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Maternal source of variability in the embryo development of an annual killifish

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Abstract

Organisms inhabiting unpredictable environments often evolve diversified reproductive bet-hedging strategies, expressed as production of multiple offspring phenotypes, thereby avoiding complete reproductive failure. To cope with unpredictable rainfall, African annual killifish from temporary savannah pools lay drought-resistant eggs that vary widely in the duration of embryo development. We examined the sources of variability in the duration of individual embryo development, egg production and fertilization rate in *Nothobranchius furzeri*. Using a quantitative genetics approach (North Carolina Type II design) we found support for maternal effects rather than polyandrous mating as the primary source of the variability in the duration of embryo development. The number of previously laid eggs appeared to serve as an internal physiological cue initiating a shift from rapid to slow embryo developmental mode. In annual killifish extensive phenotypic variability in progeny traits is adaptive, as the conditions experienced by parents have limited relevance to the offspring generation. In contrast to genetic control, with high phenotypic expression and heritability, maternal control of traits under natural selection prevents standing genetic diversity from potentially detrimental effects of selection in fluctuating environments.

Keywords: genetic benefit, good genes, mating system, diapause, erratic rainfall

Introduction

Unpredictable, temporally fluctuating environments facilitate the evolution of diversified reproductive bet hedging strategies (Cohen, 1966; Seger and Brockmann, 1987; Philippi and Seger, 1989; Childs *et al*., 2010; Simons 2011). Diversified bet hedging describes the situation when a parent produces a range of offspring phenotypes such that at least some are adapted to the prevailing conditions and are able to survive to reproduce. The variant

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phenotypes are generated regardless of actual environmental conditions (Olofsson *et al*., 2009). Most definitions of diversified bet hedging require that offspring variability is generated by the same genotype (e.g. Childs *et al*., 2010; Simons, 2011). However, the genetic diversity bet-hedging hypothesis has also been proposed, predicting that polyandrous females gain fitness benefits through increasing genetic diversity in the offspring. In this case, variability in parental genotypes results in adaptive variation in offspring phenotypes (e.g. Garcia-Gonzales *et al*., 2015; Yasui and Garcia-Gonzales, 2016).

Reproductive bet hedging is adaptive because it avoids complete reproductive failure (i.e. reduction in fitness variance, increase in geometric mean fitness), though this comes at the potential expense of a reduction in mean fitness through parental investment in phenotypes that are mis-matched with the environment (Krug, 2009; Garcia-Gonzales *et al*., 2015). Classical examples of reproductive bet hedging include annual plants adapted to environments with intermittent water availability. In these cases the risk of failure is spread through an intrinsic asynchrony in seed germination, with a portion of seeds germinating after early season rainfall, but with others remaining dormant until they experience more sustained precipitation (Evans and Dennehy, 2005). In the former case seedlings can potentially avoid competition through early germination, but risk drought stress if rainfall is not sustained. Later germinating seedlings face a lower risk of desiccation, but are disadvantaged in competition for space and resources with earlier-germinating seedlings. The overall outcome is that some seedlings survive irrespective of water availability (Evans and Dennehy, 2005).

Offspring phenotypic diversity in unpredictable environments can be generated in a number of ways. One mechanism is through mate choice combined with multiple mating partners ('polyandry'), to control the paternal contribution of genes to offspring. The contribution of multiple parental genotypes may be key to generating phenotypic diversity in progeny (Yasui, 1998; Simons, 2011; Garcia-Gonzales *et al*., 2015). Evidence to support this

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hypothesis is scarce but several studies have demonstrated that the genetic background of the male (Chen *et al*., 2012), female (McWatters and Saunders, 1997) or both parents (McWatters and Saunders, 1997; Yang *et al*., 2007; Chen *et al*., 2012) can directly influence variability in particular bet-hedging traits.

Another mechanism for generating offspring diversity is through modulating specific traits passed on through cytoplasmic factors that influence offspring development (Marshall and Uller, 2007; Sprenger *et al*., 2010). For example, in the quacking frog (*Crinia georgiana*), which reproduces in temporary pools, variable maternal egg provisioning when conditions in the spawning habitat are unpredictable generates phenotypic variability in offspring and reduces fitness variance among females (Dziminski *et al*., 2009). There is growing recognition that the production of specific phenotypes in sexually reproducing taxa can arise through processes acting above the direct effect of inherited genes, and are instead contingent on a set of processes that involve the activation, limitation or disabling of the activity of genes, such as DNA methylation, histone modification or regulation by small RNA molecules (Bossdorf *et al*., 2008; Smith and Ritchie, 2013).

Annual killifish of the genus *Nothobranchius* inhabit temporary pools on the East African savannah that are distinct examples of an unpredictable habitat. The pools only exist during the rainy season and the fish populations persist throughout the dry season exclusively as embryos enclosed in the egg envelope buried in the substrate of the pool. With the onset of rainfall and filling of the pool with water, killifish eggs hatch and the fish grow and mature rapidly, reaching maturity within less than three weeks (Blažek *et al*., 2013). *Nothobranchius* spp. are promiscuous income breeders (Vrtílek and Reichard, 2015; Wootton and Smith, 2015). They perform multiple spawning acts every day throughout their adult lives. During each spawning act, the female lays a single egg and can produce 30-50 eggs daily, each egg potentially fertilized by a different male. The eggs do not complete development in the

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presence of the adults (Inglima *et al*., 1981) and the lifespan of the adult fish is determined by the persistence of their natal pool, which is highly variable among years (e.g. Reichard *et al*., 2009; Watters, 2009, Reichard *et al*., 2014). An outcome of this breeding system is the complete temporal segregation of the parental and offspring generations.

Non-overlapping generations and the capricious character of their habitat mean that the optimum timing of the completion of embryo development in *Nothobranchius* is unpredictable. The timing of the onset and magnitude of seasonal rainfall are highly variable in some regions (Mazuze, 2007; Terzibasi *et al*., 2013; Furness *et al*., 2015) and there are no reliable environmental cues signalling that hatching at a particular point is optimal. Synchronized embryo development at the population level could easily result in the failure of an entire generation if eggs hatch prematurely, such as following brief periods of rainfall prior to the start of the rainy season proper (Wourms, 1972; Pinceel *et al*., 2015; Furness *et al*., 2015; supplementary Fig. 1). Annual killifish have evolved a system of three facultative diapauses, introducing unusual variability in the duration of embryonic development. Each of the three developmental arrests can be skipped, but can also vary considerably in duration (Wourms, 1972; Podrabsky *et al*., 2010; Furness, 2015; Pinceel *et al*., 2015). For example, the embryos of *Nothobranchius furzeri* Jubb, living at the southern margin of the *Nothobranchius* distribution, where annual rainfall is extremely erratic (Mazuze, 2007), may develop into the pre-hatching stage within just a couple of days or after several years (Watters, 2009; Polačik *et al*., 2014; Furness *et al*., 2015; Cellerino *et al*., 2016). This 'multiplier effect' (Wourms, 1972) in annual killifish embryo development has been termed an adaptive bet-hedging strategy, which has an outcome of supporting long-term population persistence (Wourms, 1972; Furness *et al*., 2015; Pinceel *et al*., 2015).

We examined the sources of variance in the duration of individual embryo development, egg production and fertilization rate in *N. furzeri*. This species is an emerging

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model in aging and evolutionary research and there is a substantial research effort to understand the mechanisms controlling its development (reviewed in Cellerino *et al*., 2016). We examined two alternative hypotheses. First, the *Adaptive Polyandry Hypothesis* predicts that multiple (polyandrous) mating by *N. furzeri* females is a primary source of variability in the duration of embryo development. This assumption implies that paternal genetic information effectively influences the duration of embryo development. Females potentially acquire genetic benefits of mating by increasing offspring developmental variability and consequently maximizing their between-generation geometric mean fitness (Yasui, 1998; Garcia-Gonzales *et al*., 2015). Alternatively, according to the *Maternal Effects Hypothesis*, embryo development may not be affected by paternal genetic background, but maternal effects enable flexible manipulation of embryo development time.

To understand the heritability of embryo development time and to identify the sources of variance we used a quantitative genetics approach and employed a cross-classified breeding design. We predicted a significant sire contribution to variation in embryo development, either through additive or non-additive genetic effects, under the *Adaptive Polyandry Hypothesis*. In contrast, we predicted a significant maternal contribution to embryo development variability under the *Maternal Effects Hypothesis*, with multiple mating unrelated to developmental bet hedging. We additionally compared egg production and fertilization rate to test whether multiple mating by females is adaptive in terms of female mate preference (more eggs laid with a preferred male) or male 'quality' (higher fertilization success of specific males or male-female pairings).

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Materials and Methods

The study system

Experimental fish were the F2 generation of 20 *N. furzeri* males and 40 females imported under the collecting code MZCS 222 (GPS: S 21°52'24.84", E 32°48'2.34") from southern Mozambique in March 2011. F2 fish were obtained from aquarium matings using standard rearing protocols and hatched simultaneously by moistening the substrate (Polačik *et al*., 2016). Juveniles were housed in rearing aquaria until achieving sexual maturity at about four weeks. A total of 48 sexually mature individuals (24 males and 24 females) were haphazardly selected and transferred to a recirculation housing system (Fish Box, Aqua Medic, www.aqua-medic.de) and housed individually in 2 L aquaria. Water temperature was maintained at 28 °C with a light: dark regime of 14:10 h. Fish were fed twice each day to satiation with live and frozen bloodworms and *Tubifex*. Approximately 30% of aquaria water was changed twice each week. Experimental work began once the fish were eight weeks old. *Nothobranchius* fishes can mature rapidly (Blažek *et al*., 2013), but are also characterized by high inherent intra-population variability in maturation and growth rate (Polačik *et al*., 2016). The age at which monitoring the reproductive output of experimental fish began corresponded with a threshold when all individuals were sexually mature, the initial phase of rapid growth had ceased (Polačik *et al*., 2014), and they were at maximal reproductive performance. Before the onset of the study, female reproductive condition was standardized by allowing each female to spawn all the mature eggs in her ovary with a non-experimental male. After 48 h females were ready to spawn again and experimental crosses commenced (Polačik and Reichard, 2009; Reichard and Polačik, 2010). After a bout of spawning to generate experimental crosses, females were always separated for 48 h before subsequent spawnings to permit completion of oogenesis. Upon completion of all experimental crosses, total length of all fish was measured using a digital camera and image analysis software (ImageJ).

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Heritability

The heritability of embryo development time was measured with a North Carolina Type II breeding design to generate a series of replicated half-sib families. This experimental design permits the relative contribution of additive and nonadditive genetic effects for a trait of interest to be measured and to identify maternal and paternal contributions to additive genetic variance (Lynch and Walsh, 1998).

Repeated successive pairings of *N. furzeri* males and females following a pre-determined mating schedule were undertaken. The 48 experimental fish were divided into eight blocks, each with a set of 3 × 3, male × female factorial crosses. Within each block all three males were crossed with all three females, with three replicates of each spawning. Thus, this design generated 3 replicates of 9 families of maternal and paternal half-siblings, in each of eight blocks, with a total of 72 replicated families in the final combined design (Fig. 1).

To generate crosses, a pair of fish was gently introduced into a 2 L plastic container with a 5 mm layer of fine sand as a spawning substrate. The fish were left to spawn for 2 h and separated. For the subsequent cross each female was assigned to the next male from the same block following a predetermined schedule (Fig.1). After 16 h spawned eggs were sieved from the substrate, counted and the proportion fertilized estimated by inspection under a light microscope following the protocol of Polačik *et al*. (2016). To estimate the duration of embryo development 20 eggs from each clutch were randomly selected and monitored. Although fertilized eggs were obtained from every experimental cross, 13.9% did not yield the target number of 20 eggs. However, only 4.2% of spawnings generated less than 10 eggs. A total of 4,091 fertilized eggs from 216 spawnings were incubated in plastic Petri dishes with a grid of small (80 mm^3) isolated compartments, with a single egg per compartment. Eggs were covered with damp peat (Kera, www.kera.cz) (Polačik *et al*., 2016). This incubation protocol contrasts with a more common method in which eggs are placed on the

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surface of peat (e.g. Genade, 2005; Pinceel *et al*., 2015). Placing the eggs under a layer of incubation media better reflected natural conditions, especially as oxygen availability (Inglima *et al*., 1981; Watters, 2009) and illumination (Markofsky and Matias, 1977; Levels and Denucé, 1988; Furness *et al*., 2015) can affect *Nothobranchius* spp. development; it is known that surface incubation can artificially shorten developmental time (Genade, 2005; Polačik *et al*., 2016). A standard level of moisture and quality of peat in all Petri dishes was ensured by using substrate from a common source prepared before the start of the study. Eggs were reared in a laboratory incubator (Q-Cell, www.pollab.pl) at 25 °C.

The developmental status of every egg was checked visually on 10 occasions over a period of approximately 25 weeks following fertilization (mean = 173 days, SE = 1.16, range 168 – 179 days). Inspections were made through the bottom of the Petri dishes using a focused light source. The position of eggs in the substrate sometimes precluded direct observation. In these cases (approximately 2% of eggs), the incubation substrate was disturbed briefly and the egg inspected directly. A total of 14,512 checks on developing eggs were made with a mean interval 17 days (SE = 0.4) between successive inspections (supplementary Table S1). The first inspection was made 25 days post fertilization (dpf) in all clutches. Two stages of embryo development were scored: (1) *Fully developed* embryos that reached their pre-hatching stage. These were recognized based on full body formation, conspicuous golden eyes and an opaque appearance to the egg (Wourms, 1972; Podrabsky *et al*., 2010; Furness *et al*., 2015; Pinceel *et al*., 2015). (2) *Undeveloped* embryos (diapausing or not) that had not attained the pre-hatching state at a given inspection. Additionally, the presence of dead embryos (white and opaque) was recorded.

Based on the time that post-fertilization eggs were detected at these stages they were assigned as: a) *escape* embryos (rapid developmental rate, *sensu* Wourms, 1972; Podrabsky *et al*., 2010) scored as *fully developed* at the time of first inspection at 25 dpf; b) *intermediate*

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embryos (intermediate developmental rate) scored as *fully developed* at any inspection other than the first; and c) *slow developing* embryos (slow developmental rate) scored as *undeveloped* at the last (10^{th}) inspection (173-179 dpf).

Data analysis

All statistical analyses were performed in the R environment (R Core Development Team, 2015). Mixed-effects Cox proportional hazard models (*coxme* package ver. 2.2-5) were used to test for a temporal effect on embryo developmental time. Replicate order (1 to 3, each lasting 6 days and involving full replication of all possible pairings) was the fixed factor and male, female and pair identity (equalling clutch identity in this analysis) were treated as random effects to control for any genetic effects on embryo developmental time. Qualitatively identical results were obtained when temporal aspects of egg production were modelled using day (9 levels) rather than replicate as the fixed factor (data not shown). A total of 3,007 embryos from 216 pairings were entered in the analysis. All dead embryos were censored from the last day when observed alive. Embryos that died during the first 25 dpf; i.e. that were found dead during the first inspection, were not entered in the analysis of developmental time. All *undeveloped* embryos were censored from the date of their last inspection (168-179 dpf). The contribution of random effects to variation in development time was calculated from the proportion of variance attributed to each random effect. Significance of replicate order (a fixed effect) was estimated with a likelihood ratio test. To examine the temporal source of variation in development time, we additionally tested the proportion of *fully developed* embryos during the first and second checks (25 and approximately 45 dpf, respectively) using proportional tests and a mixed-effects Cox proportional hazard model with left-truncated dataset (including only information after the first and second checks, i.e. after the age of 45 dpf). Individual female consistency in embryo development time among trials was estimated

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with a repeatability score (Lessells & Boag, 1987), measured as the intraclass correlation coefficient (ICC) using the *ICC* package ver. 2.3.0 in the R statistical environment (R Development Core Team, 2015). Repeatability was scored as proportion of total variation in development time attributable to variance among females, which ranged from 0 to 1, with a value close to 1 demonstrating high repeatability. To determine whether repeatability deviated significantly from zero, the fitted model was compared with a null model with female identity missing, using a likelihood ratio test.

Generalized Linear Mixed models (GLMM; *lme4* package ver. 1.1-10) were used to test for temporal effects (fixed effect: replicate, random intercepts: female ID, maleID, pair ID) on the number of spawned eggs and the proportion of fertilized eggs. Poisson and binomial error distributions were used for the number of eggs spawned and fertilization rate respectively. The fit of all models was examined based on examination of residual plots. We note that using random intercepts only (rather than in combination with random slopes) may lead to overconfident estimates of random effects (Schielzeth and Forstmeier, 2009). However, sample sizes were insufficiently large to run models with both random slopes and intercepts. The heritability of embryo development time was analyzed using the *agricolae* package ver. 1.2-3. Using North Carolina Design II, each 3 × 3 factorial block was used to compare effects of sire, dam and their interaction on the duration of embryo development and estimate heritabilities (Lynch and Walsh, 1998).

Results

Egg production, fertilization success and embryo development

The mean total number of eggs produced per female *N. furzeri* during the experiment was 466.2 (SE = 24.6). Within females the mean number of eggs varied among spawning replicates (GLMM with Poisson distribution, $Z = 10.21$, $P < 0.001$, N = 216), being the lowest

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in the first replicate and comparable between the second and third replicate (Fig. 2a). Regarding random effects, most variation in egg number was explained by female ID (74% of the total of 0.075 of total random-effects variance). Male-female combination (pairID) explained 17% (variance 0.017) and male ID explained 10% of the variation assigned to random effects (variance: 0.010).

The mean proportion of fertilized eggs was 85.2% (SE = 2.7). The proportion of fertilized eggs was lower during the first spawning replicate but increased in subsequent replicates (GLMM with binomial distribution, $Z = 9.24$, $P < 0.001$, N = 216, Fig. 2b). Female ID explained much more variation in the random part of the model (variance: 0.404; 84%) than male ID (variance: 0.078; 16%). Male-female combination did not explain any variance (pairID variance <0.0001).

A total of 2,112 (51.6%) embryos completed development during the study; i.e. within approximately 180 days, with 727 (17.8%) still undeveloped at completion of the study. The remaining 1,252 (30.6%) embryos died during incubation. There was high intra-population variability in the length of embryo development, despite standardized laboratory conditions for female rearing, egg production and embryo development. Some females tended to produce mostly embryos that developed rapidly while others produced a high proportion of slowly developing embryos. Females with a balanced proportion of fast and slow developing embryo types also occurred frequently (Fig. 3).

The same pattern of intra-population variability in the length of embryo development was also apparent within individual females over the course of the experiment. Production of *escape*, *intermediate* and *slow developing* embryos showed a high degree of variability (Fig. 4). Females typically produced a combination of embryo types at varying proportions over the course of the nine successive crosses (Fig. 4a). Some females produced mainly *escape* (Fig. 4b) or *intermediate* to *slow developing* embryos (Fig. 4c). Repeatability of embryo

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development time for individual females was estimated as 0.31 (95% CI 0.19 - 0.44). The hypothesis that this result did not differ from zero was rejected with $P < 0.001$. There was no association between the mean length of embryo development and female body size (Pearson correlation, $t_{22} = 0.02$, $r = 0.004$, $P = 0.980$).

Temporal patterns

Within females the length of embryo development increased across the three successive replicates (mixed-effects Cox proportional hazard model: estimate: -0.44 (SE = 0.028), $Z = 15.33$, $P < 0.001$), with no effect of female size ($Z = 0.08$, $P = 0.940$). The proportion of undeveloped embryos decreased, on average, by a factor of 0.65 in each subsequent replicate.

Completion of development at the last (10^{th}) inspection declined across the three successive replicates (replicate 1: 87%, replicate 2: 74%, replicate 3: 62%; proportional tests, all pairwise comparisons, $P < 0.001$, Fig. 5). Hence, the embryos from later laid eggs took longer to develop, in support of the *Maternal Effects Hypothesis*. Differences among replicates were apparent as soon as at the first inspection (25 dpf) (proportion of developed embryos; replicate 1: 33%, replicate 2: 23%, replicate 3: 16%; proportional test, all pairwise tests, $P < 0.001$), with a further increase in differences at 45 dpf (proportion of developed embryos; replicate 1: 71%, replicate 2: 55%, replicate 3: 34%). There was no significant difference in the length of embryo development for a left-truncated subset of eggs that developed later than 45 dpf (3^{rd} – 10^{th} check) (mixed-effects Cox proportional hazard model: estimate: 0.036 (SE = 0.068), $Z = 0.53$, $P = 0.590$, Fig. 5). In contrast, the difference was highly significant for a subset including the first 45 days (1^{st} – 2^{nd} check). This outcome indicates that most of the variation in duration of embryo development was expressed in the course of the first and second month of development.

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Heritability

We found no support for the *Adaptive Polyandry Hypothesis*. There were highly significant additive female effects on the duration of embryo development, with approximately 27% of variation in development time explained by female identity (Table 1a). There were also significant female effects on the number of eggs laid, with 19% of variation explained (Table 1b) and egg fertilization success, with 12% of variation explained (Table 1c). No additive male effects on these traits were detected (Table 1) and there was no evidence for a non-additive male × female interaction (Table 1). The results of the heritability analysis were congruent with the random component of the model of temporal aspects of egg production, fertilization success and embryo development.

Discussion

We demonstrated significant maternally mediated intra-population and intra-individual variability in the duration of embryo development in *N. furzeri*. Parental influence on embryo developmental duration, number of eggs and fertilization success was asymmetric, with no significant additive or non-additive paternal effects and with all additive variance in these traits attributed solely to female identity. These findings provide no support for the *Adaptive Polyandry Hypothesis*. Instead our data provide support for maternal control of embryo development in *N. furzeri*; i.e. the *Maternal Effects Hypothesis*. The duration of embryo development increased steadily over the course of the experiment, implying a strong non-genetic maternal effect, with most variation confined to the first 45 days of embryo development. Interestingly, females varied in their propensity to produce embryos with short or long duration of the embryo development.

The expression of all possible developmental scenarios under constant environmental conditions points to the existence of a diversified bet-hedging strategy in *N. furzeri* (*sensu*

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Wourms, 1972; Furness *et al*., 2015; Pinceel *et al*., 2015), though additional lines of evidence are needed for its unambiguous confirmation (Simons, 2011). Diversified bet-hedging through the production of phenotypically variable offspring is a functional life-history strategy in fluctuating environments where sources of mortality are stochastic and condition independent, and where there are no reliable cues to predict environmental conditions (Pigliucci, 2001; Childs *et al*., 2010). In *N. furzeri*, non-overlapping generations and highly unpredictable future habitat states provide the environment predicted to favour the evolution of diversified bet-hedging (Starrfelt and Kokko, 2012; Crowley *et al*., 2016).

A key finding of the present study was that the mean duration of embryo development increased over the course of the experiment. This result implies a non-genetic (cytoplasmic or otherwise) rather than genetic source of developmental variability. Maternal effects are known to influence the duration of embryo development in several taxa with diapausing stages (e.g. rotifers: Martinez-Ruiz and Garcia-Roger, 2015; crustaceans: Van Dooren and Brendonck, 1998; Allen, 2010; insects: Mousseau and Dingle, 1991; Lacour *et al*., 2014). Maternal effects in the form of age-related differential maternal provisioning, changes in maternal hormonal levels and/or other regulatory substances such as miRNA or cytoplasmic factors have been proposed as modulating developmental trajectory in terms of facultative onset or omission of diapause in the Neotropical annual fish *Austrofundulus limnaeus* (Podrabsky *et al*., 2010). In contrast to genetic control with high phenotypic expression and heritability, control of traits under natural selection by a process that does not affect DNA sequence prevents standing genetic diversity from potentially detrimental effects of selection if the environment changes (Kawecki, 2000). In our study all mothers were of the same age and spawned at the same time. To circumvent any possibility that developmental time was under offspring control, an asynchronous experiment, with females laying their clutches at different times would be required. Given that such a design would compromise our condition

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for a common environment, a separate experiment will be needed to test the interaction of laying order with maternal effects on embryo development. Therefore, on the basis of the results of the present study we cannot unambiguously conclude that mothers solely dictated the developmental time of their offspring, and the observed shift in the duration of development may be under the control of the embryo.

The difference in the duration of embryo development among replicates was predominantly expressed in the first two months of incubation. At 25 °C *Nothobranchius* spp. embryos either develop directly as escape embryos without any developmental arrest within 17-21 dpf or, in the case of a longer period of development, predictably enter a diapause (Wourms, 1972; Podrabsky, 2010; Blažek *et al*., 2013; Furness *et al*., 2015, Polačik *et al*., 2016). The incubation period during which we detected differences between subsequent experimental replicates extended beyond the point required for direct development (Blažek *et al*., 2013; Polačik *et al*., 2016). An implication is that the maternal effects influencing embryo development in *N. furzeri* not only determine entry or skip of a developmental arrest (Podrabsky *et al*., 2010), but may also regulate the duration of the dormant phase.

While early pairings yielded embryos with a relatively shorter duration of embryo development, the embryos produced in later experimental pairings took longer to develop. Maternally manipulated change in the duration of embryo development that correlates with maternal age may have an adaptive basis. Older females have also been reported to produce embryos with relatively longer embryo development in a diapausing cricket *Ephippiger ephippiger* (Hockham *et al*., 2001) and the annual fish *A. limnaeus* (Podrabsky *et al*., 2010). In each case the observed shift in the duration of development was attributed to age-related differential maternal provisioning and explained as an adaptive response to the likelihood of life cycle completion (Hockham *et al*., 2001; Podrabsky *et al*., 2010). Thus in *N. furzeri*, young females may benefit from producing rapidly developing embryos early in the

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reproductive season as these may complete their entire life cycle in the same rainy season. Rapid development can permit offspring to exploit secondary pools that sometimes develop after the original killifish pool desiccates but subsequently re-fills (Polačik *et al*., 2014). Multiple generations are thereby completed in the course of a single reproductive season. Production of offspring that develop at different rates, with the effect of spreading their hatching times, might also limit sibling rivalry for limiting resources (Mock and Parker, 1998).

Alternatively, the age-related shift in development time may be non-adaptive and arise from a physiological constraint, stemming from a progressive decrease in maternal resources. For example, younger females of the rotifer *Brachionus plicatilis* had higher energy reserves and produced offspring with relatively longer development compared to their later produced offspring. In this case, developmental duration depended on the laying order (Martinez-Ruiz and Garcia-Roger, 2015). While our results are consistent with Hockham *et al*. (2001) and Podrabsky *et al*. (2010), in that female age correlated positively with the duration of embryo development, our data also permit the alternative explanation that developmental trajectory involved a mechanism based on laying order *sensu* Martinez-Ruiz and Garcia-Roger (2015), rather than maternal chronological age. In the present study all the eggs were collected within a period of 17 days, representing only approximately 12% of the reproductive lifespan in *N. furzeri* (Terzibasi *et al*., 2008, Blažek *et al*., 2017, but see also Valdesalici and Cellerino, 2003). In contrast, Podrabsky *et al*. (2010) observed a shift in production from rapid to slow-developing embryos over the full reproductive lifespan of the relatively longer-lived *A. limnaeus* (approximately 60 weeks vs. 23 weeks in *N. furzeri*; Podrabsky, 1999, Terzibasi *et al*., 2008; Podrabsky *et al*., 2010). The modest age increment associated with a significant change in the duration of embryo development in the present study implies that maternal age may not alone be the primary factor regulating embryo developmental pathway.

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A more parsimonious interpretation of this finding is that *N. furzeri* females spread the risk of offspring failure by switching from rapid to slow-developing embryos once a threshold number of fast-developing embryos has been produced. While the sensitivity of the 'endogenous counter' may vary among females (Figs 3 and 4, significant ICC), its existence is clearly apparent at the population level (Fig. 5). Although this mechanism does not mitigate future environmental uncertainty, it is adaptive in acting as an internal cue that reliably spreads the risk of offspring mortality on a biologically meaningful temporal scale; i.e. from the onset to cessation of spawning rather than as a function of female longevity, which may not be realized and is less sensitive to environmental change than the physiological processes associated with oogenesis (Wootton and Smith, 2015). Notably, Sprenger *et al*. (2010) suggested that even the number of mating partners can represent a social cue that triggers females to alter offspring phenotype (egg size) in the marine gastropod *Chelidonura sandrana*. Hence we propose that the endogenous counter may similarly operate as a functional switch point for modulating adaptive progeny phenotype.

We detected no significant additive paternal contribution to the number of eggs produced, nor was there a non-additive male x female interaction effect. Sexual reproduction allows natural selection to proceed more effectively because it increases genetic variation (Goddard *et al*., 2005). As such, both male and female parents are expected to contribute to offspring fitness (e.g. Moshgani and Van Dooren, 2011). Female reproductive investment in terms of egg number and quality is plastic, and females are predicted to allocate their resources according to the perceived 'quality' of their mating partners (e.g. fish: Kolm, 2001; frogs: Reyer *et al*., 1999; birds: Horváthová *et al*., 2012). Male 'quality' and its fitness consequences are often contingent on environmental context (e.g. Kasumovic and Andrade, 2009; Teyssier *et al*., 2014). In *Nothobranchius* spp. habitat characteristics can vary greatly across and within seasons and the lack of an additive or non-additive male effect on egg

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number has been a consistent result across several studies on these fishes (Haas, 1976a,b; Polačik and Reichard, 2009; Reichard and Polačik, 2010), including the present one. The unpredictable environment of temporary pools makes the fitness value of male traits uncertain. Intense male coercion during mating, combined with a mating system that involves daily multiple spawning events, means that female mate choice is consistently undermined (Polačik and Reichard, 2011) and it is notable that when female *Nothobranchius* spp. are permitted to select mating partners they mate indiscriminately, at least in the laboratory (Haas, 1976b; Polačik and Reichard, 2009; Reichard and Polačik, 2010, Polačik & Reichard, 2011). Thus, in unpredictable habitats strong mate choice may not bring any benefits and relaxed female mate choice is a viable alternative reproductive strategy (Sprenger *et al*., 2010).

A surprising finding of the present study was that variability in fertilization success was an exclusively female effect. We predicted males to have a substantial impact on fertilization rate through the quality and number of spermatozoa released (Bobe and Labbé, 2010; Caspers *et al*., 2014). A significant female effect on fertilization rate can be explained by consistent differences in egg quality among females (Evans *et al*., 2007). Both fertilization success and number of eggs were relatively lower in the first replicate compared to the second and third. This effect is potentially an experimental artefact arising from the forced separation of the sexes before the first experimental spawning (see Methods). This separation can have the effect of decreasing egg number and quality due to the retention of the eggs in the ovary, which become overripe (Fauvel *et al*., 1992; de Gaudemar and Beall, 1998; Azuma *et al*., 2003). However, this effect did not undermine our overall conclusion relating to the duration of embryo development as the effect of an endogenous counter was consistent well beyond the initial pairings (Fig. 4).

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Developmental bet hedging may function as a mechanism for the long-term persistence of *Nothobranchius* spp. populations in the unpredictable temporary pools that they occupy. A highly variable range of offspring phenotypes is needed since the habitat conditions experienced by the parents have limited relevance to their offspring. We demonstrate an unusually high degree of intra-population, but also intra-individual variability in the duration of embryo development of *N. furzeri*. We detected no evidence for the *Adaptive Polyandry Hypothesis* since there was no male genetic contribution to variation in embryo development. Instead, the source of variance was wholly maternal, supporting the *Maternal Effects Hypothesis*. Females possibly affected the duration of embryo development, as suggested by a modal shift in embryo developmental duration over the course of the experiment. Feedback on the laying order of an egg obtained through an endogenous counter may increase the likelihood that some embryos achieve a match with their environment, and may additionally spread mortality risk. The absence of a significant male effect on the number of eggs spawned by females implies limited benefits of female mate choice for this trait, with indiscriminate mating possibly representing an additional form of reproductive bet-hedging that maximizes offspring diversity.

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Table 1. Parental sources of variability for duration of embryo development (a), egg number (b) and fertilization success (c). The experiment involved North Carolina Type II breeding design and data were analysed using *agricolae* package (nested factorial ANOVA). Variables in **bold** were statistically significant.

Source	df	SS	MS	F	P	Variance	%
a) Duration of embryo development							
Female	16	121357	7585	8.86	<0.001	406.3	27
Male	16	16439	1027	1.2	0.32	0	0
Male x Female	32	27407	856	0.79	0.779	0	0
Error	140	151833	1085			1084.5	73
b) Egg number							
Female	16	26884	1680	5.78	<0.001	82.6	19
Male	16	5012	313	1.08	0.413	0	0
Male x Female	32	9305	291	0.81	0.753	0	0
Error	144	51686	359			358.9	81
c) Proportion of fertilized eggs							
Female	16	3.37	0.21	5.45	<0.001	0.009	12
Male	16	0.72	0.04	1.16	0.345	0	0
Male x Female	32	1.24	0.04	0.58	0.964	0	0
Error	144	9.63	0.07			0.067	88

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Figure legends

Fig. 1. Pairing order scheme for a single experimental group (one block out of eight). Within the group, every male was paired with every female with each pairing combination replicated three times.

Fig. 2. Mean (SE) number of eggs (A) and fertilization rate (B) over the course of the experiment in the three experimental replicates.

Fig. 3. Intra-population variability in *N. furzeri* embryo development within the experimental population. Escape embryos – scored as *fully developed* at the time of first inspection at 25 dpf; intermediate embryos – entered a diapause but completed development before the end of the experiment; slow embryos – did not complete their development by the end of the experiment.

Fig. 4. Examples of female intra-individual consistency and variability in embryo development in *N. furzeri*. Each graph shows nine pairings (crosses) of a single female. For description of column shading see Fig. 3. Most of the females produced a range of developmental categories at varying proportions throughout their nine crosses (A). Specific females consistently produced predominantly escape (B) or slow-developing (C) embryos.

Fig. 5. Shift in the duration of *N. furzeri* embryo development over the course of the experiment. Note that the differences among replicates arise from the developmental trajectories within the first 45 dpf and no further increase occurs from the age 45 dpf onwards.

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Supplementary figure

Fig. S1. Data from a thermal data logger showing multiple inundations (arrows) in a *Nothobranchius* pool in Mozambique. Diurnal thermal amplitude is higher when the pool is empty and it declines when the pool is inundated.

Fig. 1

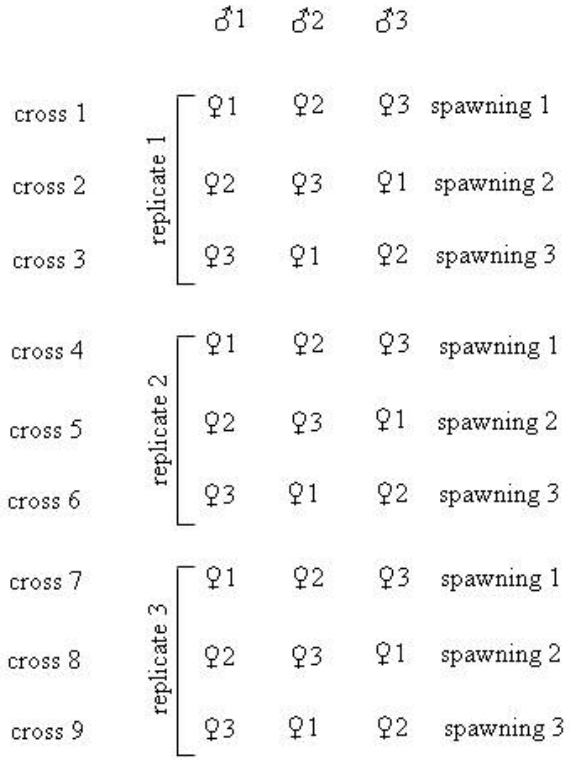

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Fig. 2

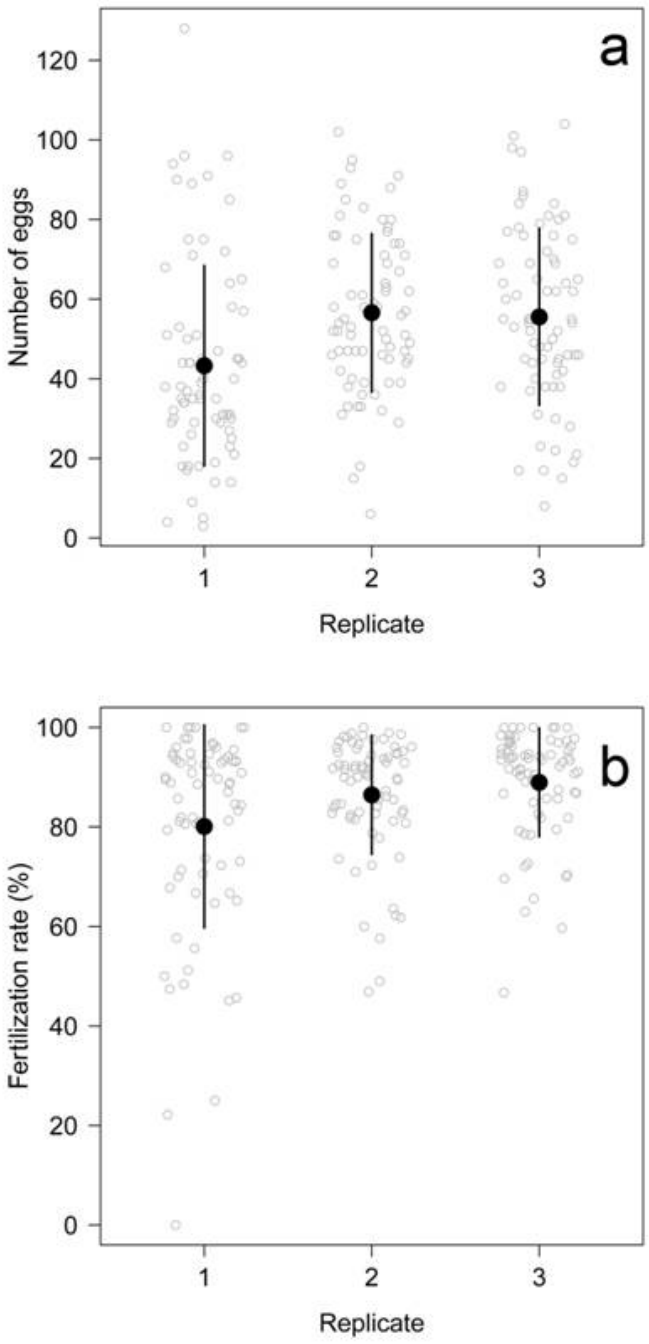

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Fig.3

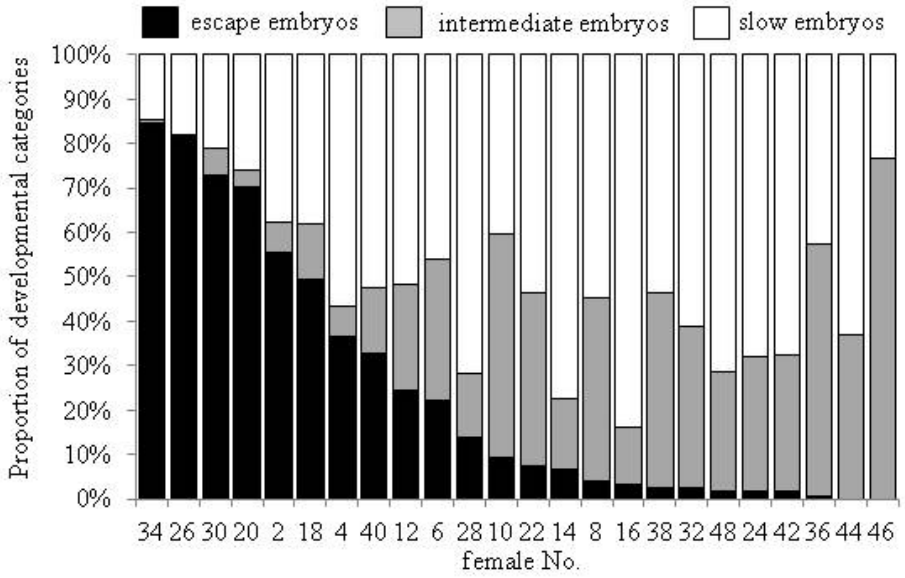

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Fig.4

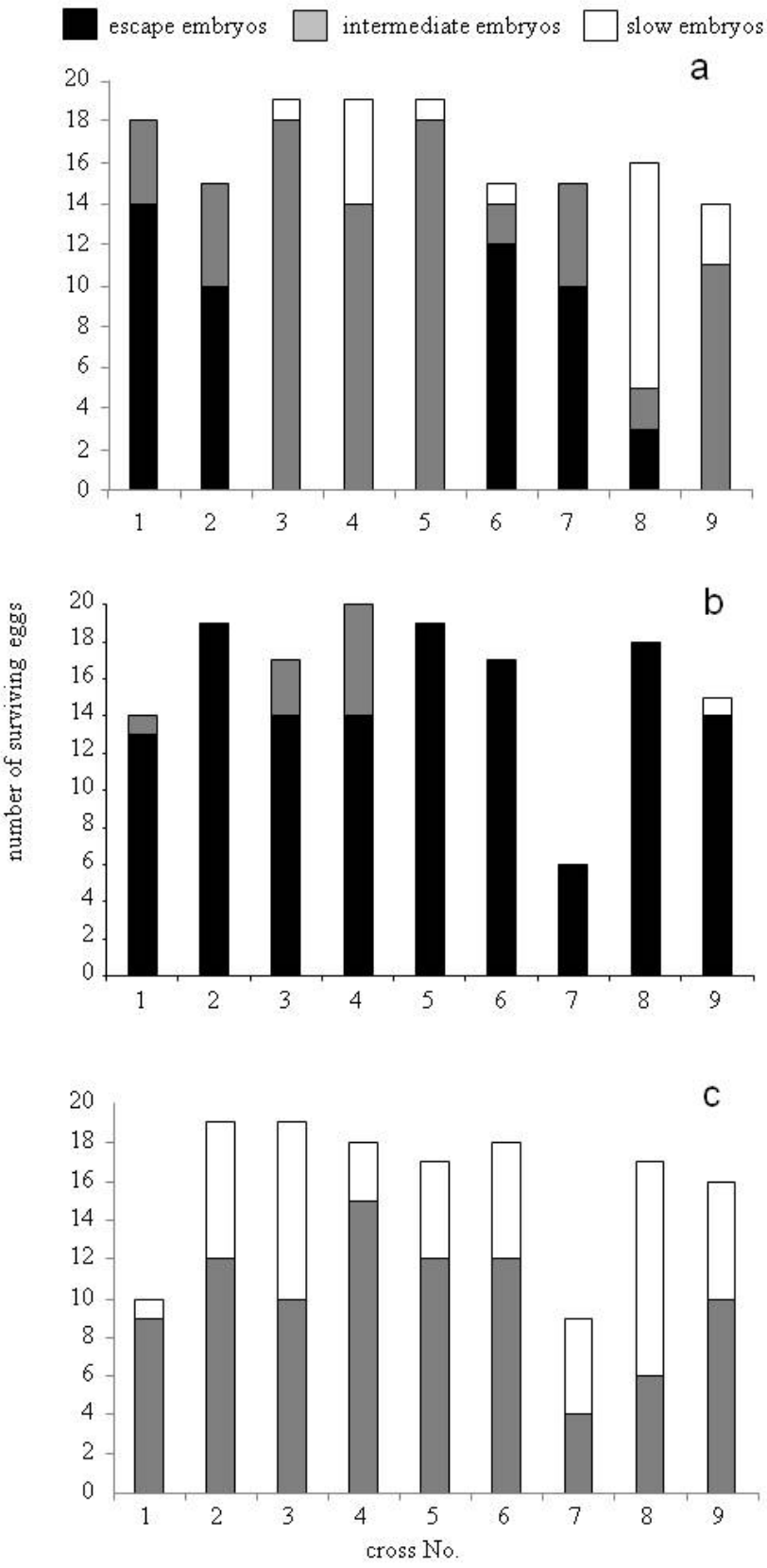

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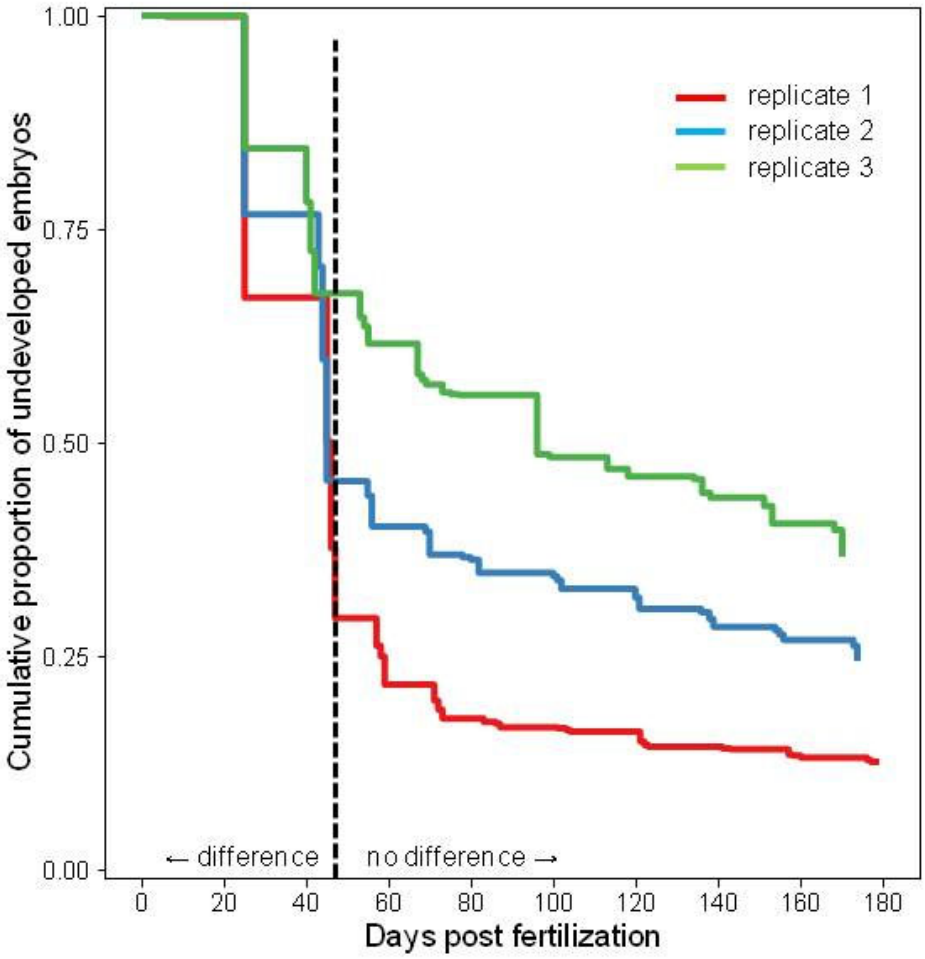
Fig. 5



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