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RESEARCH ARTICLE

The Role of Energetic Reserves During Embryonic Development of an Annual Killifish

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Background: Females can significantly improve their fitness by utilizing a range of maternal effects. Embryos of annual killifish survive the dry season in ephemeral pools encased in dry substrate for several months. Here, we experimentally test the association between energetic provisioning and maternally controlled duration of embryonic development in the African annual killifish *Nothobranchius furzeri* (Cyprinodontiformes). Results: We found that embryonic energetic reserves do not limit duration of development. However, differences in energetic reserves affect the size at which embryos hatched, with larger yolk size resulting in larger hatchling size. Conclusions: These findings suggest uncoupling of the two traits examined (i.e., embryonic energetic reserves and development duration) and emphasize the strong buffering role of diapause in the energetic balance of embryonic development in the annual killifish. *Developmental Dynamics 246:838–847, 2017*. © 2017 Wiley Periodicals, Inc.

Key words: *Nothobranchius furzeri*; egg size; hatching; maternal effects; development duration

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Introduction

Maternal effects entail nongenetic transfer of information from the mother to her offspring (Mousseau and Fox, 1998). The particular character and magnitude of such maternal effects often differ among individual females (Green, 2008) and may change with a female's age (Berkeley et al., 2004). Maternal effects are frequently directed toward increasing offspring fitness, and thereby fitness of the female (Marshall and Uller, 2007). The mechanisms engaged in maternal effects are diverse and can include regulatory chemical substances (Giesing et al., 2011), variable nutrient provisioning (Berkeley et al., 2004), and behavioral components (Smith et al., 2000; Warner et al., 2010).

One of the most studied of maternal effects is female reproductive resource allocation as expressed through egg size (Bernardo, 1996; Fox and Czesak, 2000). Large eggs are advantageous under a whole array of conditions (Einum and Fleming, 1999; Gagliano et al., 2007; see Régnier et al., 2013) and are generally favored under natural selection. As an example, egg size is typically positively correlated with hatchling size (Green, 2008; Krist, 2011), a trait that has a crucial impact on offspring survival (Kaplan, 1992; Mitchell et al., 2014). Abundant energetic reserves may also significantly improve survival of embryos per se, especially when development is prolonged (hatching is postponed) and additional energy is subsequently required (Martin, 1999).

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Diapause is a special case of dormancy, developmental arrest controlled by interplay of endogenous and environmental cues (Podrabsky and Hand, 2015). Diapausing stages are resistant to harsh environments (Schiesari and O'Connor, 2013), and females normally produce diapausing offspring in anticipation of adverse conditions, such as the onset of winter (Bradford and Roff, 1993). In terms of embryonic diapause, females may produce a range of offspring phenotypes (even within a single clutch), including non-diapausing individuals and diapausing individuals with variable diapause duration. Such a strategy ensures that at least part of the offspring will match upcoming conditions. Diversification in developmental duration ultimately results in a "germ bank," a reservoir of embryos constantly containing a proportion of ready-to-hatch individuals (Evans and Dennehy, 2005; García-Roger et al., 2014).

Variable duration of embryonic development is a crucial feature in the life history of annual killifish (genera of the families Nothobranchiidae and Rivulidae; Cyprinodontiformes) adapted to temporary pools (Berois et al., 2015). These fishes hatch at the start of a rainy season, mature rapidly, lay drought-resistant eggs, and die when the pool desiccates; only the embryos surviving the extended dry period in the bottom substrate (Wildekamp, 2004; Cellerino et al., 2016). The dry period regularly spans several months, and the only energy available to the annual killifish embryos during this time is stored in their yolk. The extended embryonic period is facilitated through a series of three facultative developmental arrests known as Diapause I, II and III (Wourms, 1972).

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TABLE 1. Summary of two spawning sessions (Age) for two experimental populations, with the complete number of spawned eggs subsequently selected for incubation, embryo survival and hatching success given. Note that hatching success is calculated only for embryos that completed development during the experiment (n = 965). We also present the proportion of long-developers that completed development during the experiment (Long-developers I; n = 965) and compare it with data including undeveloped embryos (Long-developers II; n = 999)

Population	Age	Females	Males	Spawned	Incubated	Survival	Long-developers I	Long-developers II	Hatched
414	young	16	9	1434	415	249 (60%)	22 (8.8%)	22 (8.8%)	171 (68.7%)
	old	12	9	2392	459	289 (63 %)	23 (8%)	26 (9%)	219 (75.7%)
422	young	15	9	1547	409	218 (53.3%)	39 (17.9%)	43 (19.7%)	176 (80.7%)
	old	12	9	2339	489	208 (42.5%)	12 (5.8%)	39 (18.8%)	181 (87%)
			SUM	7712	1772	965	96	130	747

Diapause I presumably occurs before the pool desiccates, after the embryo finishes epiboly, at which point it is still represented by dispersed undifferentiated cells (Wourms, 1972). Under laboratory conditions, however, embryos generally skip Diapause I (Wourms, 1972). Diapause II commences in the early stages of morphogenesis, when the embryo possesses a rudimentary nervous system and a beating heart (Wourms, 1972). Metabolic energetic demands during Diapause II are significantly reduced (~90%) compared to pre-diapause O_2 consumption (Podrabsky and Hand, 1999). Embryos in Diapause III have a considerably higher metabolic rate (approximately 15–20×) than those in Diapause II (Podrabsky and Hand, 1999; Furness et al., 2015a), as they enter Diapause III just prior to hatching and are fully developed (Wourms, 1972; Podrabsky and Hand, 1999). Diapause II, therefore, represents the major physiological adaptation of annual killifish for saving energy through the extended desiccated period. The timing of entrance and time spent in Diapause II depends on both environmental conditions (Levels and Denucé, 1988; Furness et al., 2015b) and maternal effects (Podrabsky et al., 2010; Polačik et al., 2017). Regardless of incubation conditions, there will be embryos within a single clutch that skip Diapause II and develop directly (within approximately 3 weeks), and others that enter Diapause II for variable periods lasting up to several years (Wourms, 1972). Such a bet-hedging strategy is important due to the largely unpredictable onset, intensity, and duration of the rainy season in their natural environment (Furness et al., 2015b; Cellerino et al., 2016; Polačik et al., 2017). It is currently unknown whether embryos with abundant energetic reserves enter Diapause II preferentially and what effect the energetic reserves have on survival of annual killifish embryos during extended incubation. Interestingly, embryos entering Diapause II have been shown to hatch larger and with smaller residual yolk than those developing directly (Polačik et al., 2014), though no record of their initial energetic reserves is available.

In this study, we focus on the association between embryonic energetic reserves, survival, duration of development, and hatchling size using a common garden experiment on embryos of an annual killifish *Nothobranchius furzeri* Jubb. This species lives at the southern margin of *Nothobranchius* spp. distribution in Africa, a region where onset and duration of the rainy season are hard to predict (Reichard et al., 2017). Consequently, *N. furzeri* embryos display naturally erratic development (e.g. Polačik et al., 2014; Furness et al., 2015b). Here, our principal aim is to test the effect of energetic reserves on embryonic survival and development duration. We hypothesize that large energetic reserves are necessary for survival during extended embryonic development in order to reach the prehatching stage (Diapause III), and that there is a trade-off between energy spent before the prehatching stage is reached and hatchling size or their residual energetic reserves. In other words, we assessed how total energetic budget (yolk) is partitioned into development and growth and survival.

Specifically, we predict that embryos with small energetic reserves going through an extended embryonic period will have a lower survival than those with large energetic reserves, as their energetic reserves will be depleted before the prehatching stage. In contrast, we do not expect any difference in survival between embryos with small and large energetic reserves following the short embryonic pathway. We further predict that embryos with greater energetic reserves will hatch with a larger body size. For a given amount of energetic reserves, embryos with long development should hatch smaller, or with smaller residual energetic reserves, than embryos with short development due to depletion of energetic reserves during the extended embryonic period.

Results

General Patterns in Embryonic Development and Hatching Success

The 12-month inspection period was sufficiently long to record complete data on the fate of 98.1% of the 1,772 embryos incubated. By the end of the experiment, 54.5% of all embryos had completed their development, the percentage of undeveloped embryos being only 1.9% (excluded from analysis, see Experimental procedures). The remaining embryos (43.6%) died during the experiment.

The duration of embryonic development showed a clear bimodal pattern. The majority (90.1%) of embryos that finished development within the experimental period were short-developing embryos (or short-developers), having developed over the first 45 days postfertilization (dpf). Development virtually ceased over the next 140 days, and only five embryos finished development between 45 and 185 dpf. Resumed development was more protracted, with long-developing embryos (long-developers; 8% of all embryos) completing development over the next 165 days (185–350 dpf).

Successful hatching was achieved in 77.4% of all developed embryos. Hatching rate did not differ between short- (77.4%) and long-developers (77.1%). See Table 1 for a general summary of the experiment.

TABLE 2. Yolk sac size (n = 1772). Coefficient estimates (Est.), standard errors (SE), and corresponding 95% confidence intervals (CI) for fixed effects on yolk sac size are derived from model averaging. Terms with 95% CI not overlapping 0 are emphasized in bold. For a full version of the variable abbreviations, see the legend in Table 6

Term	Est.	SE	95% CI	z-score
Intercept	0.854	0.010	(0.834, 0.874)	84.978
AGE/old	0.005	0.008	(-0.011, 0.021)	0.656
POP/422	**-0.049**	**0.012**	**(-0.073, -0.025)**	**4.024**
Female size	−0.005	0.004	(-0.014, 0.004)	1.146
Fecundity	0.000	0.002	(-0.005, 0.004)	0.176
POP/422×AGE/old	−0.007	0.006	(-0.019, 0.004)	1.212
Fecundity × POP/422	0.000	0.001	(-0.002, 0.002)	0.083

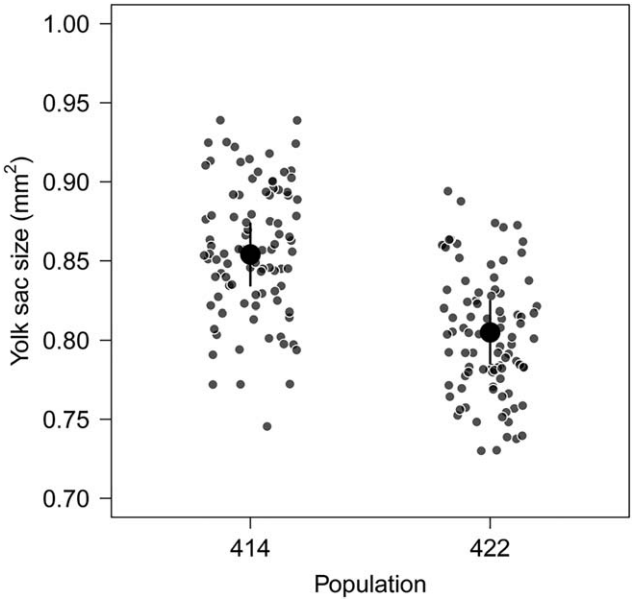

Fig. 1. Yolk sac size variation in the two study populations. The filled black points denote the population mean, and the vertical lines represent the 95% confidence intervals. Gray points indicate mean yolk sac size for individual females per spawning replicate.

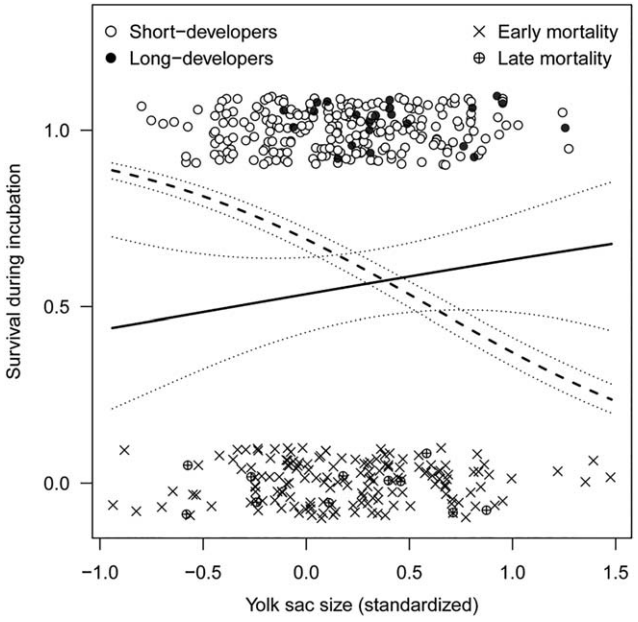

Fig. 2. Survival during incubation. Survival in the early incubation phase (dashed line) decreased strongly with increasing yolk sac size, while the relationship between survival and energetic reserves was weak in the advanced incubation phase (solid line). The empty and filled points denote raw values for short-developers and long-developers, respectively. The crosses and crossed circles show embryos that died in the early or advanced phase of incubation, respectively. Note that the plot shows the relationship for young females of population 414 only, as the trend lines for older females and for population 422 differed only very slightly in their intercepts.

Maternal Provisioning and the Effect of Energetic Reserves on Survival and Hatchling Size

Our data showed an interpopulation difference in maternal provisioning, with population 414 females providing their offspring with more energy via a larger yolk sac size (mean: 0.854 vs. 0.805 mm^2, 95% confidence intervals [CI] [0.834, 0.874] vs. [0.785, 0.825]; Table 2, Fig. 1). Female identity explained 39.8% (SD = 0.033) of yolk sac size random-effect variation, while variation between spawning replicates accounted for 4.8% (SD = 0.011). Embryos from population 414 also show a higher overall survival than population 422 (62% vs. 49%; Fig. 2, Table 3).

The effect of maternal provisioning on embryo survival differed between incubation phases (m3 with Akaike weight = 0.952). Large yolk sac size was associated with higher mortality during the early incubation phase (effect estimate from m3 model: -1.331, 95% CI [-1.665, -1.007]) but had no effect during the advanced incubation phase (effect estimate from m3 model: 0.406, 95% CI [-0.529, 1.341]; Fig. 2, Table 3).

In general, larger yolk sac size resulted in larger hatchling size, and there was no difference between long-developers (effect estimate from m3a model: 4.187, 95% CI [4.134, 4.241]) and short-developers in hatchling size (effect estimate from m3a model: 4.159, 95% CI [4.117, 4.200]) when controlling for maternal provisioning and residual energetic reserves (yolk sac size and residual yolk sac size; Table 4, Fig. 3). Note that the effect estimates given here and in Tables 3 and 4 differ, as the tabulated summary output gives contrasts of different groups from the reference, while we specifically calculated group estimates.

Effect of Maternal Age, Provisioning, and Female Identity on Duration of Embryonic Development

The effects of both maternal age and yolk sac size on development duration varied between populations. Maternal age influenced development duration only in population 422, with young

TABLE 3. Survival during incubation (n = 1738). Coefficient estimates (Est.), standard errors (SE), and corresponding 95% confidence intervals (CI) for fixed effects (logit scale) on survival during incubation are derived from the best candidate model (m3, Akaike weight = 0.952). Terms with 95% CI not overlapping 0 are emphasized in bold. For a full version of the variable abbreviations, see the legend in Table 6

Term	Est.	SE	95% CI	z-score
Intercept	**0.803**	**0.276**	**(0.262, 1.344)**	**2.913**
INC/advanced	−0.662	0.408	(-1.462, 0.138)	−1.622
Yolk	**-1.331**	**0.168**	**(-1.660, -1.002)**	**-7.905**
POP/422	**-1.148**	**0.338**	**(-1.810, -0.486)**	**-3.392**
AGE/old	−0.153	0.208	(-0.561, 0.255)	−0.735
INC/advanced×yolk	**1.736**	**0.484**	**(0.787, 2.685)**	**3.590**
INC/advanced×POP/422	**1.572**	**0.534**	**(0.525, 2.619)**	**2.944**

TABLE 4. Hatchling size (n = 747). Coefficient estimates (Est.), standard errors (SE), and corresponding 95% confidence intervals (CI) for fixed effects on hatchling size are derived from model averaging. Terms with 95% CI not overlapping 0 are emphasized in bold. For a full version of the variable abbreviations, see the legend in Table 6

Term	Est.	SE	95% CI	z-score
Intercept	4.159	0.021	(4.117, 4.201)	193.916
Yolk	**0.124**	**0.014**	**(0.096, 0.153)**	**8.573**
Resyolk	0.029	0.018	(-0.006, 0.065)	1.624
DUR/long	**0.024**	**0.012**	**(0.001, 0.047)**	**2.014**
POP/422	0.020	0.022	(-0.024, 0.064)	0.874
DUR/long×yolk	0.002	0.011	(-0.019, 0.023)	0.187
DUR/long×resyolk	0.065	0.036	(-0.005, 0.135)	1.819

females producing a larger proportion of long-developers than old females. No age-linked effect was observed in population 414 (Table 5). Large yolk sac size was associated with short development duration in population 414, but no such relationship was observed in population 422 (Table 5).

Individual females produced short- and long-developers at variable proportions in both study populations. We observed a considerable level of variation in development duration among individual females, comparable to the main effect of population (female identity SD in model m3 = 3.389 compared to the effect of different population in young females in m3 = 4.418).

Discussion

We demonstrated that whereas low energetic reserves did not reduce survival until the prehatching stage in embryos with extended development, initial embryonic mortality increased with high energetic reserves. There was no positive relationship between duration of embryonic development and the amount of energetic reserves. Development was shorter in embryos with larger yolk sacs, but only in one of the two populations. Initial energetic reserves were important for embryo size at hatching. We showed that extended embryonic development in annual killifish is not costly in terms of smaller hatchling size.

No Effect of Energetic Reserves on Long-term Embryonic Survival or Duration of Development

Though we predicted that long-term embryonic survival would be enhanced by higher energetic reserves, we observed no effect of yolk sac size on survival during the advanced incubation phase. Similarly, we found no association between yolk sac size and duration of development. Hence, yolk reserves do not appear to be a physiological constraint for long-term survival in annual killifish embryos, despite representing the only source of energy during several months when the embryo may be encased in dry bottom substrate. Available information suggests that Diapause II and Diapause III (probably also Diapause I due to its occurrence in the early developmental stage) are accompanied by severe metabolic restriction (Levels et al., 1986; Podrabsky and Hand, 1999; Furness et al., 2015b), with metabolism decreased by 90% during Diapause II and by 84% in Diapause III compared to the respective pre-diapause stages (Podrabsky and Hand, 1999). Such metabolic efficiency clearly has the potential to allow for considerable plasticity in the duration of embryonic development. The results of our study suggest that such plasticity is largely independent of available energetic reserves.

The major part of the yolk reserve is spent between the end of Diapause II and the onset of Diapause III (the prehatching stage) (Podrabsky and Hand, 1999). Given the low proportion of remaining reserves, however, survival at Diapause III will be time-limited (approximately 120 days; Wourms, 1972). This could have resulted in a potential caveat in our experiment. Embryo inspections were scheduled for every seven days, and all developed embryos were removed for hatching long before low yolk reserves could have compromised survival. Diapause I is thought to occur when embryos are still in water, embryonic development being inhibited by presence of adult fish or anoxic conditions in the bottom substrate (Wourms, 1972, Levels and Denucé, 1988). As such, our incubation method (placing eggs on top of damp

substrate) may have prevented embryos from entering Diapause I. Consequently, the decoupling between maternal provisioning, embryo survival, and duration of their development is largely explained by the duration of Diapause II, the most common and perhaps the most important dormant stage in the embryonic development of African annual killifish.

Large Hatchlings From Eggs with Large Energetic Reserves

We demonstrated that embryos with large yolk sac were larger upon hatching. Female investment into large eggs commonly results in larger offspring (Duarte and Alcaraz, 1989; Semlitsch and Gibbons, 1990; Hutchings, 1991; Krist, 2011). Adaptive interpretations of such a maternal effect entail superior viability of large offspring (Marshall and Keough, 2008; Warner and Lovern, 2014), enhanced avoidance of predators (Janzen, 2000), an ability to exploit larger prey (Krebs and Turingan, 2003), earlier maturity (Santo et al., 2001), and larger size at first reproduction (Uller and Olsson, 2010). Annual killifish start feeding exogenously during the very first hours after hatching; hence, direct dependence on posthatch yolk reserves is weak. This contrasts with many other fish, which often start feeding a few days after hatching and obtain energy exclusively from their yolk sac in the meantime (Kamler, 2005). Larger hatchling size decreases gape limitation and allows fish to capture larger variety of prey during the initial phase of natural pool succession (Meintjes, 1996). In *Nothobranchius* spp., increased food intake greatly enhances growth rate (Vrtílek and Reichard, 2016) and, consequently, promotes more rapid maturation (Blažek et al., 2013). This may be crucial for successful reproduction in ephemeral habitats where desiccation risk is imminent. It appears that increased maternal investment per offspring does not result in higher female fitness through increased offspring survival at the embryonic stage. Instead, increased maternal provisioning could improve offspring survival during the early posthatch period.

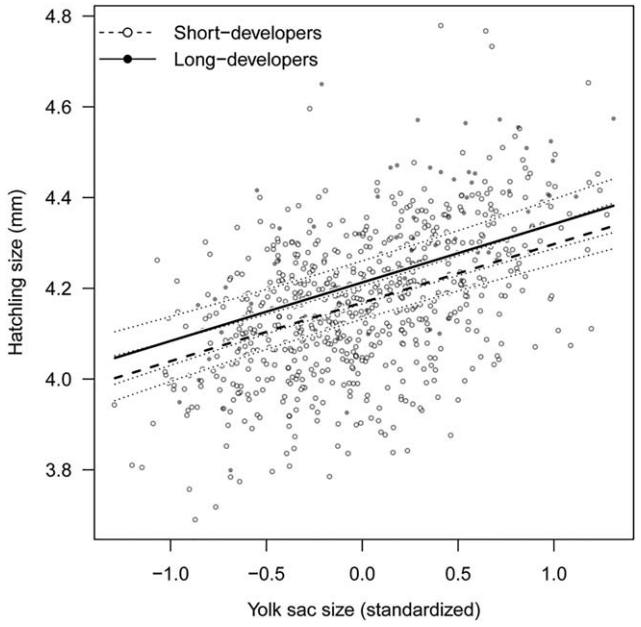

Fig. 3. Hatchling size. The relationship between hatchling size and yolk sac size was generally positive, and long-developers (solid line) hatched relatively larger than short-developers (dashed line) with the same yolk sac size, though their confidence intervals overlap. Points denote raw values for individual hatched embryos, with empty points denoting short-developers and filled points long-developers.

Better provisioned embryos hatched larger, irrespective of whether they were short- or long-developers. This implies that duration of the period that embryos spend inside an egg with limited yolk reserves is unimportant, as the relationship (slope and intercept) between initial amount of yolk (which correlates with egg size) and hatchling size appears to be conserved. Our findings, therefore, tend to substantiate the role of diapause stages during the embryonic development of annual killifish.

An alternative explanation, however, may be that qualitative changes occurred in the yolk without affecting the residual amount of yolk. Although we controlled for quantity of yolk (a commonly used proxy measure of embryonic energetic reserves; Bernardo, 1996) we did not measure qualitative changes in egg yolk composition. Until now, it has been assumed that energetic metabolism during diapause (sole maintenance) and active development (growth, development and maintenance) is most probably dependent on the common pool of phospho- and lipoprotein resources (Podrabsky and Hand, 2015). We suggest that qualitative changes in yolk composition during annual killifish embryonic development warrant further study to better understand the developmental dynamics and energetic balance of diapausing embryos.

Absence of Maternal Age Effect

We observed no effect of maternal age on duration of embryo development. Annual killifish females have previously been

TABLE 5. Duration of embryonic development (n = 965). Coefficient estimates (Est.), adjusted standard errors (SE), and corresponding 95% confidence intervals (CI) for fixed effects on duration of embryonic development (logit scale) are derived from model averaging. Terms with 95% CI not overlapping 0 are emphasized in bold. For a full version of the variable abbreviations, see the legend in Table 6

Term	Est.	SE	95% CI	z-score
Intercept	−7.248	1.759	(-10.696, -3.8)	4.120
Yolk	**-1.934**	**0.892**	**(-3.681, -0.186)**	**2.169**
AGE/old	−0.232	1.047	(-2.283, 1.819)	0.222
POP/422	**4.283**	**1.712**	**(0.928, 7.638)**	**2.503**
Yolk×POP/422	**2.367**	**1.142**	**(0.128, 4.605)**	**2.072**
AGE/old×POP/422	−1.199	0.814	(-2.795, 0.397)	1.472

TABLE 6. Specifications for the *a priori* defined models and the outcome of model selection procedure for all response variables. The best model was selected using Akaike Information Criterion for small sample-size (AICc). Strength of support for the best model is expressed as relative change in AICc (ΔAICc) and Akaike weight (Weight). R^2_{GLMM} shows the amount of variability explained by the fixed part only (R^2_{GLMMm}) and when also accounting for random effects (R^2_{GLMMc}) (Nakagawa and Schielzeth 2013)

a)

Model	Fixed effects	AICc	ΔAICc	Weight	R^2_{GLMMm}	R^2_{GLMMc}
m2b	yolk ~ AGE + POP + female size + fecundity + POP×AGE	−1409.2	0.0	0.678	0.212	0.548
m0a	yolk ~ POP	−1407.2	2.0	0.246	0.203	0.545
m1	yolk ~ AGE + POP + female size + fecundity	−1404.0	5.1	0.052	0.204	0.547
m2a	yolk ~ AGE + POP + female size + fecundity + POP×fecundity	−1402.4	6.7	0.023	0.204	0.547
m0	yolk ~ Intercept	−1394.2	15.0	0.000	–	0.546
m0b	yolk ~ AGE	−1392.2	17.0	0.000	0.000	0.546
Random effects: (1|female identity) and (1|spawning replicate)						

b)

Model	Fixed effects	AICc	ΔAICc	Weight	R^2_{GLMMm}	R^2_{GLMMc}
m3	SURV ~ INC + yolk + INC×yolk + POP + INC×POP + AGE	2222.3	0.0	0.952	0.088	0.262
m2a	SURV ~ INC + yolk + INC×yolk + POP + AGE	2228.9	6.6	0.036	0.082	0.245
m1a	SURV ~ INC + yolk + POP	2231.5	9.2	0.010	0.076	0.246
m2	SURV ~ INC + yolk + INC×yolk	2234.2	11.9	0.002	0.073	0.295
m1	SURV ~ INC + yolk	2238.3	16.0	0.000	0.072	0.298
m1b	SURV ~ INC + yolk + AGE	2239.7	17.4	0.000	0.073	0.298
m0	SURV ~ Intercept	2292.1	69.8	0.000	–	0.153
Random effects: (1|female identity), (1|spawning replicate/dish number) and (1|dish position)						

c)

Model	Fixed effects	AICc	ΔAICc	Weight	R^2_{GLMMm}	R^2_{GLMMc}
m3a	HatchSL ~ DUR + yolk + resyolk + DUR×resyolk + POP	−998.7	0.0	0.871	0.218	0.422
m2	HatchSL ~ DUR + yolk + resyolk + POP	−993.3	5.4	0.059	0.201	0.412
m3b	HatchSL ~ DUR×yolk + yolk + DUR + resyolk + POP	−993.1	5.62	0.052	0.206	0.413
m1	HatchSL ~ yolk + resyolk	−990.9	7.8	0.018	0.194	0.422
m0	HatchSL ~ Intercept	−912.3	86.4	0.000	–	0.405
Random effects: (1|female identity), (1|spawning replicate/dish number) and (1|dish position)						

d)

Model	Fixed effects	AICc	ΔAICc	Weight	R^2_{GLMMm}	R^2_{GLMMc}
m3	DUR ~ yolk + AGE + POP + yolk×POP + AGE×POP	426.9	0.0	0.771	0.227	0.845
m2a	DUR ~ yolk + AGE + POP + yolk×POP	430.0	3.1	0.160	0.237	0.841
m2b	DUR ~ yolk + AGE + POP + AGE×POP	432.2	5.3	0.054	0.209	0.810
m1	DUR ~ yolk + AGE + POP	435.3	8.4	0.011	0.221	0.806
m0	DUR ~ Intercept	439.0	12.1	0.002	–	0.749
m0a	DUR ~ yolk	439.5	12.6	0.001	0.006	0.753
Random effects: (1|female identity), (1|spawning replicate/dish number) and (1|dish position)						

* yolk, yolk sac size; resyolk, residual yolk sac size; SURV, survival (development completed or death); INC, incubation phase (early or advanced); DUR, development duration (short- or long-developer); HatchSL, hatchling size; POP, population (414 or 422); female size, female size; fecundity, female fecundity (number of eggs per single spawning); AGE, age (young [75 days] or old [111 days])

reported as producing an increasing proportion of long-developing embryos with advancing age, interpreted as an adaptation to the erratic character of their natural habitat (Podrabsky et al., 2010). In some regions, rainfall is so stochastic that a temporary pool may even desiccate during the rainy season and may refill again with later rains as a "secondary pool" (Polačik et al., 2014; Reichard et al., 2017). If developed embryos are present in the germ bank of the pool, these can hatch into the secondary pool within the same season (Polačik et al., 2014). As such, it would appear adaptive for young females to produce a higher

proportion of short-developing embryos ready to exploit the secondary pool within the same rainy season. In contrast, old females that survive into the advanced part of the rainy season have only a negligible prospect of a secondary pool emergence and, therefore, should produce long-developing embryos suited to survival throughout the dry season (Podrabsky et al., 2010; Polačik et al., 2017).

Here, "young" females (11 weeks old) from both populations produced mainly short-developers, whereas the proportion of long-developers did not increase in "old" females five weeks later. By that time, the experimental females had reached 16 weeks, an age when many pools in the semi-arid region would be dry already (Terzibasi Tozzini et al., 2013; Polačik et al., 2014). Thus, an age-related switch from producing short-developers to long-developers does not appear to be a general feature in *N. furzeri* females. Alternatively, the maternal effect may have been overridden by specific incubation conditions. Whereas aquatic medium incubation (Podrabsky et al., 2010) or incubation under a layer of substrate (Polačik et al., 2017) has been used in previous studies demonstrating such a maternal effect, we placed the experimental embryo directly on top of the incubation substrate. Annual killifish embryos display a plastic response to environmental factors (Wourms, 1972; Levels and Denucé, 1988; Podrabsky and Hand, 1999; Furness et al., 2015b); hence, while it is possible that our mode of incubation produced an artificially increased proportion of escape embryos (Polačik et al., 2016), the method is unlikely to have influenced the main findings of our study (i.e., that survival over the extended embryonic period is unconstrained by the amount of energetic reserves and does not carry a cost in terms of hatchling size or residual yolk).

In conclusion, the degree of maternal provisioning and developmental duration varied considerably in *N. furzeri* embryos. Our results demonstrate that these two life-history traits were decoupled, with the amount of energetic reserves not constraining developmental duration through survival limitation. In effect, females do not provision their offspring in accordance with their final developmental pathway (i.e., to enter or skip diapause). These findings do not align with our predictions; rather, they emphasize the strong buffering role of diapause in the energetic balance of annual killifish embryonic development. As maternal provisioning positively affects the size of hatched fish, this would appear to represent an investment into postembryonic survival and posthatching development.

Experimental Procedures

Our approach consisted of recording embryonic development in *N. furzeri* from eggs of variable size. In order to clarify the relative importance of influencing factors (egg size, female age), we used two populations with contrasting mean egg size and compared the effects in relation to age of parental fish. Finally, we related egg size and duration of the embryonic development to the size of freshly hatched fish.

Parental Stock

The parents of the experimental embryos were F2 generation of the strain founders, imported from southern Mozambique in 2013. Vrtílek and Reichard (2016) have demonstrated natural interpopulation variability in *N. furzeri* egg size. Hence, we specifically chose to use individuals from the large-egg-producing population 414 (collection code MZCS 414, GPS: S22° 33′ 16.7″, E32° 43′ 38.1″; 12 wild-caught pairs imported) and the small-egg-producing population 422 (collection code MZCS 422, GPS: S22° 06′ 37.0″, E33° 46′ 28.7 ″; 15 wild-caught pairs imported). Pilot measurements (10 fertilized eggs per female from 1 different females in each population) indicated a mean ± standard error (SE) egg diameter in the large-egg population 414 of 1.251 (±0.011) mm, and 1.202 (±0.011) mm in the small-egg population 422.

The parental stock consisted of nine males and 16 females from population 414, and nine males and 15 females from population 422. The parental fish were housed individually in a customized recirculation system (Fish Box, Aqua Medic GmbH) and fed frozen bloodworms (*Chironomus* sp.) once per day to satiation. Water temperature was maintained at 25 °C, 30% of water-volume was changed twice a week, and the light regime was set to 14:10 (light:dark).

Egg Production

The fish were spawned in two major spawning sessions, each including a set of repeated pairings (replicates). The two spawning sessions represented contrasting age periods in the life of the parental fish: young and old age.

The young-fish spawning session started when the fish were 75 days old (11 weeks). All males of each strain were paired with a female, and the clutches produced were collected and incubated. Since the number of males was approximately half that of females in both strains, the same set of males was also paired with the remaining females of the respective strain the next day (after 24 hours). The respective pairs were identical throughout the period of egg production. In each case, each male was always paired with the same females (i.e., with two females or a single female if a pair could not be formed due to the odd number of females). A pairing of a single male with the two assigned females (or a single female in the case of an odd number of females) within two days represented a replicate. For young fish, three replicates were conducted over six days.

The spawning session of old fish started when they reached 111 days (16 weeks) and followed a schedule identical to that of young fish, with the exception that four spawning replicates (taking eight days) were necessary to obtain a comparable number of eggs to those produced by young fish. An extended spawning schedule was required due to female mortality between the two spawning sessions (see Table 1 for details).

Actual spawning was achieved by introducing a pair of fish into a 2L plastic tub with a mesh-covered bottom, the mesh separating the fish from the eggs and preventing their consumption (see Polačik et al., 2016 for details). The fish were allowed to spawn for 2-hr, a period sufficient for releasing all ovulated eggs (Polačik and Reichard, 2009). After spawning, the pair was photographed in a dish with a scale to record body size and returned to their home tanks.

Egg Collection and Incubation

After spawning, the egg's chorion was allowed to harden for 4 hr in the spawning tub. Subsequently, a Pasteur pipette was used to transfer each clutch into a Petri dish containing a methylene blue solution to help prevent the emergence of mold (Polačik et al., 2016). All clutches were then placed into a laboratory incubator

(Q-Cell, Pol-Lab Ltd.) for 24 hr at 25 °C, following which all eggs in each clutch were counted.

The pairing schedule resulted in a total of 192 pairings, producing 7,712 eggs with a mean ± standard deviation (SD) of 40.2 (± 20.4) eggs per spawning. A random sample of 10 fertilized eggs (identified under a dissecting microscope based on their double-layered structure; Polačik et al., 2016) was subjected to further incubation. Fewer eggs were used in cases where a clutch yielded < 10 fertilized eggs (5.7% of all pairings). Overall, 90.1% of clutches produced the full number of eggs.

We measured each of the sampled eggs using an Olympus SZX10 dissecting microscope (25 × magnification, Olympus Corp.) and ImageJ image analysis software (NIH). Two structures were measured at the longest and perpendicular axis, the external chorion layer, and the yolk sac, resulting in each egg being characterized by four dimensions (length and width of the egg, length and width of the yolk sac). The section surface delimited by the chorion (egg) and by the vitelline membrane (yolk sac) was then calculated using the formula for an ellipse (i.e. a/2*b/2*π, where "a" is length and "b" is width, thereby giving values for egg size and yolk sac size.

After measuring, the eggs were placed onto the surface of moistened and firmly pressed gardening peat (prepared according to Polačik et al., 2016) in compartmented Petri dishes (8 cm^3 per cell). The position of the eggs was randomized to control for any potential effect of position within the dish. During data collection, we recorded both the identity of each Petri dish and egg position. To prevent excessive loss of humidity, each batch of eggs was sealed in a plastic zip-lock bag before being placed in an incubator at 25 °C.

Collection of Data on Egg Development and Hatchling Size

Data on the duration of embryonic development were obtained through 24 visual inspections over 12 months. During the first six weeks of incubation, inspections were performed on a weekly basis. The embryos were scored as a) undeveloped (eggs translucent, no internal structure visible to the naked eye), b) developed (eggs opaque, embryo with conspicuous, golden pigmented eyes filling the internal space), or c) dead (egg white, no sign of embryonic structure).

A sharp decline in the proportion of developed embryos was recorded during the advanced phase of the experiment (see Results), prompting a modification in the inspection schedule. Starting from inspection no. 7, inspections were conducted on a monthly basis up to inspection no. 12. The inspection schedule was switched back to weekly checks following the reappearance of developed embryos (see Results) during inspection no. 12, continuing until inspection no. 18. At this point, the rate of development again decreased, prompting a shift to biweekly checks for the final six inspections (nos. 19–24).

We attempted to hatch all embryos scored as "developed" in order to obtain data on hatchling size. Each developed embryo was left intact for an additional seven-day period to ensure full completion of development (note that the golden pigment in an embryo's eyes may still be present 1–3 days before hatching; Wourms, 1972). We recorded survival before the prehatching stage (Diapause III) was reached, as fully developed embryos may spend an undefined period (weeks) waiting for inundation (Wourms, 1972).

Since our primary aim was to collect data on hatchling size, and hatching rate is known to vary in *N. furzeri*, we employed a technique known to increase hatching success (Polačik et al., 2016). Each egg was placed in an empty cell of a compartmented Petri dish and wetted with 10 mm of 20 °C water, whereupon it was immediately covered with a 10-mm layer of fine glass beads. Three to four hours later, all hatched fish were collected, anesthetized with clove oil, and measured for standard length and surface of residual yolk sac, using the same method used for measuring egg dimensions (see above - section Egg collection and incubation). The residual yolk sac was measured as a polygon surface as, unlike eggs or the initial yolk sac, the hatchling yolk sac is irregular in shape.

Data Analysis

Four response variables were used for data analysis: yolk sac size, survival, development duration, and hatchling size. As yolk sac size and egg size were highly correlated (Pearson's r = 0.871), we only used yolk sac size for all further analyses. Yolk sac size was used in preference, as the space between the chorion layer and yolk sac varied considerably, and yolk sac size served as a more precise proxy measure of embryonic energetic reserves (Jardine and Litvak, 2003).

Survival, representing the status of the embryo at final inspection, was expressed using two categories: development completed or dead. We considered undeveloped embryos at the end of the experiment as embryos with an extremely long development. These were not used during analysis of survival or development duration, as their future fate (death or development completion) was uncertain. Including them in the analysis of survival and development duration as successfully developed embryos with long development did not change the inferences. Undeveloped embryos were not characteristic by specific egg size, nor did they come from a single population, a single female, or females of a specific age.

We used binomial coding instead of the exact number of incubation days for development duration, as the distribution was clearly bimodal. Short-developers developed between 23 and 45 dpf. We also included five embryos developing at 51, 80, 109, and 110 dpf in this category. All other incubated embryos completing development within the experimental period did so between 189 and 314 dpf, with just two embryos developing at 349 and 350 dpf. These were categorized as long-developers.

Yolk sac size (continuous), residual yolk sac size (continuous), development duration (short- or long-developers), incubation phase (early = < 135 days of incubation; advanced = > 161 days), population (POP414 or POP422), age (young or old), female fecundity (continuous), and female size (continuous) were used as explanatory variables. Continuous explanatory variables were standardized to a mean at zero and to half standard deviation (SD/2) (Schielzeth, 2010). Female identity (31 levels), spawning replicate (seven levels), dish identity nested in spawning replicate (20 levels per replicate), and dish position (18 levels) were used as random factors.

Data was analyzed using the lme4 (Bates et al., 2015) and MuMIn (Bartoń, 2016) packages in R software version 3.1.3 (R Development Core Team, 2015). Binomial response variables (survival, development duration) were analyzed using generalized mixed-effects models fitted with the logit-link function using Laplace approximation (Bolker et al., 2009). We used linear

mixed-effects modeling for continuous response variables with approximately normal distribution (yolk sac size, hatchling size). The candidate model set for each response variable was specified *a priori* (see Table 6; Burnham and Anderson, 2002), with the information-theoretic approach (Burnham and Anderson, 2002) used to compare fit and parsimony between individual candidate models. Relative support for each model was compared using the Akaike Information Criterion corrected for small sample-sizes (AICc) (Zuur et al., 2013) and related Akaike weights under maximum likelihood model parameterization. R^2_{GLMM} (Nakagawa and Schielzeth, 2013) was used as a measure of explained variability (Table 6).

Model sets with a high level of selection uncertainty (Akaike weight of the best model < 0.95) were averaged across all models in the set (Grueber et al., 2011), with averaged estimates and unconditional (adjusted) SEs tabulated for each fixed-effect term. The influence of individual model terms (including interactions) was assessed by estimation of the 95% CI, $CI = 1.96 \times SE$. Terms with a narrow CI not containing zero were considered as strongly influential. The effect of terms with a CI marginally overlapping zero was interpreted as a tendency to affect the response variable.

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