* 121, *N. kadleci* 430) and asynchronous development (high I_{QV} values: *N. furzeri* 2, *N. kadleci* 108, *N. kadleci* 109; Fig. 4). Similarly, no correlation was observed between the climatic factors and mean rank duration of embryo development [Table II and Fig. 3(b)]. There was, however, a significantly decreasing proportion of escape embryos being produced along the gradient of mean annual rainfall [Table II and Fig. 3(c)] together with a similar trend along the gradient of rainfall variability (Table II). The production of slow developing embryos was not correlated to climatic factors [Table II and Fig. 3(d)].

TABLE II. Relationship between the parameters of embryo development of the eight study populations of *Nothobranchius furzeri* and *N. kadleci* and climatic variables

Variable	Correlation with mean annual rainfall		Correlation with rainfall variability		Correlation with mean annual temperature	
	Spearman's ρ	P	Spearman's ρ	P	Spearman's ρ	P
Index of qualitative variation (I_{QV})	0.238	>0.05	−0.524	>0.05	−0.619	>0.05
Mean rank of embryo development	0.405	>0.05	−0.024	>0.05	0.548	>0.05
Proportion of escape embryos	−0.857	<0.05	0.667	>0.05	0.191	>0.05
Proportion of slow embryos	0.405	>0.05	−0.024	>0.05	0.548	>0.05
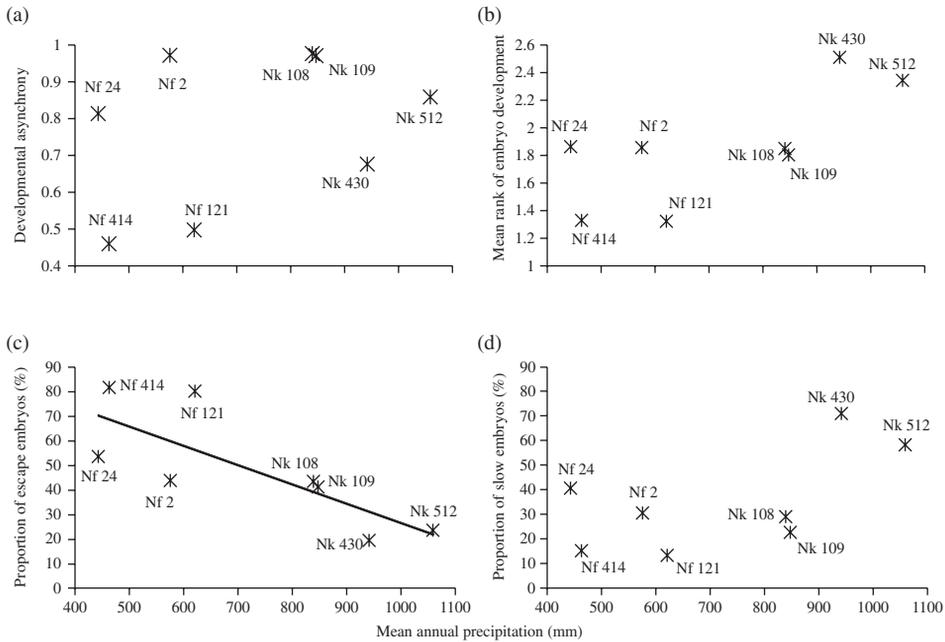
FIG. 3. (a) Developmental asynchrony in the eight experimental populations of *Nothobranchius furzeri* (Nf *n*) and *N. kadleci* (Nk *n*) in relation to mean annual precipitation. Asynchrony is expressed as index of qualitative variation (I_{QV}). An I_{QV} of 1 indicates maximum variability, while 0 denotes absolute synchrony. (b) Mean rank of embryo development (1 = escape, 2 = intermediate, 3 = slow) in the eight experimental populations in relation to mean annual precipitation. (c) Proportion of escape embryos in the eight experimental populations in relation to mean annual precipitation. The correlation was significant ($y = -0.0786x + 105.38$, Spearman's rank correlation, $\rho = -0.857$; $P < 0.05$). (d) Percentage of slow developing embryos in the eight experimental populations in relation to mean annual precipitation. The trend-lines were omitted from panels where the relationship was non-significant.

DISCUSSION

In this study, the degree of asynchrony in duration of embryo development in *N. furzeri* and *N. kadleci* populations from sites along a gradient of rainfall intensity and predictability was evaluated. There was considerable inter-population variability in the parameters tested. The degree of developmental asynchrony and duration of embryo development did not conform to the experimental predictions, with no negative relationship observed between these parameters and precipitation characteristics. An increase however, in the proportion of fast-developing, escape embryos [<30 dpf; Fig. 2(c)] with decrease in the mean annual rainfall and its predictability was observed [Table II and Fig. 2(c)].

Intra-population variability in the duration of embryo development in each experimental population was recorded. Asynchrony under constant conditions appears to be an inherent characteristic of embryo development in many (if not all) annual killifish species (Wourms, 1972; Podrabsky *et al.,* 2010; Cellerino *et al.,* 2016). Though seasonal rains arrive more reliably in some parts of *Nothobranchius* spp. distribution than others (Geurts & Van den Berg, 1998; Mazuze, 2007; Watters, 2009), any temporary pool still represents an unpredictable ecosystem by its very nature. As parental

Nf 24   Nf 414   Nf 2   Nf 121   Nk 108   Nk 109   Nk 430   Nk 512
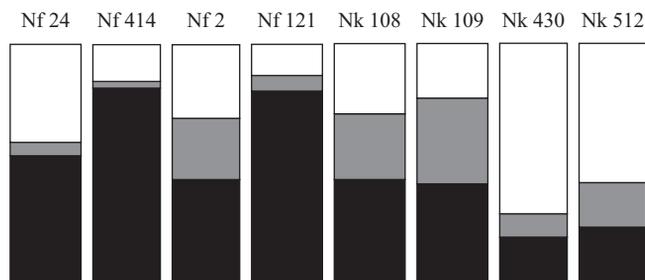
FIG. 4. The relative proportions of escape (■), intermediate (▨) and slow (□) developing embryos in each study population of *Nothobranchius furzeri* (Nf *n*) and *N. kadleci* (Nk *n*).

generations lack any reliable cues on the future environment of their offspring (Furness *et al.,* 2015; Pinceel *et al.,* 2015; Polačik *et al.,* 2017), the environmental stochasticity is accounted for by developmental bet-hedging in order to avoid reproductive failure (Crean & Marshall, 2009).

While higher developmental asynchrony in fish from more arid parts of the species' distribution range (Khatchikian *et al.,* 2009; Watters, 2009) was predicted, no such pattern was observed in the data [Fig. 2(a)]. Developmental asynchrony (as a bet-hedging strategy) is generally believed to be positively correlated with unpredictability of local conditions across a range of organisms (plants: Hacker, 1984; invertebrates: Khatchikian *et al.,* 2009; vertebrates: Watters, 2009). Anecdotal experience from captive breeding of *Nothobranchius* spp. suggests that embryos of species originating from arid regions display a more staggered developmental pattern, while those from areas with higher and more regular rainfall display more uniform embryonic development (Watters, 2009). Results of this study however, suggest only weak selection on the trait by precipitation in the geographic area sampled. Although the difference in mean annual rainfall between the driest and most humid sample sites in our study was *c*. 2·5 fold (Table I), this may not have been sufficient to induce local adaptations in the mode of embryonic development. On the other hand, it is possible that such a negative correlation between precipitation and developmental asynchrony exists at a more coarse geographic resolution, *i.e.* between populations or species in even more contrasting environments (Khatchikian *et al.,* 2009; Zhao *et al.,* 2015).

Despite the lack of any clear correlation between asynchrony and precipitation, the proportion of escape embryos decreased significantly with increasing annual rainfall. This could be interpreted within the context of exploitation of an alternative ecological niche such as secondary pools, *i.e.* temporary pools that fill with rainwater desiccate and refill again within the same rainy season. In brief, embryo developmental asynchrony in annual fish includes an escape (direct) pathway whereby an embryo skips diapauses I and II (Podrabsky *et al.,* 2010) and develops to the pre-hatching stage over a very short period (about 3 weeks in *N. furzeri* and *N. kadleci*; Blažek *et al.,* 2013; Polačik *et al.,* 2014*a*; Furness *et al.,* 2015). Since regular dry seasons last for much longer, escape development is considered an adaptation for exploiting such secondary pools that allow for a second generation of fish (those with direct embryo development) within a single season (Podrabsky *et al.,* 2010; Polačik *et al.,* 2014*a*). The appearance of secondary pools together with a new generation of fish has been confirmed in the wild (Reichard *et al.,* 2017*a*). Fish developing along each of the two alternative pathways also follow

different post-hatching life history strategies (Polačik *et al.,* 2014*a*). The likelihood of primary pool desiccation is relatively low in the more humid regions of the area sampled (Geurts & Van den Berg, 1998; Blažek *et al.,* 2017); hence, populations in such humid areas become adapted to local conditions by investing less into production of escape embryos and redirecting their resources to production of slow developing embryos that diapause throughout the dry season.

Locally adjusted duration of embryo development with incubation temperature as the main determinant has been confirmed across a range of taxa (insects: Tikkanen *et al.,* 2006; Stålhandske *et al.,* 2015; reptiles: Du *et al.,* 2010, Zhao *et al.,* 2015; fish: Whitney *et al.,* 2014; amphibians: Drakulić *et al.,* 2016). In the geographic area studied, the mean annual temperature shows negligible variability across the tested populations (Table I). To date, studies on the local effects of amount and reliability of precipitation have focused mainly on desert plants. While some studies have noted local genetic adaptations in the form of a negative correlation between rainfall and proportion of germinating seeds (Hacker, 1984; Shem-Tov *et al.,* 2002), others have emphasized the role of maternal effects (Philippi, 1993) or phenotypic plasticity during germination (Clauss & Venable, 2000). Although erratic embryo development in annual killifish undoubtedly represents a bet-hedging strategy with a clear intrinsic component (Wourms, 1972; Furness *et al.,* 2015; Pinceel *et al.,* 2015; Polačik *et al.,* 2017), it is also modulated by incubation conditions (Markofsky & Matias, 1977; Inglima *et al.,* 1981; Levels *et al.,* 1986) which differ *e.g.* by substantial diurnal temperature fluctuations in the wild (Reichard *et al.,* 2009). While the performed common-garden experiment yielded strong differences at the inter-population level, thereby confirming the significant role of genetic background on developmental characteristics, it is possible that the direction and magnitude of laboratory-based results may not fully mirror the situation under natural conditions, Nevertheless, the high proportion of escape embryos in populations from drier regions [Fig. 3(c)] represents a logical step in an adaptation towards rainfall unpredictability by a higher degree of diversification of available resources in *Nothobranchius* spp.

Embryo development in annual fishes reflects the unpredictable nature of the temporary pools they inhabit. Evolution of a local adaptation can sometimes be prevented by substantial inter-population gene flow (Rytkönen & Orell, 2001), though this is not the case in the well-isolated study populations (Bartáková *et al.,* 2013, 2015). The *Nothobranchius* spp. showed partial signs of the expected adjustment to local conditions. Though they inhabit regions of different mean annual rainfall amount and reliability, it is the environmental stochasticity, a common feature for all the pools located in the southernmost margin of *Nothobranchius* spp. distribution in Africa that appears to play the most important role. To further unravel potential for local adaptation in annual killifish embryo development, future studies should focus on populations or species across even a greater geographical gradient than that examined in this study.

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