Desiccation plasticity and diapause in the Argentinian pearlfish *Austrolebias bellottii*

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Abstract

Background: The annual life history strategy with diapauses evolved repeatedly in killifish. To understand their and to characterize their variation between species, patterns of desiccation plasticity seem central. Plasticity might have played a role in the origin of these developmental arrests, when annual fish evolved from non-annual ones. The consequences of desiccation on survival and developmental rates of embryos of annual fish are poorly known. Using detailed demographic modelling of embryonal life histories, we investigate plasticity for desiccation in the Argentinian pearlfish *Austrolebias bellottii*. The treatment protocol contains changing environmental conditions with successive phases of mild desiccation and rewetting.

Results: We observed no clear diapause II and thus no increased incidence caused by mild and prolonged desiccation. Embryos arrest development in the pre-hatching stage (DIII) or in the dispersed cell phase (DI) irrespective of environmental conditions. There are limited effects of desiccation on survival, limited developmental delays and an acceleration of development into the pre-hatching stage. We found significant parental variance components on developmental rates, but hardly any effect of parental age. Hatching probabilities increased with age, when embryos had been in air at 100% RH and increased further when embryos were rewetted a second time after a two month interval.

Conclusions: Mild desiccation and rewetting affect survival, rates of development and hatching probability, but not the fractions of embryos that arrest development in particular stages. We can conclude that the incidences of diapause have become relatively independent of the occurrence of mild desiccation, as if they have become assimilated. In contrast to the responses to mild desiccation observed in the non-annual rivulids, *Austrolebias* accelerates development into the pre-hatching stage.

46 Introduction

47

48 Diapause, where the progress of development is interrupted or halted, is a trait used to explain that
 49 evolution can favour delaying strategies and bet-hedging (Philippi and Seger 1989, Stearns 2000,
 50 Evans and Dennehy 2005). Diapause is not an instantaneous, easily reversible response to adversity
 51 (Danks 1987), but a developmental and metabolic arrest that can also occur in the absence of
 52 adverse environmental conditions. A decrease in metabolic activity during diapause allows embryos
 53 to survive such periods for long. These two main characteristics differentiate diapause from other
 54 types of developmental arrest or delaying strategies such as quiescence (which only happens in the
 55 presence of adverse environmental conditions) or delayed hatching (which does not encompass a
 56 metabolic arrest and is eventually deleterious for the embryo, Darken et al. 1998). Individual
 57 variability in such strategies can have components of bet-hedging and (transgenerational) plasticity
 58 together with genetic variation (Haccou and Iwasa 1995, Van Dooren and Brendonck 1998, Furness
 59 et al. 2015a, Polacik et al. 2017), adapting trait determination and rates of development to regimes
 60 of environmental variability. The study of individual life histories where diapause can occur
 61 therefore presents an excellent opportunity to link proximate drivers and properties of
 62 developmental processes with demography and long-term fitness or even phylogenetic evolutionary
 63 processes of species selection (Helmstetter et al. 2016).

64

65 Annual killifish are oviparous fish species from the suborder Aplocheiloidei (Berois et al. 2015)
 66 which use diapause to persist in temporary ponds of Africa and South America. These ponds dry
 67 out seasonally, killing all adults and juveniles present. Diapausing embryos buried in the soil
 68 survive the dry period and can hatch when the opportunity presents itself. This "annualism" life
 69 history has evolved repeatedly (Furness et al. 2015b, Helmstetter et al. 2016). Because fish eggs are
 70 transparent, the developmental dynamics of these embryonal life histories can be studied well in a
 71 non-invasive manner. Diapause in annual killifish can occur in three developmental stages
 72 (Wourms 1972a,c). Diapause I occurs between epiboly and the onset of embryogenesis, diapause II

73 in the long-somite embryo, and diapause III occurs when the embryo is completely developed and
74 ready to hatch. Diapause I takes place in a unique developmental stage after epiboly where the
75 blastomeres individually disperse, and diapause II can coincide with a delay in the development of
76 anterior structures with respect to the rest of the body (Podrabsky et al. 2010, Furness et al. 2015b).
77 It is currently impossible to synonymize annualism with the occurrence of any of the separate three
78 diapauses, because data on their occurrence and obligate or facultative character are lacking for
79 many annual fish species. Among annual species studied thusfar, the presence of diapause I, II, and
80 III and the different environmental cues that affect their onset, duration, and termination seem
81 variable (Wourms 1972a,b,c, Markofsky and Matias 1977, Markofsky et al. 1979, Inglima et al.
82 1981, Matias and Markofsky 1984, Levels and Denuce 1988, Furness et al. 2015b).
83 Wourms (1972c) and Varela-Lasheras and Van Dooren (2014) have suggested that the evolution of
84 plasticity has played a role in the evolution of the diapauses in annual fish and therefore that they
85 might have originated in a scenario of genetic assimilation (Waddington 1942). Such a scenario is
86 characterized by a temporary increase in the strength of phenotypic plasticity (reaction norm slope,
87 Lande 2009), which then becomes reduced again or disappears. We previously found that non-
88 annual Rivulids slow down early development in response to mild desiccation, and that hatching
89 can be delayed over a prolonged period (Varela-Lasheras and Van Dooren, 2014; see also Furness
90 2015). Embryos with delayed hatching and decreased activity responded to a cue which provokes
91 hatching in annual killifish such that the hatching delay observed showed several characteristics of
92 diapause III. According Furness (2015), true annualism involves the occurrence of diapause I and
93 II, and delayed hatching and some desiccation resistance would be commonly present in annual and
94 non-annual species that encounter edge or marginal habitats with a risk of desiccation. To
95 understand the evolution of the diapauses in killifish and to characterize variation between species,
96 patterns of desiccation plasticity therefore seem central.

97

98 For annual killifish, however, there are no data allowing a detailed analysis of rates of development

and survival in different developmental stages during and after a desiccation period. Podrabsky et al. (2001) found that embryos of the annual *Austrofundulus limnaeus* in diapause II can survive desiccation when air relative humidity (RH) is below 85% for over a month, whereas embryos in other stages did not survive for more than two weeks. Mortality was low and differences between developmental stages seemed small to negligible when RH was 85% or higher. This study did not assess effects of desiccation on the further development of the embryos that experienced desiccation and embryos were not returned to more humid conditions. Wourms (1972c) contains a brief statement that eggs of the Argentinian pearlfish *Austrolebias bellottii* (Steindachner 1881), an annual killifish from the sister taxon of non-annual rivulids, enter diapause I and II more often when subjected to partial desiccation, but data were not presented.

To investigate responses to desiccation of an annual rivulid related to the non-annuals investigated previously, we exposed embryos of the Argentinian pearl killifish *Austrolebias bellottii* in different stages of development to a period of mild desiccation and analyzed the effects on stage-specific survival and developmental rates, and on diapause incidence. Embryos were rewetted after desiccation, to investigate effects on hatching probability, and lagged effects on the further life histories of unhatched embryos after rewetting. The approach used to analyse the data is akin to the modelling proposed by Atchley et al. (1994), in that we mainly investigate and model rates of development (Varela-Lasheras & Van Dooren 2014). However, this is combined with modelling of survival patterns to obtain an integrative demographic model for the developing individuals.

Methods

Housing and data collection

The twenty-eight individuals of *Austrolebias bellottii* used in this experiment (14 males and 14

124 females from different family lines) were descendants from a population of 50 individuals collected
 125 in Ingeniero Maschwitz, Argentina in 2004 and hatched from eggs collected in 2008. There were
 126 two groups of pairs with an age difference of about 4 months (hatched two or six months before the
 127 start of egg collection, on April 20, 2010). Pairs were kept in 20-L aquaria in a climate room at
 128 19°C with a 12-hour light/dark cycle. The aquaria contained some plants (e.g. *Elodea densa*,
 129 *Vesicularia dubyana*, *Lemna minor*) and a black, plastic 2L container half-filled with boiled coco
 130 peat where the fish could hide and lay eggs.

131 The day before collecting eggs, a clean container with 300 ml glass beads and 300 ml peat granules
 132 (both sieved and boiled) replaced the other container (as in Moshgani and Van Dooren 2011).
 133 Twenty-four hours later, this container was removed, the eggs collected and placed individually in
 134 wells of a 24-well plate with 1 mL of UV-sterilized tap water (DUNEA Leiden, The Netherlands)
 135 per well. Each clutch was divided over two different multiwell plates and placed in a different
 136 climate room with a temperature difference of 1 degree between them (19.5C vs. 20.5C). Next to
 137 the control treatment, which contained eggs continually incubated in water, we exposed the eggs to
 138 different “desiccation” treatments, limited to the range where expected survival of most stages
 139 would allow developmental responses to be observed (Podrabsky et al. 2001). We set up desiccators
 140 with demineralized water and saturated salt solutions of KNO₃, NH₄H₂PO₄ and KCl that would
 141 generate air humidities from 100 % relative humidity (Water), 94-93%, 93% and 86 %, respectively
 142 (O'Brien 1948). The humidity levels were verified with dataloggers (Gemini Tinytag PlusII,
 143 <https://www.geminidataloggers.com>) which could only confirm for KCl that the relative humidity
 144 was below 100% and at 85%. Some loggers might have failed to capture near 100% humidities
 145 reliably, due to wear on the probe or limited precision at high humidities, or some solutions were
 146 insufficiently saturated and did not produce the expected regime. For this reason, we decided to
 147 analyse differences between the different desiccation regimes with categorical variables and not by
 148 means of regressions on RH levels. Developmental states were determined minimally once per
 149 week for each individual embryo, except for one longer interval in summer (Figure 1). Eggs were

150 transferred to the different desiccation treatments at varying ages between 17 days and 50 days. The
 151 desiccation treatments lasted between 51 and 94 days. Treatments and control were terminated by
 152 replacing the water in the wells with a mix of tap water, demineralized water and peat extract to
 153 promote hatching (Varela-Lasheras and Van Dooren 2014), and this event was followed-up over
 154 successive days. Alevins that hatched were removed from their well on the same day.
 155 Approximately thirty days later the water in the wells was replaced with sterilized water again for
 156 the eggs that had not hatched and there were no further observations made during a period of 61
 157 days. The hatching procedure was then repeated (112 to 119 days after the first rewetting) and
 158 hatching scored again. In all, embryos experienced environmental sequences with up to four
 159 changes and were followed for up to 244 days.

160 Based on previous studies (Wourms 1972a, Varela-Lasheras and Van Dooren 2014), individual
 161 state was scored as "dead" or as being alive in one of five developmental stages that can be
 162 distinguished well (Fig. 1). Briefly, embryos were assigned to stage one when collected. A neural
 163 keel and the first somites appearing delineate the start of stage two, the presence of optic cups the
 164 transition to stage three and the pigmentation of the eyes the transition to stage four. Finally, stage
 165 five starts when the embryo completely surrounds the yolk sac and ends when the fry hatches or
 166 dies. For embryos in stages four and five, we noted at each observation whether the tail coiled over
 167 the left or right side of the head.

168

169 **Statistical analysis**

170 Developmental life histories consist of time-to-event data. The age intervals embryos spend in each
 171 developmental stage and environment before either moving into the next stage, hatching, or dying
 172 are the durations on which our analysis was based (Fig. 1). Events (deaths, developmental
 173 transitions) are assumed to have occurred just before they are observed. The data were analyzed

174 using Cox proportional hazard models with random effects, using libraries survival and coxme in R
 175 (Therneau and Grambsch 2000, Therneau 2015). We modeled all transition rates per stage
 176 separately, that is, the mortality rates per stage and per-stage developmental transition rates to the
 177 next stage (Varela-Lasheras & Van Dooren 2014). The models per stage in the main text combine
 178 all durations in the different environmental conditions, so that effects of environmental conditions
 179 can be tested by removing them from the model. In the supplement, results are presented for models
 180 where the durations were analysed separately per environment (Control/Desiccation
 181 Treatments/Rewetted) or censored before the long inter-observation summer interval. In some
 182 environment/stage combinations, we had very few individuals at risk and therefore either did not fit
 183 any separate models to the data, or Cox models with fixed effects only. We also analysed some
 184 zero-one responses or proportions of states present at specific time points using logistic regressions.
 185 In order to give an overview of individuals in different stages depending on age, we used multi-state
 186 estimation of fractions per stage and made plots of these fractions using the mstate library for R (de
 187 Wreede et al. 2011).

188 **Survival.** Per stage, we first fitted a Cox model with period (control "Wet"/ desiccation "Dry"
 189 /"Rewetted") and salt treatment ("H2O" / "KNO3" / "NH4H2PO4" / "KCL") fixed effects, their
 190 interactions and temperature and parental age (categorical, "Young" / "Old") fixed effects. For
 191 stages two to five, we added the duration spent in the preceding stage as a covariate. The model
 192 contained a parental pair random effect, a random effect of collection date and a plate effect. This
 193 model was then simplified by removing effects that were not significant in a chi-squared likelihood
 194 ratio test. We started model simplification by inspecting the significance of random effects,
 195 followed by tests on the fixed effects. We sequentially tested and deleted non-significant effects in a
 196 fixed order: first the tests for pair variation, collection date, plate variation; then previous duration
 197 effects, salt treatments, the period effect, temperature and age effects. When an effect was
 198 significant in a likelihood ratio test but the parameter estimate had a very large standard error or
 199 there were data lacking to estimate a subset of parameters, we also removed that effect from the

200 model or analysed a subset of the data. We encountered numerical problems fitting models with
 201 random effects that had different variances per period. Therefore we could not test directly whether
 202 these variances depend on the environmental state and analysed the data per period separately as
 203 well. The models in the Supplement fitted to the data per separate stage and period (i.e. without
 204 period effects) underwent the same model selection procedure.

205

206 **Development.** The data on developmental rates were analysed starting from models containing the
 207 same maximal models as for survival and we tested effects in the same order. For each separate
 208 analysis per developmental stage, age within stage is used as the appropriate time scale.
 209 Spontaneous hatching from stage five was modeled as a developmental transition, while hatching
 210 induced by rewetting caused censoring.

211

212 **Fraction in diapause.** We investigated whether the distribution of embryos across stages at the
 213 moment of rewetting and at the end of the experiment depended on previous individual history. We
 214 fitted generalized linear models (glm's) to the data, analysing the multinomial data distribution with
 215 a set of binary logit models, each for the fraction of embryos in an earlier developmental stage
 216 relative to the number in stage five (baseline category logit models, Agresti 2013). Mixed binary
 217 models did not converge well here, we therefore restricted the analysis to fixed effects of
 218 desiccation treatment, time spent in the treatment and their interaction, and added age and
 219 temperature main effects. We used the inverse of the time spent in a treatment as an explanatory
 220 variable such that the intercepts per treatment became the expectation for when embryos would
 221 have been in the treatment for an infinite amount of time. Model selection was carried out using
 222 likelihood ratio tests, as the data were 0/1 individual responses with no need for overdispersion
 223 corrections.

224

225 **Coiling.** For embryos in stage four and five we repeatedly observed the direction in which they
 226 coiled their tail over the head, and used the rate of change in direction as a proxy for activity
 227 (Varela-Lasheras and Van Dooren 2014). We estimated how the probability of a transition between
 228 left and right-coiling depended on treatment, parental pair, and time spent in stages four or five. We
 229 used glm's for the occurrence of a change in coiling (positive response) and with a complementary
 230 log-log link, so that by means of an offset we could correct for the duration between observations
 231 (Harney et al. 2013).

232

233 **Hatching.** The probabilities of hatching when water with peat extract was added are reported. We
 234 analysed these probabilities of hatching using binary logit generalized linear models. Individuals
 235 that hatched within three days of adding peat water were scored as a positive response, the ones that
 236 remained in stage five as non-hatchers. Individuals that were found dead in this time interval were
 237 not included in the analysis, as it was difficult to determine whether they had actually hatched or
 238 not. This model contained as explanatory variables effects of temperature, collection date, date
 239 where hatching water was added, desiccation treatment, parental pair, total age, age in stage five,
 240 time spent in treatment and interactions of desiccation treatment effects with all three time
 241 variables. Model selection was carried out using likelihood ratio tests.

242

243 **Results**

244

245 We collected and incubated 2161 eggs in total, 539 embryos were subjected to desiccation
 246 treatments (including air at 100% humidity) and 599 embryos from the Control and desiccation

247 treatments were rewetted. The median number of observations per embryo was 16 and the
248 maximum 31. Table 1 shows that the desiccator with KCl contained fewer embryos at risk than the
249 other treatments. We decided to do that to minimize individual losses at the strongest level of
250 desiccation in this experiment.

251

252 **Survival.** The data represented in Figure one are first used to draw survivorship curves (Figure two,
253 A-E). For age intervals where these curves are steep, mortality rates are high. Where they become
254 flat, mortality becomes negligible. Figure two shows that, overall, survival is highest for pre-hatching
255 embryos (Fig. 2E) and lowest for the earliest developmental stage (Fig. 2A). What is also apparent
256 is that the death rate decreases with age to become zero in most stages and treatment groups. By the
257 age where all surviving embryos are in the rewetting phase, there are hardly any new deaths
258 occurring. Table 2 summarizes which variables were retained in the models for survival per stage,
259 For comparison, Table S1 does that for a dataset restricted to the observations made before the start
260 of the single long interval in between observations (days 96-133, Fig.1). For stage two and stage
261 five, there are significant fixed effects. Other treatment effects suggested by Fig. 2 were not
262 significant. Embryos that are desiccated in stage two have an increased death rate (c.i. [0.11, 1.49])
263 and embryos which have previously spent a longer duration in stage one have a decreased death rate
264 (c.i. slope [-0.12, -0.02]). In stage five, there is negligible mortality in the wet phase (Fig. 2 and
265 Figure Surv5 in the Supplement). Death rates in the pre-hatching stage are increased by any of the
266 desiccation regimes, even when eggs were in air with 100% RH (increase in mortality rate for H₂O
267 c.i. [0.33, 3.00])). Confidence intervals for mortality rates of KNO₃ (c.i. [2.45, 4.81]) and
268 NH₄H₂PO₄ ([2.51, 4.87]) desiccation regimes overlap with H₂O, for KCL it does not (c.i. [3.68,
269 6.31])). It needs to be noted that the order of mortality rates is according the intended strengths of
270 desiccation. In the pre-hatching stage, mortality increases with temperature (c.i. [0.89, 1.81]). The
271 supplementary material contains Table S3 and figures of survivorship curves per stage and per

272 period (Wet/Dry/Rewetted). These results suggest that the lower survival in the driest desiccator
 273 was caused by a briefly elevated mortality at the start of the rewetting phase. There are no
 274 significant effects of parental age (Table 2). However, in stage five and when each period is
 275 analysed separately (Table S3), we recover parental age effects not found when the data are jointly
 276 analysed across periods. Pre-hatching embryos with older parents have lower mortality in the dry
 277 period (parameter estimate -1.49 (0.42)) but a higher mortality in the following rewetted period
 278 (1.43 (0.75)) resulting in no net overall effect.

279 When we inspect the results on the random effects, then variances between parental pairs and
 280 between plates are significant in stage one only, while random effects of collection date are present
 281 in three out of five stages (stages one, three and four).

282

283 **Development.** Figure 2 F-J represents the proportions of embryos that have made a developmental
 284 transition depending on the time already spent in each stage. For cohorts of embryos in stages one
 285 and five, the rate of development or the probability of spontaneous hatching become zero before all
 286 individuals have made a developmental transition (Fig. 2F, J), showing that diapause I and III occur
 287 irrespective of mild desiccation. There is a small fraction of abortive hatchings from stage five.
 288 Diapause II is not observed as a levelling off in the fraction that made a transition (Fig. 2H), even
 289 with prolonged but mild desiccation. What we do see is that the curves for development in stage
 290 two, three and four all show a period of about twenty days between days 20 and 50 with fewer
 291 developmental transitions. We found out that this is due to the occurrence of the single long time
 292 interval between observations. When data were censored before that interval, there is no such period
 293 and we conclude that there are no slow/fast alternative developmental trajectories in these stages.
 294 For the shortened dataset and relative to the Control, embryos in saturated air develop significantly
 295 slower (-0.37, s.e. 0.17) from stage one into two. However, the effect is not clearly visible or strong
 296 in graphs, there are no significant effects for the other treatment levels and the effect is not detected

when the complete data are analysed. We therefore denote it as spurious. Desiccation changed the developmental rate from stage two into stage three relative to wet conditions (Table 3 and parameter estimates H₂O 0.27 (s.e. 0.23); KNO₃ -0.21 (0.25); NH₄H₂PO₄ -0.57 (0.26)); KCL 1.51 (s.e. 1.06). This pattern of estimates suggests a non-linear effect of desiccation intensity, but that suggestion strongly depends on the estimate for KCL which has large sampling variance and is therefore the least reliable. The pairwise difference from the control group is only significant for the NH₄H₂PO₄ treatment. These differences are not discernible in Fig.2G where individuals in Control, desiccation and rewetted regimes are pooled for most of the time points. However, they are visible in Figure SDev2 in the Supplement. Individuals that spent more time in stage one have a slower development rate from stage two into tree (Table 3, slope -0.030 (0.006)). Figure Dev4 in the supplement suggests that for developmental rates from stage four into five, KCL and the control groups have a lower rate of development than other treatments. The mixed Cox models confirm this, with development into stage four significantly increased relative to Control in a desiccator with H₂O (1.73 (0.41)) KNO₃ (1.49 (0.43)) and NH₄H₂PO₄ (1.18 (0.46)). The rate of development in stage four tends to be accelerated for embryos that have spent more time in the preceding stage (slope 0.044 (0.024), Table 3, TableS2, S4). The rate of abortive and spontaneous hatchings from stage five is significantly smaller for embryos that are in the desiccators (parameter estimate -2.24 (0.72)) and there are no abortive hatchings in the rewetting phase (Figure SDev5 in the Supplement).

We find significant variation between pairs for the first and fourth stage (Table 3). Plate effects for developmental rates occur in slightly more developmental stages than for survival rates (Table 3, S2, S4).

319

Multistate overview We made an overview figure of fractions of embryos in different states across time with different panels for the different periods (Figure 3). The panels again show the decreases in rates when embryos have been a while in a treatment – the boundaries between states become

323 more horizontal. The amount of events is overall small in the rewetting period. Among the
 324 individuals that didn't hatch, stage five embryos are the most numerous at the end of the
 325 experiment, suggesting that in the environments we investigated, diapausing individuals are mainly
 326 of diapause type III after some time. Note also that among the rewetted embryos, there is a small
 327 fraction of individuals in stage three. This could not be seen in Fig. 2H, where the age-within-stage
 328 intervals for desiccated and rewetted individuals overlapped nearly completely for that stage.

329

330 **Distributions across stages.** At first rewetting, 42 individuals were in stage one, 6 in stage two, 19
 331 in stage three, 7 in stage four and 525 in stage five. We only analysed fractions in stages one and
 332 three relative to stage five using glm's. For the fraction of embryos in stage one relative to stage five
 333 at rewetting, we found a trend for the *time in treatment* $\times$ *treatment* interaction (chisq 10.79, df = 5,
 334 $p = 0.06$). This suggests that the speed at which the proportion of embryos in stage one stabilizes
 335 relative to the fraction in the pre-hatching stage differs between treatments. However, there are no
 336 intercept terms that differ significantly. There is therefore is no parental age effect on the eventual
 337 fraction in stage one, if the time spent in treatment is taken into account. The fraction of embryos
 338 that are in stage three at rewetting is smaller for older parents (effect estimate -2.06 (s.e. 0.53);
 339 chisq 17.8, df = 1, $p < 0.001$). The parental age effect on the fraction in stage three persists when
 340 time in treatment is included in the model. For the distributions over stages at the end of the
 341 observation period, we find the same effects. We can interpret this at most as different speeds
 342 within treatments at which the remaining individuals settle in diapauses I and III, but no consistent
 343 treatment differences in the eventual fractions. When we represent the distribution over stages at
 344 first rewetting in a graph (Figure 4), it can be seen that the older parents contributed more
 345 individuals overall and in particular in the pre-hatching stage, as they produced larger clutch sizes
 346 sooner. At the end of the period where we observed individual state regularly, 137 of the stage five
 347 individuals had hatched or died.

348

349 **Hatching** On average, the hatching probability of the Control group where embryos were in water
 350 at all times is 12%. For the H₂O group it is 27%, in the KNO₃ desiccator the hatching probability is
 351 13%, from NH₄H₂PO₄ 12 % and there was no hatching in the KCL dessicators. Mixed models for
 352 hatching probabilities did not converge well, and we refitted them as glm's. For the earliest set of
 353 days where peat water was added, we found significant effects of collection date ($\text{chisq}(11) = 40.29$,
 354 $p < 0.001$), a time in treatment $\times$ treatment interaction ($\text{chisq}(5) = 29.46$, $p < 0.001$), and a negative
 355 parental age effect ($\text{chisq}(1) = 5.08$, $p = 0.02$). In the H₂O treatment, the intercept term is increased
 356 relative to the control (H₂O 6.33 (s.e. 3.06)).

357 When we inspected embryos before the second rewetting (Table 4), a fraction of the eggs had
 358 developed further starting from stages one to four. Other embryos had remained in the stages where
 359 they were two months before, which was the case for stages one, two, three and five. Survival did
 360 not differ significantly between stages. Overall survival was 83 % across this period. When peat
 361 water was added, 48% of the stage five individuals hatched. We found significant effects of
 362 temperature ($\text{chisq}(1) = 5.02$, $p = 0.02$) and of the date where the embryos were rewetted before
 363 ($\text{Chisq}(3) = 30.52$, $p < 0.0001$). For embryos stored at 20.5 degrees, the hatching probability was
 364 reduced (parameter estimate effect -0.56 (s.e. 0.25)). Two first rewetting dates had significantly
 365 reduced hatching probabilities at the second rewetting (parameter differences approx. -1.5).

366

367 **Coiling.** We estimated rates of change of the tail coiling orientation using logistic regression, with a
 368 glm as in Harney et al. (2013). In stage four, we found a significant treatment $\times$ time in stage
 369 interaction ($\text{chisq}(4) = 908.84$, $p < 0.0001$) and differences between parental pairs ($\text{chisq}(6) = 13.59$,
 370 $p = 0.035$), but none of the parameter estimates were significantly different from zero. When
 371 simplifying the model to a single effect of time within age, we found that it was non-significant.
 372 In stage five, there is a significant treatment $\times$ age within stage interaction ($\text{Chisq}(3) = 9.072$, $p =$
 373 0.0284), but again no separate parameters could be shown to be significantly different from zero.

374 For the group desiccated with $\text{NH}_4\text{H}_2\text{PO}_4$ the probability tends to be lower (-1.48, 0.82, $p = 0.07$).
 375 Simplifying to a single covariate - age within stage five - we find that the probability of changing
 376 direction decreases with age in stage five (parameter estimate (-0.092 (s.e. 0.038)). According to
 377 this simple model, at age-within-stage zero the probability to change coiling direction for stage five
 378 is 5% per day, twenty days into stage five it is 0.8% and at day forty 0.1 %. For stage four, the
 379 probability to change coiling direction is 7% per day.

380

381 Discussion

382

383 We observe that irrespective of whether environmental switches were imposed, a general slowdown
 384 in rates of events and no further deaths nor developmental transition events were observed when the
 385 environment remained constant for a prolonged time. It is as if all rates become as good as zero and
 386 the developmental system slows down for an extended period of time, while mortality is arrested.

387

388 In the Argentinian pearlfish, embryos enter diapause I and III irrespective of the presence of mild
 389 desiccation and before transition rates become zero overall. Diapause II is at best rare and does not
 390 increase in frequency with desiccation. We do observe embryos that spend substantial periods in
 391 stages two and three, after water has been replaced two months before the second rewetting, but is
 392 unclear whether that is due to overall low rates of development in that period. Our data suggest that
 393 long observation periods combined with repeated environmental changes will be necessary in
 394 *Austrolebias* to determine whether diapause II truly occurs. Whether an embryo has been
 395 developing slowly through a developing stage can have effects on subsequent speeds of
 396 development, but the effects are not systematically magnifying the range of variation in timings. We
 397 observe an acceleration of development and growth from stage four into five with mild desiccation.
 398 For the pre-hatching stage, the rate at which coiling changes gradually decreased with age-within-

stage, suggesting that overall levels of activity decrease during late development. This is in agreement with intensifying diapause III, while there were no significant desiccation effects. We observed an increased probability of hatching when embryos have been in the 100% RH environment. When the hatching protocol is repeated two months later, there are no remaining lagged effects of the desiccation treatment and embryos hatch again with a higher probability. In our experiment that was at an age of about six months. Is desiccation not provoking any delayed effects? We found overall effects of desiccation when data were analysed across periods, so we cannot exclude that desiccation has delayed effects during rewetting. When data are analysed per period separately, desiccation effects are not demonstrable in the rewetting period, due to the low number of events observed there, as all processes have already slowed down. We note that survival effects of desiccation were significant only for the pre-hatching stage, hence involving diapause III embryos.

Annual vs. non-annual rivulids

Striking here in comparison with previous results on species from the non-annual rivulid sister group (Varela-Lasheras & Van Dooren 2014) is that passing through a desiccation period has limited effects and can accelerate development. In non-annuals, delays were early in development and desiccation provoked an increased incidence of delayed hatching (similar or equal to diapause III) in stage five in some species. In the annual, the speed of developing near head formation was slowed after rewetting for one desiccation level, or showing a positive response to desiccation late in development. Regarding early development in the first stage, we only found a significant effect in a reduced dataset and for the weakest desiccation level, casting doubt on its existence. The desiccation regimes in this study are much prolonged in comparison to Varela-Lasheras and Van Dooren (2014), where the median duration was eight days, with visible effects after one hour. In non-annuals, desiccation significantly increased mortality overall in embryos in stage three and four and decreased in *C. magdaleneae* again after return to water. In the annual, developing embryos are quite resistant to the desiccation levels we applied as survival effects are limited to the pre-hatching

stage and we could impose mild desiccation as a prolonged treatment. Note that there are no escape embryos in the sense of Polacik et al. (2017) that reach the pre-hatching stage within a month. Both *Austrolebias* and non-annual rivulids show diapause III-like characteristics. This trait, very similar to delayed hatching, might be ancestral in the group. Slowing down during early development in non-annuals in response to desiccation could be mostly an immediate responses therefore quiescence, even while there are some lagged effects. If genetic assimilation of diapause has occurred in killifish via the evolution of plasticity to desiccation, then there has clearly been further plasticity evolution after the emergence of diapause-like characteristics. The plasticity now involves an accelerating effect.

The activity of pre-hatching annual embryos seems overall lower from the start in comparison to non-annuals where initially over 10% of individuals changed coiling per day. Also notable is that hatching probabilities were relatively low at the first rewetting and doubled at the second rewetting.

The three diapauses

Diapause II might be rare in *Austrolebias*. According to Wourms (1972c) diapause I is common in South-American *Austrolebias* killifish, whereas diapause III is obligatory and diapause II facultative, as in *Nothobranchius* (Wourms 1972c, Furness et al. 2015a). This would render the first and last diapauses the most defining characteristics of the annual strategy for the *Austrolebias* genus. Several papers recently brought new data and ideas on the diapauses in annual fish. Furness (2015) believes diapause I to have a short duration in benign conditions and to be mainly brought about by harsh conditions. Diapause II, after the phylotypic stage, would contribute most to variability in developmental duration (Furness 2015). Podrabsky et al (2001) found that *Austrofundulus* embryos resisted desiccation best in this stage. Diapause II at low temperatures is not nearly obligate as in *Austrofundulus limnaeus* (Podrabsky et al 2010), but at best facultative in *Austrolebias*. Given the results here, it is certainly not the most prominent stage of arrest in annual

452 fish as proposed by Furness (2015). In the Argentinian pearl killifish and in presumably benign
 453 conditions virtually all embryos showed behaviour classified as diapause III, a fraction diapause I,
 454 and diapause II is at best rare and potentially only occurs in older eggs.

455 Hatching of the stage five embryos was limited at the first wetting, the fraction hatching increased
 456 several months later. This calls into question the hypothesis that diapause III capitalizes on within-
 457 season hatching opportunities (Furness 2015), if the hatching response depends on age in this
 458 manner.

459 At first rewetting we had fractions of individuals in all stages. The stage three individuals might be
 460 in diapause II but flipped survivorship curves (Fig. 2H) suggest that there are no individuals
 461 remaining in stage two or three for long at that age. Our results are in agreement with Wourms
 462 (1973), that diapause II is not constitutive for this genus. Being more facultative than diapause III, it
 463 can not be equated to the annualism syndrome (Furness et al. (2015b). Diapause II shows strong
 464 plasticity in *Nothobranchius* (Furness et al. 2015a) and has negligible incidence in *Austrolebias* at a
 465 temperature where it is common in *Nothobranchius furzeri*. In Diapause II, development is arrested
 466 at a point where maintenance costs of the thusfar developed organs and structures might be
 467 minimized over prolonged periods of time. These costs might depend on the environment and
 468 diapause I and III might be better options for *Austrolebias*.

469 Our results demonstrate a scenario where all rates gradually become zero, and a distribution over
 470 stages is obtained that remains stable for some time. The rates of events before that point determine
 471 how many individuals there will be in each category when the cohorts are in pseudo-equilibrium.

472 We did not find evidence of systematically widening developmental variation leading to two
 473 alternative slow (diapause II) and fast strategies (no diapause II). However, it has not been clear
 474 which criteria were followed to assign embryos to diapause or direct-developing pathways (Furness
 475 2015a&b) and there has been some circularity in the data analysis, as developmental process
 476 outcomes were used as explanatory variable of the same process. Data on *Austrolebias nigripinnis*
 477 in Furness et al. (2015b) suggest that developmental trajectories are not very dichotomous between

embryos with direct development to a pre-hatching stage or diapause II, whereas direct developers in *Nothobranchius* have significantly larger heads). This suggests that next to presence of developmental delays, morphological embryonal life history traits have evolved differently in the different annual groups.

Parental effects

We found a very limited number of significant effects of parental age on the survival and development of embryos. However, we did find variation between parental pairs for survival and developmental rates, which could encompass parental effects. *Austrolebias* annual fish do have substantial egg size variability (Moshgani and Van Dooren 2011), even when average egg size does not depend on parental age. This might be a different manner in which parental effects on survival and development might operate.

Pitfalls

Our incubation protocol differed from incubation methods used by others, i.e., with eggs placed either on top of or under a small layer of peat moss. Alternative setups to study desiccation effects might be relevant for annual fish, but care should be taken in controlling environmental variability. In the field, it is expected that changes in soil dryness will make availability of oxygen and dryness covary, such that an experiment designed to separate oxygen and desiccation effects would be crucial to explore plasticity and survival effects of both. We incubated embryos in normoxia and with different levels of desiccation. With crossed desiccation and oxygen levels embryos might show different desiccation sensitivities depending on oxygen availability. Such a more involved experimental design would also allow a direct comparison of annuals and non-annuals, as the environmental design would include environments characteristic for either group, especially when a range of durations of desiccation are imposed. However, the amount of individuals required for

503 such an experiment seems forbidding without automated observation of survival and developmental
504 state.

505 Our data loggers did not allow us to demonstrate that we reached levels of desiccation precisely as
506 intended, and there might be other side effects of salts than by just controlling humidity levels. The
507 desiccators with different salts clearly smelled differently and it can be expected that molecules
508 enter the medium with embryos as well. If such effects were there, they seem to have been limited.

509 We reserved the term diapause for cohorts with an absence of events for a prolonged period, as we
510 did not use accessory individual data on their metabolism or the advance of morphological
511 structures which could have provided measures of diapause depth at the individual level. We did
512 record the changes in coiling direction of individual embryos finding that it occurred at a lower rate
513 than in non-annuals. We should try to correlate different invasive and non-invasive manners of
514 measuring metabolic activity, to see which non-invasive measures can be used as fast and reliable
515 proxies across a wide range of killifish species in the future.

516

517 **Outlook**

518 Timing is important in evolution, to match life histories to environments and to accelerate or delay
519 the pace of life when advantageous. For developmental processes, this has led to the definition and
520 study of heterochrony, an evolutionary change in the rate or timing of developmental processes
521 (Horder 2013), or heterokairy, plasticity for developmental timing (Spicer & Burggren, 2003). Our
522 analysis consisted of a detailed dissection of the developmental life history of embryos, and uses
523 time-to-event analysis for analysis. The demography was followed in full, by estimating survival
524 and rates of passing into next developmental stage. Such a dissection is important to understand
525 life history evolution, but a lack of statistical power can occur, because not all cohorts occur in large
526 sample sizes and the events occurring within them in certain environments cannot be controlled
527 easily. Contrary to Furness et al (2015a&b) and Polacik et al. (2017) we did not assign individuals

528 to alternative developmental trajectories to then redo an analysis of events that occurred before the
529 individuals could be classified. Models such as the ones selected here could be used to estimate
530 sensitivities of demographic parameters to environmental and genetic modification, which would
531 allow exploring where the strongest selection gradients on developmental processes are situated.

532

533 **Conclusions**

534 Mild desiccation and rewetting affect survival, rates of development and hatching probability in
535 *Austrolebias bellottii*, but not the fractions of embryos that arrest development in particular stages.
536 We can conclude that the incidences of diapause has become relatively independent of the
537 occurrence of mild desiccation, as if they have become assimilated. In contrast to the responses
538 observed in the non-annual rivulids, *Austrolebias* accelerates development into the pre-hatching
539 stage in response to mild desiccation.

540

541 **Declarations**

542 Animal Care and handling protocols were supervised by Animal Welfare committee of Leiden
543 Faculty of Sciences and Medicine. Our data and example scripts will be made available for analysis
544 on dryad. There are no competing interests and no funding to declare.

545 **Authors' contributions**

546 TJMVD conceived the study. IVL and TJMVD performed the experiments, analyzed the data, and
547 wrote the manuscript. Both authors read and approved the final manuscript.

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677 Figure one. An overview of the "raw" data. Embryos were classified into five developmental stages
678 as explained in the text, or as dead (this absorbing state is not represented). Per egg and stage, a
679 horizontal line is drawn between the days of the first and last observation in that stage. Eggs are
680 ordered within stage according to their collection day. A blue line is drawn for eggs that were
681 observed in the same stage at second rewetting (on day 264) and at the end of the period where
682 regular observations were made (day 189). Developmental transitions are drawn as grey lines.
683 They connect the last observation in a stage with the first observation in the next stage where the
684 egg was observed. Note that no observations were made between days 96 and 133. In the insets
685 depicting the different developmental stages, small triangles indicate the location of the embryo
686 when not clearly visible.

687

688 Figure two. Survival and development represented with survivorship curves. Each panel contains
689 the data for a specific developmental stage and has a separate survivorship curve per treatment
690 group. At many instances, embryos within the same stage were distributed over different
691 environmental regimes (control / dry / rewetted). Bars above panels indicate ages were embryos
692 were in that stage (grey, survival), in the desiccation regime (red) or rewetted (black).

693

694

695

696 Figure three. Proportions of embryos in different states (stages of development, death) in
697 dependence on their age. Proportions were estimated using multistate survival analysis. Wet, dry
698 (humid air) and rewetted periods are detailed in separate panels. Each panel only shows the
699 fractions of embryos in the different developmental stages for the focal environmental regime. In
700 the left panel, embryos that entered the dry and rewetted regimes are categorized separately as being
701 in that state. In the middle panel, the distribution of embryos in the dry regime over different states
702 (including death) and the fraction that entered the rewetted state are shown. In the right panel, the
703 distribution of embryos over stages in the rewetted regime are detailed.

708 Figure four. Distributions over stages at rewetting, for embryos in control (Left Panel) and
709 desiccation regimes (Rigt Panel). Embryos of older and younger parental pairs are represented
710 separately. There is a bar for each developmental stage we scored. These are ordered from one to
711 five, from left to right.

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713
714

Table1. Number of individuals at risk (number of developmental transition events, number of deaths within stage) per stage and period/salt used for desiccation.

Stage	Wet	KCL	KNO3	NH4H2PO4	H2O	Rewetted
1	2161 (595/1091)	8 (4/2)	62 (43/15)	48 (34/11)	101 (75/15)	42 (2/3)
2	535 (410/18)	6 (4/2)	57 (49/7)	54 (44/5)	111 (103/6)	13 (7/1)
3	422 (273/13)	9 (7/0)	91 (84/6)	77 (74/3)	145 (134/7)	21 (2/0)
4	242 (126/10)	21 (18/0)	107 (93/12)	96 (84/8)	154 (139/14)	15 (8/2)
5	199 (8/0)	28 (0/1)	111 (0/20)	106 (1/21)	162 (2/4)	536 (0/58)

Table 2. Survival analysis per stage across periods. "NS" indicates non-significant effects. Period × treatment interactions were never significant and therefore not listed. Significant effects are indicated by Chi-squared statistic and p-value. For the salts used, those which had significantly different parameter estimates from desiccators with water are given differently shaded cells.

Stage	Random effects			Fixed effects							
	Parent	Date	Plate	KCL	KNO3	NH4H2PO4	Salts	Periods	Temp	Age	Preceding
1	134.25 < 0.001	71.70 < 0.001	12.41 < 0.001				NS	NS	NS	NS	
2	NS	NS	NS				NS	5.19 0.023	NS	NS	11.67 <0.001
3	NS	9.08 0.003	NS				NS	NS	NS	NS	NS
4	NS	4.78 0.029	NS				NS	NS	NS	NS	NS
5	NS	NS	NS	+			157.02 <0.001	NS	37.25 <0.001	NS	NS

Table 3. Analysis of developmental rates per stage, across periods. "NS" indicates non-significant effects. Period $\times$ treatment interactions were never significant and therefore not listed. Significant effects are indicated by Chi-squared statistic and p-value. For the salts used, those which had significantly different parameter estimates from desiccators with water are given differently shaded cells. For stages written in bold, we did not include time intervals in the rewetted period in the analysis.

Stage	Random effects			Fixed effects							
	Parent	Date	Plate	KCL	KNO3	NH4H2PO4	Salts	Periods	Temp	Age	Preceding
1	79.68 <0.001	8.9 7 0.003	NS				NS	NS	NS	NS	
2	NS	8.55 0.003	4.53 0.033				10.34 0.016	NS	NS	NS	30.31 <0.001
3	NS	NS	7.46 0.006				NS	NS	NS	NS	NS
4	6.64 0.010	7.34 0.007	4.61 0.032				9.56 0.023	NS	NS	NS	3.21 0.073
5	NS	NS	NS				NS	11.39 0.003	NS	NS	NS

Table 4. Developmental stages at second rewetting. A two month-period separated the follow-up of embryos after a first rewetting from the second rewetting. Numbers of embryos are given for each combination of states at the end of the follow-up period and at the moment of rewetting.

	Stage at second rewetting					
	1	2	3	4	5	Dead
State at end of follow-up after first rewetting						
1	21	1	1	0	5	11
2 somites	0	3	0	0	1	1
3 head formed	0	0	8	1	7	3
4 pigmented	0	0	0	0	3	2
5 pre-hatching	0	0	0	0	309	79

Supplementary Material

Table S1. Survival analysis per stage censored before the long interval between observations. "NS" indicates non-significant effects. Period $\times$ treatment interactions were never significant and therefore not listed. Significant effects are indicated by the Chi-squared statistic and p-value. For the salts used, those which had significantly different parameter estimates from desiccators with water are given differently shaded cells. Period effects could not be fitted in most models.

Stage	Parent	Date	Plate	KCL	KNO3	NH4H2PO4	Salts	Temp	Age	Preceding
1	54.47 < 0.001	70.68 < 0.001	10.10 0.001				NS	NS	NS	
2	NS	5.08 0.024	NS				NS	NS	NS	8.51 0.004
3	NS	NS	NS				NS	NS	NS	NS
4	NS	NS	NS				NS	NS	NS	NS
5	NS	NS	NS				NS	NS	NS	NS

Table S2. Analysis of developmental rates per stage, censored before the long interval. "NS" indicates non-significant effects. Period $\times$ treatment interactions were never significant and therefore not listed. Significant effects are indicated by Chi-squared statistic and p-value. For the salts used, those which had significantly different parameter estimates from desiccators with water are given differently shaded cells. For stages written in bold, we did not include time intervals in the rewetted period in the analysis.

Stage	Parent	Date	Plate	KCL	KNO3	NH4H2PO4	Salts	Temp	Age	Preceding
1	76.09 <0.001	9.38 0.002	NS				13.98 0.007	NS	NS	
2	NS	NS	7.81 0.005				17.52 <0.001	5.36 0.021	NS	NS
3	NS	NS	NS				NS	NS	NS	NS
4	NS	38.52 <0.001	NS				NS	NS	NS	8.92 0.003
5	NS	NS	NS				NS	NS	NS	NS

Table S3. Survival analysis, per stage and period separately.. Significant effects are listed, based on likelihood ratio Chisq tests. Significant effects are indicated by p-values. When the effect was not tested, cells are left empty. For the salts used, those which had significantly different parameter estimates are given differently shaded cells.

Stage	Parent	Date	Plate	KCL	KNO3	NH4H2PO4	Salts	Temp	Age	Preceding
1 Wet	<0.001	<0.001	0.001					NS	NS	
1 Dry	NS	NS	0.043				NS	NS	NS	
1 Rewetted							NS	NS	NS	
2 Wet	NS	0.016	NS					NS	NS	0.001
2 Dry	NS	NS	NS				NS	NS	0.004	0.021
2 Rewetted							NS	NS	NS	NS
3 Wet	NS	NS	NS					NS	NS	NS
3 Dry	NS	NS	NS				NS	NS	NS	NS
3 Rewetted							NS	NS	NS	NS
4 Wet	NS	NS	NS					NS	NS	NS
4 Dry	NS	NS	NS				NS	NS	NS	NS
4 Rewetted							NS	NS	NS	NS
5 Wet	No events									
5 Dry					+	+	<0.001	0.002	0.001	NS
5 Rewetted	NS	0.023	NS	+			<0.001	<0.001	0.022	NS

Table S4. Development per period and per stage. Significant effects are indicated by p-values. When the effect was not tested, cells are left empty. For the salts used, those which had significantly different parameter estimates are given differently shaded cells.

Stage	Parent	Date	Plate	KCL	KNO3	NH4H2PO4	Salts	Temp	Age	Preceding
1 Wet	<0.001	0.006	NS					NS	NS	
1 Dry	0.004	0.001	0.027				NS	NS	NS	
1 Rewetted							NS	NS	NS	
2 Wet	NS	0.001	0.033					NS	NS	0.003
2 Dry	NS	NS	NS				NS	NS	NS	0.001
2 Rewetted							NS	NS	NS	NS
3 Wet	NS	NS	NS					NS	NS	NS
3 Dry	NS	NS	0.036				NS	NS	NS	NS
3 Rewetted							NS	NS	NS	NS
4 Wet	NS	0.032	NS					0.027	NS	<0.001
4 Dry	0.016	<0.001	NS				0.010	NS	NS	0.024
4 Rewetted							NS	NS	NS	NS
5 Wet	NS	NS	NS					NS	NS	NS
5 Dry	NS	NS	NS				NS	NS	NS	NS
5 Rewetted	No events									

Fig Surv1. Survival stage one different periods. From left to right: before desiccation, during desiccation, and when water was added again. In the two rightmost panels age is rescaled to the time where the regime started (i.e. start of dry period, rewetted). The control group is added to each panel as well, with the average age at which the regime started subtracted from the actual age of embryos. This was done to facilitate comparisons between treatments. Black, thin: Control group, blue, thin: dessicator with H2O, red thick KNO3 blue thick NH4H2PO4 black, thick: KCL.

790 Fig Surv2. Survival stage two different periods. From left to right: before desiccation, during
791 desiccation, and when water was added again. In the two rightmost panels age is rescaled to the
792 time where the regime started (i.e. start of dry period, rewetted). The control group is added to each
793 panel as well, with the average age at which the regime started subtracted from the actual age of
794 embryos. This was done to facilitate comparisons between treatments. Black, thin: Control group,
795 blue, thin: dessicator with H2O, red thick KNO3 blue thick NH4H2PO4 black, thick: KCL.
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802 Fig Surv3. Survival stage three in different periods. From left to right: before desiccation, during
803 desiccation, and when water was added again. In the two rightmost panels age is rescaled to the
804 time where the regime started (i.e. start of dry period, rewetted). The control group is added to each
805 panel as well, with the average age at which the regime started subtracted from the actual age of
806 embryos. This was done to facilitate comparisons between treatments. Black, thin: Control group,
807 blue, thin: dessicator with H2O, red thick KNO3 blue thick NH4H2PO4 black, thick: KCL.
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Fig Surv4. Survival stage four different periods. From left to right: before desiccation, during desiccation, and when water was added again. In the two rightmost panels age is rescaled to the time where the regime started (i.e. start of dry period, rewetted). The control group is added to each panel as well, with the average age at which the regime started subtracted from the actual age of embryos. This was done to facilitate comparisons between treatments. Black, thin: Control group, blue, thin: dessicator with H2O, red thick KNO3 blue thick NH4H2PO4 black, thick: KCL.

Fig Surv5. Survival stage five in different periods. From left to right: before desiccation, during desiccation, and when water was added again. In the two rightmost panels age is rescaled to the time where the regime started (i.e. start of dry period, rewetted). The control group is added to each panel as well, with the average age at which the regime started subtracted from the actual age of embryos. This was done to facilitate comparisons between treatments. Black, thin: Control group, blue, thin: dessicator with H2O, red thick KNO3 blue thick NH4H2PO4 black, thick: KCL.

Fig Dev1. Development from stage one into stage two in the different periods in the experiment. On the y-axis is plotted the fraction in the population that made the transition. From left to right: before desiccation, during desiccation, and when water was added again. In the two rightmost panels age-within-stage is rescaled to the time where the regime started (i.e. start of dry period, rewetted). The control group is added to each panel as well, with the average age at which the regime started subtracted from the actual age-within-stage of embryos. This was done to facilitate comparisons between treatments. Black, thin: Control group, blue, thin: dessicator with H2O, red thick KNO3 blue thick NH4H2PO4 black, thick: KCL.

849 Fig Dev2. Development from stage two into stage three in the different periods in the experiment.
850 From left to right: before desiccation, during desiccation, and when water was added again. In the
851 two rightmost panels age-within-stage is rescaled to the time where the regime started (i.e. start of
852 dry period, rewetted). The control group is added to each panel as well, with the average age at
853 which the regime started subtracted from the actual age-within-stage of embryos. This was done to
854 facilitate comparisons between treatments. Black, thin: Control group, blue, thin: dessicator with
855 H2O, red thick KNO3 blue thick NH4H2PO4 black, thick: KCL.
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862 Fig Dev3. Development from stage three into stage four in the different periods in the experiment.
863 From left to right: before desiccation, during desiccation, and when water was added again. In the
864 two rightmost panels age-within-stage is rescaled to the time where the regime started (i.e. start of
865 dry period, rewetted). The control group is added to each panel as well, with the average age at
866 which the regime started subtracted from the actual age-within-stage of embryos. This was done to
867 facilitate comparisons between treatments. Black, thin: Control group, blue, thin: dessicator with
868 H2O, red thick KNO3 blue thick NH4H2PO4 black, thick: KCL.

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874 Fig Dev4. Development from stage four into stage five in the different periods in the experiment.
875 From left to right: before desiccation, during desiccation, and when water was added again. In the
876 two rightmost panels age-within-stage is rescaled to the time where the regime started (i.e. start of
877 dry period, rewetted). The control group is added to each panel as well, with the average age at
878 which the regime started subtracted from the actual age-within-stage of embryos. This was done to
879 facilitate comparisons between treatments. Black, thin: Control group, blue, thin: dessicator with
880 H2O, red thick KNO3 blue thick NH4H2PO4 black, thick: KCL.
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887 Fig Dev5. Proportions of individuals in stage five that do not hatch spontaneously. From left to
888 right: before desiccation, during desiccation, and when water was added again. In the two rightmost
889 panels age-within-stage is rescaled to the time where the regime started (i.e. start of dry period,
890 rewetted). The control group is added to each panel as well, with the average age at which the
891 regime started subtracted from the actual age-within-stage of embryos. This was done to facilitate
892 comparisons between treatments. Black, thin: Control group, blue, thin: dessicator with H2O, red
893 thick KNO3 blue thick NH4H2PO4 black, thick: KCL.
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