Variation in developmental rates is not linked to environmental unpredictability in annual killifishes

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ABSTRACT

Comparative evidence suggests that adaptive plasticity may evolve as a response to predictable environmental variation. However, less attention has been placed on unpredictable environmental variation, which is considered to affect evolutionary trajectories by increasing phenotypic variation (or bet-hedging). Here, we examine the occurrence of bet-hedging in egg developmental rates in seven species of annual killifish, which originate from a gradient of variation in precipitation rates, under three treatment incubation temperatures (21°C, 23°C, and 25°C). In the wild, these species survive regular and seasonal habitat desiccation, as dormant eggs buried in the soil. At the onset of the rainy season, embryos must be sufficiently developed in order to hatch and complete their life-cycle. We found substantial differences among species in both the mean and variation of egg development rates, as well as species-specific plastic responses to incubation temperature. Yet, there was no clear relationship between variation in egg development time and variation in precipitation rate (environmental predictability). The exact cause of these differences therefore remains enigmatic, possibly depending on differences in other natural environmental conditions than precipitation predictability. Hence, if species specific variances are adaptive, the relationship between development and variation in precipitation is complex, and does not diverge in accordance with simple linear relationships.

INTRODUCTION

Organisms may cope with fluctuations in environmental conditions by means of phenotypic plasticity or bet-hedging (Crean and Marshall 2009; Simons 2011; Furness et al. 2015a). While there is abundant theoretical literature on both plasticity and bet-hedging (e.g. Cooper and Kaplan 1982; DeWitt and Langerhans 2004; Marshall and Uller 2007; Scheiner and Holt 2012; Tufto 2015), only more recently have empirical studies focused on validating these predictions (i.e. Bradford and Roff 1993; Richter-Boix et al. 2006; Marshall et al. 2008; Simons 2014; Furness et al. 2015a; Shama 2015). Understanding the evolution of these two adaptive strategies is especially important in the face of global climate change, which is likely to change not only the environment, but importantly, the predictability of environmental conditions (Robeson 2002; Thornton et al. 2014; Berg and Hall 2015).

The evolution of plasticity and bet-hedging requires variation in the fitness of individuals under different environmental conditions (i.e. that phenotypes differ in which environment they perform best) (Simons 2011). Phenotypic plasticity is an environment-dependent trait expression, and given genetic variation in reaction norms (i.e. that genotypes differ in their associated phenotypes depending on environmental conditions, Simons 2011), adaptive phenotypic plasticity is considered to evolve as a response to predictable environmental changes, for which there are reliable cues (Ghalambor et al. 2007). However, phenotypic variation *per se* can be adaptive, as over a range of environmental conditions, by chance alone, the phenotypes of some individuals may be close to the environment dependent fitness optima (i.e. bet-hedging) (Crean and Marshall 2009). Therefore, when environmental conditions are either not predictable, or non-transducible into developmental regulators, production of highly variable phenotypes will spread the risks associated with unsuitability to particular environmental conditions. Under this scenario, bet-hedging is considered to be an adaptive strategy (Clauss and Venable 2000; Crean and Marshall 2009).

The random nature of bet-hedging helps to ensure that there will be at least some surviving offspring, resulting in reduced among-generation variation in reproductive success (Crean and Marshall 2009). Bet-hedging has been suggested to be a costly strategy, as there will be constant selection against a non-trivial part of the population that is not suited to current environmental conditions (Kussell & Leibler 2005; Beaumont et al. 2009). Further, while bet-hedging can theoretically evolve in any trait, it is hypothesized to be particularly relevant in traits related to juvenile establishment, in highly fecund organisms that do not engage in parental care, and in organisms that inhabit areas

prone to rapid environmental fluctuations (e.g. "*r*-selected" species, following MacArthur and Wilson 1967).

One textbook example of bet-hedging can be observed in annual desert plants, which produce seeds with highly variable germination times, in response to unpredictable rates of precipitation (Venable 2007). In this case, if there is a drought in any given year, at least a subset of seeds will still likely germinate in the following years, and ensure survival of the lineage (e.g. Phillipi 1993; Evans et al. 2006; Venable 2007). Similarly, in animals, important upcoming environmental events such as the arrival of winter or the next rainy season may be difficult to predict. For example, bet-hedging in diapause length (an adaptation to surviving undesirable seasonal conditions) has been reported in many animal taxa, such as insects, crustaceans, rotifers, and in fish (Cáceres and Tessier 2004; García-Roger et al. 2014; Furness et al. 2015a; Hopper 2018). These animal examples include increased variation in the length of the diapause (Furness et al. 2015a; Hopper 2018) and variation in the proportion of eggs that fall into diapause or direct development (Cáceres and Tessier 2004; García-Roger et al. 2014; Furness et al. 2015a; Hopper 2018).

Bet-hedging is hence an adaptive increase in phenotypic variation, and under a bet-hedging scenario, trait variation is expected to correlate to environmental variation (Crean and Marshall 2009). Species often inhabit different environmental conditions, which differ in both variability and predictability. Thus, by comparing different species or populations, which inhabit environments which vary in the predictability of particular environmental conditions or events, we can test for the generality of bet-hedging strategies (van Kleunen et al. 2014; Hopper 2018). In plants, such comparative studies have repeatedly demonstrated bet-hedging in seed dormancy (e.g. Philippi 1993; Evans et al. 2006; Venable 2007). Moreover, evidence from several studies suggests that differences in bet-hedging responses occur among animal populations and species (Marshall et al. 2008; Krug 2009; Nevoux et al. 2010; García-Roger et al. 2014; Polačik et al 2018). Yet, questions remain regarding the adaptive nature of these patterns, as few studies have assessed differences across species or populations that occur over a gradient of environmental predictability (but see García-Roger et al. 2014; Polačik et al. 2018).

Here, we investigate mean and variation in the developmental time of eggs in seven species of annual killifishes (Cyprinodontiformes, Aplocheiloidei). These species inhabit ephemeral freshwater bodies in Africa and South-/Central America, where both within- and between-season conditions often are highly unpredictable (Inglima et al. 1981; Genade et al. 2005; Furness 2016). Several species of killifish have independently evolved specific adaptations to survive in ephemeral

habitats. For instance, in these annual clades, eggs stay dormant (i.e. in diapause) in the substrate of the dried-out pools until the next wet season (Furness et al. 2015b). However, even within annual species, the duration of egg diapause is variable and not always obligatory, so that one clutch of eggs may consist of both directly developing eggs, and eggs entering diapause (of varying duration) (Wourms 1972b). This variation in development time has been suggested to constitute a bet-hedging strategy that maximizes fitness by spreading the risks associated with variability in the hydrological dynamics of the ephemeral pools (Wourms 1972b; Polačik et al. 2014; Furness et al. 2015a). Furthermore, variation in the duration of killifish diapause has been found to be both under maternal control and governed by plasticity during embryonic development (Podrabsky et al. 2010; Polačik et al. 2016a; Pri-Tal et al. 2011; Furness et al. 2015a).

Here, we measured duration of egg development in a standardized laboratory setting and correlated species-specific means and variances of egg development time, with measurements of variation in precipitation rate, in the species native ranges, during the onset of the rainy season. Eggs were reared under three different temperatures to assess the extent to which development time variation was stable across thermal conditions. The seven species included in this study represent five of the independent clades that have evolved an annual life cycle in killifishes. These species were also chosen because they originate from a gradient of environmental conditions, with differences in precipitation predictability. We predicted that species that have evolved under more unpredictable environmental conditions will display a larger variation in egg development rates, compared to those species that originate from more predictable environments.

MATERIALS AND METHODS

Animal models

We used seven annual killifish species (*Gnatholebias zonatu*s Myers, *Millerichthys robustu*s Miller and Hubbs, *Nematolebias whitei* Myers, *Nothobranchius guentheri* Pfeffer, *No. kadleci*; Reichard, *Pituna schindleri* Costa, and *Simpsonichthys constanciae* Myers), collected from 12 locations/populations (see Table S1, supplementary information 1 for details), which include representatives from five of the major independent evolutionary transitions between a non-annual and annual life cycle (Furness et al. 2015b; Gonzalez-Voyer et al. unpublished data). The experimental procedures were approved by the Ethical Committee in Stockholm, Sweden (license N132/15).

Rearing of parental generation

The parental generation were hatched from eggs sourced from dedicated hobbyists, or from our own breeding groups. Eggs were kept in dry peat, for each species, the peat was wetted in half-filled 5-liter containers until eggs hatched. Fry were then transferred individually into 0.75 liter plastic boxes with ram's horn snails (Planorbidae), which removed uneaten food, and java moss (*Taxiphyllum barbieri*) for shelter. Fry were fed (3 times/day-weekdays, and 1 time/day weekends) with freshly hatched Artemia nauplii. Parental fish were reared under standardized conditions (ambient temperature 28°C, average water temperature 24.2°C ± 0.65 s.d). The water used was tap-water (KH=4, GH=7, pH=7.5) with addition of Jbl Biotopol (JBL GmbH & Co, Nauhofen, Germany) water conditioner. At one week of age, and if feeding without any problems, the fry were pooled into groups of 3-4 individuals in one 0.75-liter plastic box. When fry reached 1cm in total length, they were transferred to 13-liter containers and fed with a mixture of de-frosted chironomid larvae and live Artemia nauplii. The 13-liter containers were furnished with gravel, an empty terracotta flowerpot and a yarn mop. Water quality was maintained with an air-driven sponge filter, and twice weekly 80% water changes in the 0.75-liter plastic boxes and weekly 25% water changes in the 13 liter containers.

Breeding procedures

When females were noticeably mature, that is, with a rounded egg-filled belly, they were paired with a randomly chosen male and placed together in a 13-liter spawning container for a period of 2-3 months. Annual killifish reproduce by either burying or dispersing eggs over substrate, therefore a 0.75-liter plastic box, filled with either glass beads (Sargenta AB, Malmö, Sweden) or coco peat (Exo Terra, Hamm, Germany) was added to each breeding container to provide substrate for egg laying. We found that glass beads facilitated more efficient egg retrieval, but these were not accepted by all species as an appropriate breeding substrate, so these species where supplied exclusively with boxes filled with coco peat. In cases of more aggressive males, they were grouped together with 2-4 females, in order to dilute male aggression among a higher number of females. Females were switched between different male containers, residing in each for 2-3 months. Eggs were gathered weekly, either by sieving the glass beads through a net, or by laying the coco peat on a white plastic board and inspecting thoroughly for the eggs.

Experimental treatment of the offspring generation

We included different temperatures to asses if developmental times are congruent across different thermal regimes. This is in line with Furness et al. (2015a), who investigated bet-hedging and developmental plasticity in *No. furzeri*. However, we chose to work within a narrower temperature

range compared to Furness et al. (2015a), since they included temperatures that could potentially be detrimental for embryonic development in some of the species that we used [i.e. sub-tropic species, as compared to the tropical *No. furzer*i used by Furness et al. (2015a)]. Fertilized eggs from each male container (per female or female group), were partitioned into three different egg incubators (Lucky Reptile Herp Nursery II, Import Export Peter Hoch GmbH, Waldkirch, Germany) set to 21°C±0.15 s.d, 23°C±0.44 s.d, and 25°C± 0.33 s.d., under a 12hr/12hr photoperiod. There are several methods for incubating killifish eggs (Polačik et al. 2016b), and in order to maximize the standardization of rearing conditions, and to avoid artificially increased within-species variation, we chose to incubate eggs submerged in a Yamamoto solution (17 mM NaCl, 2.7 mM KCl, 2.5 mM CaCl2, pH set to 7 with NaHCO3; Valenzano et al. 2009 after Rembold et al. 2006). This method has previously been successfully used by Furness et al. (2015a, b, 2016). In addition, 2 drops of 6.25 mM methylene blue and 5.33 mM acriflavine solution were added per 1 liter of Yamamoto solution, to prevent fungus and bacterial infections. For incubation, each embryo was transferred to a separate well containing the incubation medium, on a 24-well tray (TC-Plate Standard F, Sarstedt AG & Co. KG, Nümbrecht, Germany). The Yamamoto solution was changed twice a week to ensure clean and stable water conditions.

Data collection

In annual killifishes, embryo eyes become pigmented after the Type II diapause (Wourms 1972a), and the embryos were visually inspected with a magnifying glass for the appearance of pigmented eyes once a week. In killifishes, three different diapause stages are found, referred to as Type I (developmental arrest during early development, dispersed cell phase), Type II (38 somites present, beginning of organ development), and Type III (embryo developed and able to hatch) (Wourms 1972a; Furness 2015b). Type II diapause is particularly resistant to dehydration stress, and found only in annual killifish (Podrabsky et al. 2015; Furness 2015b; Furness 2016), making the time until the end of Type II diapause an important time-point for survival during the dry season (Podrabsky et al. 2015). Therefore, the period between egg laying date and appearance of pigmented eyes was used as a proxy for developmental time. In total we collected 2567 eggs, out of which 24% (N = 614) survived until the eye-pigmentation stage. The majority of mortality occurred shortly after egg collection, with half of the mortality occurring during the first week of incubation (see Table S2 in supplementary information 2 for more information on the eggs that survived), presumably due to being unfertilized or inflicted with minor damage during the collection, exposing the embryos to oomycete (Saprolegniaceae) infections. At the end of experimental period (that spanned two years; between beginning of July 2017 and the end of June 2019) there were 21 embryos that did not show eye development. The undeveloped embryos, which were evenly distributed across the temperature

treatments, belonged to *G. zonatus* and *P. schindleri* and were excluded from the analysis.

Estimation of precipitation variability

To assess the influence of precipitation variability on embryo development time and variation in development time, we identified the exact coordinates of the collection localities of our lab populations from the killi-data.org archive (Huber et al. 2016) and retrieved site-specific climate data for each locality from the Local Climate Estimator software (New_LocClim, average length of time-series is 50 years; FAO 2018). The following variables were collected: *i*) mean among-year precipitation for the three first months of the rainy season, and *ii*) the among-year standard deviations in precipitation. We chose these specific parameters, as precipitation predictability during the rainy season should be key to complete pond-filling and, hence, crucial for embryo survival. We reasoned that high variation in precipitation rates during the early rainy season may result in ponds only partially filling. Partial filling may be enough to induce killifish eggs to hatch, but possibly not provide enough time to complete their entire life cycle, as a partially filled pool might desiccate quickly in times of drought. For each species, the first month of a rainy season was assumed to be the month when precipitation increased, following the dry season. The dry season was considered as a within year period of low precipitation, with average precipitation falling under 60 mm · month^{-1} (Peel et al. 2017), during at least one month. In the case of *Ne. whitei*, the differentiation between dry and rainy season was not as obvious as in case of the other species, and indicated an existence of two rainy seasons. Hence, we averaged precipitation data for these two periods (Table S1, supplementary information 1). When our lab populations originated from more than one collection locality, we averaged the climate data for these populations (Table S1, supplementary information 1). The averaged climate data is therefore representative for the species, as different populations of the same species used in this study typically inhabit similar environments (Table S1, supplementary information 1). Annual killifishes are not bound to a certain water body, but may disperse across areas during flooding events, promoting gene flow between populations which would diminish differences in local adaptation between populations (Berois et al. 2015).

As variance scales to the mean, and as there are large divergences among the populations in the mean rainfall, we calculated species-specific coefficients of variation *(CV*) for monthly precipitation (average length of data series being 50 years), as the standard deviations multiplied by 100, divided by the mean, and averaged the monthly *CV*s for the first three months of the rainy season.

Statistical models

To examine the sources of variation in egg development time, we first fit two separate intercept-only models. The first model was fit on a subset of the whole data, when female identity was known (data from breeding containers where only one female was present, model 1a, Table S3, supplementary information 3), and included species, male, and female as random effects. The second model was fit to the full dataset including those observations where female identity was impossible to know (breeding containers with both, single females and female groups, model 1b, Table S3, supplementary information 3), and included species, male, and female/female group as random effects. Of these models, the former model gave us an opportunity to estimate female effects, while the later model provided better estimates of species effects, given the larger dataset. We then calculated medians and 95% confidence intervals of male, female/female group, within-, and among-species posterior distributions of variation.

In order to *i*) correlate environmental predictability with means and variances of development time, and *ii*) compare among-temperature means and variances of development time, we first ran four intercept-only models, one model per temperature treatment, and one with pooled temperatures. All models included species as a random effect and allowed for separate residual variance among species (models 2-5, Table S3, supplementary information 3). From these models, we obtained full posterior distributions for species-, and temperature-specific means and variances of development time, which allowed for calculation of posterior distributions for *CV*s in developmental times. The posterior distributions for the *CV*s were calculated as the square root of posterior distribution for variances, multiplied by 100, divided by the posterior distribution for the mean.

In order to assess the significance of species-specific responses to temperature, we calculated the differences among the three temperature-specific posterior distributions for each species (Fig. 2 and 4). Significance was determined as a lack of overlap with 0.

Finally, to test if the variation in development time increased with increasing variation in precipitation, as would be expected under a bet-hedging scenario, we modelled *CV*s of development time as a response variable, and a vector of precipitations *CV*s as an explanatory variable. This was done for the full posterior distribution of CV ($N_{\text{iterations}}$ = 1000), to account for uncertainties in the estimates. In addition, for each iteration, we bootstrapped over species, in order to account for uncertainty associated with the included species (where, for example *M. robustus* could be classified as an outlier). This approach yielded posterior distributions of the slope of the regression,

where significance was determined as a lack of overlap with 0. Similarly, we used the same approach to examine if means of development time were associated with precipitations *CV*s.

Potentially confounding factors

Maternal age has previously been found to affect egg development time in killifishes (Podrabsky et al. 2010; Pri-Tal et al. 2011; Polačik et al. 2016a). Therefore, we wanted to exclude the sources of maternal effects that could be viewed as experimental artefacts. Specifically, as pairing was preformed over an extended period (see Table S2 in supplementary information 2 for more information on the crossings), the age of parental fish differed across different full-sib families, which previously has been found to influence embryo development time (Podrabsky et al. 2010). Moreover, while our intention was to create full-sib offspring families, some males were aggressive and multiple females were needed to be in the mating container, which could potentially influence egg developmental trajectories. Hence, we initially ran a model with female age (continuous variable) and female group rearing (factor with two levels, single and group) as fixed effects. Species, male, and the female/female group identity were added as random effects, in order to access if female age or group/single female breeding could constitute a source of maternal effects, and influence the results. However, as these factors were non-significant, they were not included in any of our inferential models.

Model evaluation

Data was analyzed using Bayesian mixed-effect models (MCMCglmm-package for R; Hadfield 2010), in R version 3.4.4 (R Development Core Team 2015). In all models, we used flat priors for the fixed effects and locally non-informative priors for the random effects, at least $1{\cdot}10^6$ iterations, a burn-in of $5{\cdot}10^4$, and a thinning interval of 1000, which resulted in effective sampling size of 1000. We diagnosed posterior distributions and model convergence by running three parallel chains using the Gelman–Rubin convergence criterion; the upper 97.5 quantile of the Gelman-Rubin test statistic was below 1.2 in all cases. All autocorrelations were within the interval −0.1 and 0.1.

RESULTS

We found that most of the variation in development time was structured within species, as the residual variation accounted for 70% (95% CI: 36, 85) of the total variation (averaged across species). Among species variation accounted for 28% (95% CI: 12, 63) of the total variation, while genetic and maternal influences were negligible (male <1%, female < 1%, and female/female group <1%).

Species- and temperature-specific length of development time

Development time differed significantly among species (Figure 1), and among different rearing temperatures within species (Figure 2), as indicated by non-overlapping 95% CI's of the posterior distributions. However, there was no clear linear relationship between development time and precipitation *CV* (β = -0.0048; 95% CI: -0.82, 1.65; Figure 1). Furthermore, there was no general relationship between development time and rearing temperature, although in a subset of species, *S. constanciae*, *P. schindleri*, and *Ne. whitei*, higher temperature correlated with shorter development times (Figure 2).

Species- and temperature-specific variation in development time

Development time *CV*s differed significantly among species (Figure 3), and among different rearing temperatures within species (Figure 4, *S. constanciae*, *Ne. whitei*, *M. robustus*), as indicated by non-overlapping 95% CI's of the posterior distributions. There was no clear linear relationship between development time *CV* and precipitation *CV* (β = 0.077; 95% CI: -0.56, 1.13; Figure 3). There was no detectable general relationship between development time *CV* and rearing temperature (Figure 4).

DISCUSSION

We found substantial among-species differences in both the mean and the variation of egg development time in annual killifish species, which originate from environments along a gradient of precipitation variability. Under a bet-hedging scenario, we expected species from more unpredictable environments to have higher variation in embryo development times (Crean and Marshall 2009). However, we did not find any general relationship between variation in precipitation and variation in development time across species. Moreover, in several species, both the mean and the variation of development time were dependent on rearing temperature. The lack of association between variation in development time and environmental variation suggests that bet-hedging either may not be an important mechanism for persisting in these ephemeral habitats, or that other environmental factors, which the species are exposed to in the wild, could have influenced the developmental times. Hence, if species specific variances are adaptive, the relationship between development and variation in precipitation is complex, and does not diverge in accordance with simple linear relationships.

In contrast to our results, comparative analyses have shown that annual desert plant species occurring in areas with unpredictable rates of precipitation, produce seeds with highly variable germina-

tion times (Venable 2007; see also Evans and Donnehy 2005). Diapausing eggs in the killifish system could be viewed as analogous to the example of seed-banks in plants. However, although pond filling period, and thus precipitation, is crucial for the survival of annual killifishes (Watters 2009; Polačik et al. 2011; Domínguez-Castanedo et al. 2017), we did not find any clear relationship between precipitation predictability and variation in embryo development time. Our results are hence similar to a recent study that did not find any differences in development times among eight populations of two closely related annual *Nothobranchius* killifish species (*No. furzeri* and *No. kadleci*; Polačik et al. 2018). Together, these results suggest that other factors likely contribute to driving differences in egg development patterns across species. While we found no evidence for bet-hedging, our results implicate a link to environmental conditions. For example, *Ne. whitei and S. constanciae* co-occur in areas with two rainy seasons per year (Costa 2012), and despite key differences in other traits between these two species (e.g. egg size and growth rate, Huber et al. 2016; Eckerström-Liedholm et al. 2017; Sowersby et al. 2019), egg development time was similar, both in terms of the variation (relatively low), mean (relatively long), and plastic to incubation temperature. These phenotypic similarities among two co-occurring species suggests that developmental time might be shaped more by the natural conditions under which these species occur.

In a bet-hedging scenario, there will be constant selection against a non-trivial part of the population that is not suited to current environmental conditions (Kussell & Leibler 2005; Beaumont et al. 2009). However, a lack of bet-hedging, particularly in unpredictable environmental conditions, may also be costly. For example, without a bet-hedging strategy, all offspring may be ill-equipped to cope under current environmental conditions. Variation in one key trait, development time, may therefore evolve as an evolutionary trade-off between the costs and benefits of employing a bet-hedging strategy. Hence, one potential explanation for our results may be that different species exhibit different solutions to this trade-off, by either trying to match the rainy season (high risk – high payoff strategy), or by having large variation (low risk – low payoff strategy) in development rates. Either scenario could yield the pattern we observed, with large among species differences in egg development rates, which were not linked to environmental predictability.

Plastic responses and adjustments in trait variance are important for facilitating adaptation, particularly as a warming climate changes both the environment and its predictability (Robeson 2002; Thornton et al. 2014; Berg and Hall 2015). Species that utilize bet-hedging strategies are hypothesized to be better adapted to cope with increased environmental variation, for instance, due to climate change, as they use a risk-minimizing strategy (Childs et al. 2010). Hence, in our study, species with higher variation in development time (for example *P. schindleri*, *No. kadleci*, and *M. ro-*

bustus), may be predisposed to cope better with future environmental changes, however at the same time they are likely to suffer significant costs, as a large proportion of a females eggs are unlikely to develop in any particular season.

In addition to differences among species in variation in embryo development time, we found that both the mean and the variation in development time was affected by rearing temperature. In regard to mean development time, this result was as expected, as thermal plasticity is pronounced in ectotherms, and generally increases the speed of biological processes (Roff 2002; for killifish diapause specifically, see Levels and Denucé 1988; Podrabsky et al. 2010; Furness et al. 2015a). For three of the species in our study (*S. constanciae, P. schindleri,* and *Ne. whitei*), we found this expected pattern of ectotherm development, with higher rearing temperatures decreasing developmental times. In general, phenotypic plasticity is thought to correlate with thermal variability (Stillman and Somero 2000; Angilletta 2009), as species encountering a wide range of thermal conditions should be adapted to cope with a wider range. Indeed, two of the species for which we observed the expected negative correlations between diapause duration and ambient temperature are the most temperate-living, of the Neotropical species (*S. constanciae, Ne. whitei*).

While we did find some clear patterns between incubation temperature and mean development time of eggs, patterns of temperature-mediated changes in the variation of developmental time *(coefficient of variation)* were less clear. However, there were some significant differences among the variations estimated for each thermal incubation regime. Again, differences among the coefficients of within-species variation, across thermal regimes, tended to occur in more temperate species (South: *S. constanciae, Ne. whitei,* North: *M. robustus*). However, across these species there was no clear directionality in how the different temperatures affected the coefficient in variation for development time. For example, while *M. robustus* had the highest variation in the intermediate incubation temperature (23°C), *S. constanciae* had the lowest variation in this intermediate temperature. Our results hence suggest that evolution under more temperate thermal regimes both increases the evolution of thermal plasticity, and decreases the canalization of traits. However, it is difficult to make any inferences on the adaptive value of increased variations across the different temperatures.

Factors such as maternal age (Podrabsky et al. 2010), egg-laying order (Polačik et al. 2016a), and hormonal levels in mothers (Pri-Tal et al. 2011) have been reported to influence variance in developmental rates of the annual killifish offspring. We found no evidence that such maternal factors influenced the developmental times in our study (maternal age and group/single breeding). Furthermore, while we did not have sufficient statistical power to test whether females, or males,

differed in terms of variation in the development time of the eggs they produced, species variance accounted for large part of the variation in the model, with little influence of male and female effects. This suggests that species have evolved different development times due to differences in their natural environment. In addition to maternal influence, several other environmental factors have been found to induce plastic effects on the length of embryonic development, such as temperature (Levels and Denucé 1988; Podrabsky et al. 2010; Furness et al. 2015a), photoperiod (Levels and Denucé 1988; Podrabsky and Hand 1999; Furness et al. 2015a), hypoxia (Inglima et al. 1981), and the presence of other fish in aquaria with developing embryos (Inglima et al. 1981; Levels et al. 1986). While these factors are mostly standardized in our experiment, we cannot exclude the possibility that they affected our results. In our study, we kept developing embryos in water (solution), which allowed for highly standardized conditions, but which could decrease development times (Polačik et al. 2016b). We cannot exclude the possibility that species differ in how they respond to rearing in water, which could in part, explain our results. Finally, we used captive bred fish populations, which may mean that some of the species could be relatively inbred. However, all but one species has been collected in the past 11 years, suggesting that inbreeding effects have had a relatively short time to accumulate (Table S1, supplementary information 1). Further, as inbreeding is considered to decrease genetic variation, we note that virtually all of the species showed considerable levels of variation in development times (CV >20). Hence, we do not believe that inbreeding has driven the patterns observed in our results.

In conclusion, we found that means and variation in developmental times differed among seven closely related annual killifish species, irrespective of environmental predictability. Moreover, we found among- and within-species differences in response to temperature treatments, with more pronounced changes in developmental time than developmental time *CV,* and with species from more temperate areas being more plastic, compared to species from more tropical areas. Although we were unable to pin-point the exact causes of the observed differences, they could possibly depend on differences in environmental conditions.

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Data archiving

Data will be deposited to the Dryad archive together with article publication.

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Figures

1

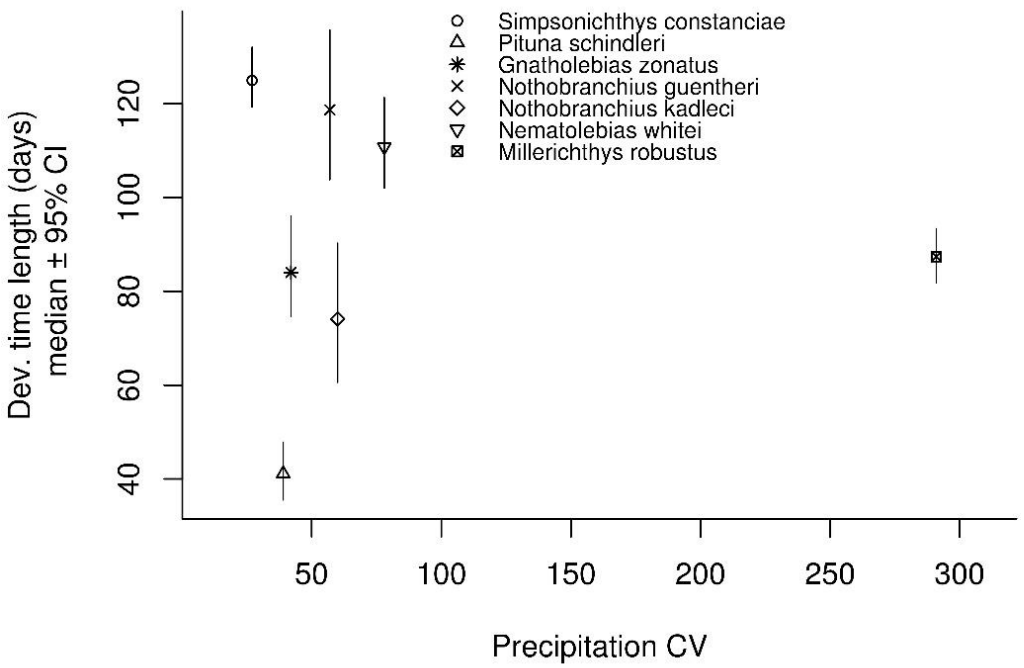

Figure 1. Species-specific medians of posterior distributions of development time length, and their 95% credibility intervals (y-axis), against precipitation CV (x-axis).

2

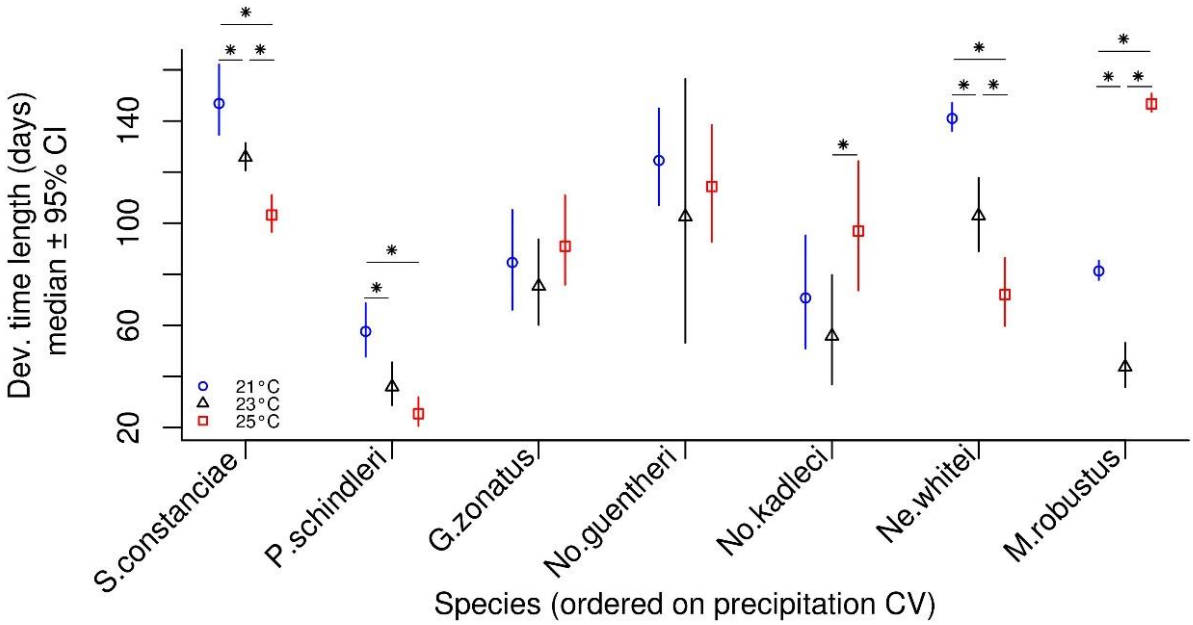

Figure 2. Species- and rearing temperature-specific medians of posterior distributions of development time length, and their 95% credibility intervals (y-axis). Species are ordered on a categorical x-axis scale, according to precipitation *CV* values, from the lowest (left) to the highest (right), for clarity of the results. Star symbols indicate significant within-species differences between the temperature treatment groups. A similar plot, but with continuous x-axis scale can be found in the supplementary material 4, Fig. S1.

3

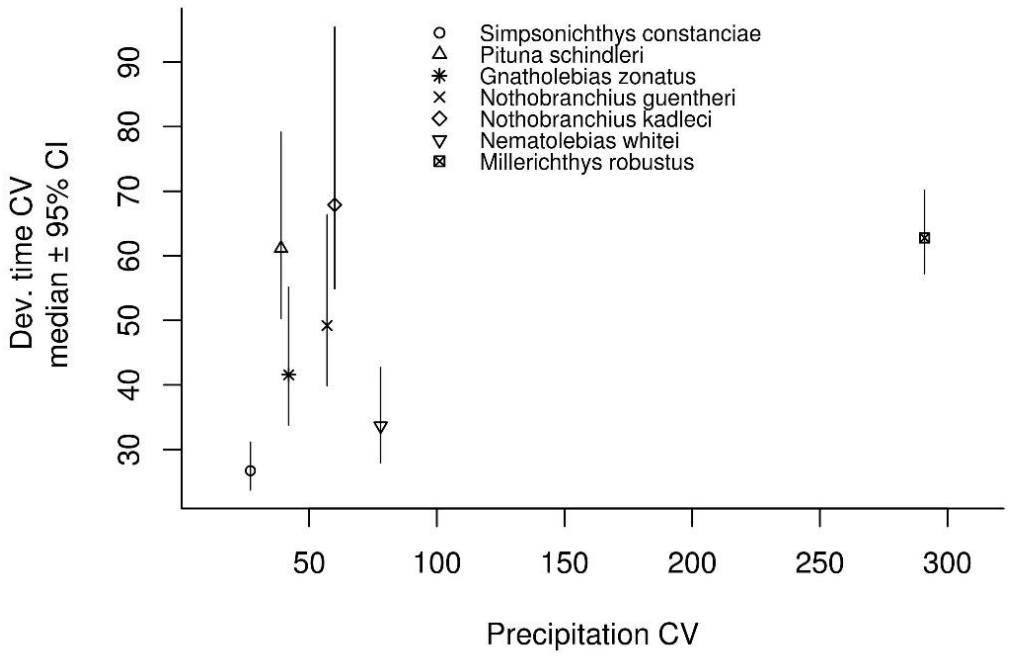

Figure 3. Medians of posterior distributions of species-specific coefficients of variation in development time, and their 95% credibility intervals (y-axis), against precipitation *CV* values (x-axis).

4

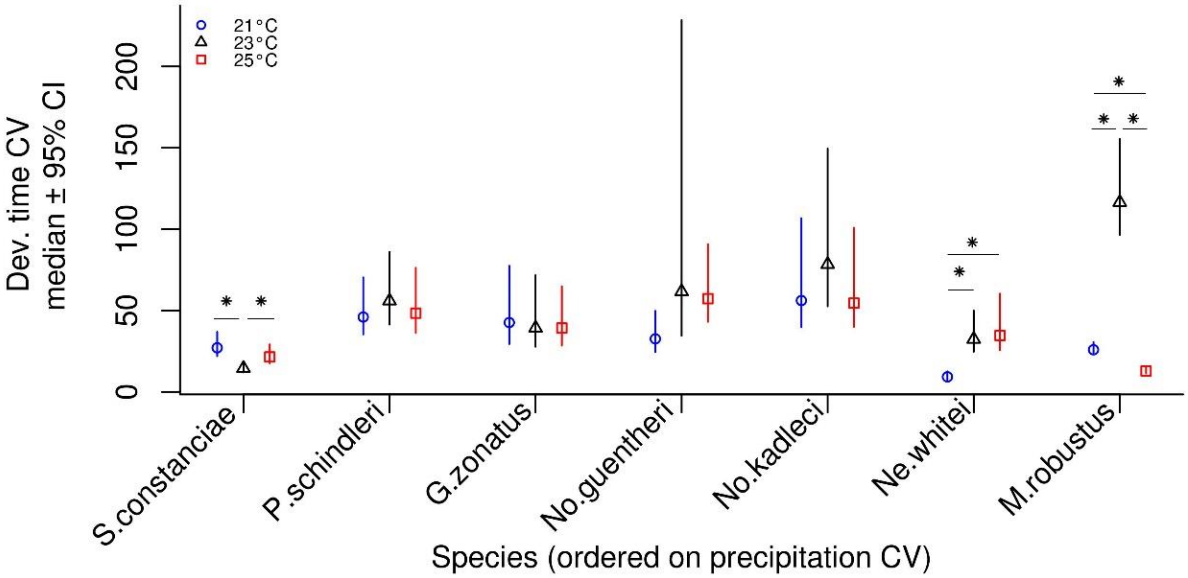

Figure 4. Medians of posterior distributions of species- and temperature-specific coefficients of variation in development time, and their 95% credibility intervals (y-axis). Species are ordered on a categorical x-axis scale, according to precipitation *CV* values, from the lowest (left) to the highest (right), for clarity of the results. Star symbols indicate significant within-species differences between the temperature treatment groups. A similar plot, but with continuous x-axis scale can be found in supplementary information 4, Fig. S2.