

Egg size does not universally predict embryonic resources and hatchling size across annual killifish species

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24 Abstract

25 Egg size has a crucial impact on the reproductive success of a mother and the performance of her offspring. It is therefore reasonable to employ egg size as a proxy for egg content when studying 26 27 variation in offspring performance. Here, we tested species differences in allometries of several egg 28 content parameters with egg area. We measured individual eggs in five species of annual killifish 29 (Cyprinodontiformes), a group of fish where egg banks permit population survival over dry season. 30 Apart from comparing allometric scaling exponents, amounts and compositions of egg components 31 across the different species, we assessed the explanatory power of egg area for egg wet and dry weight 32 and for hatchling size. We found notable species-specific allometries between egg area and the other 33 egg parameters (egg dry weight and water content, elemental composition and triglyceride content). 34 Across species, egg area predicted egg wet weight with highest power. Within species, coefficients of 35 determination were largest in Austrolebias elongatus, a large piscivorous species with large eggs. Our 36 study shows that systematically using egg area as a proxy of egg content between different species can ignore relevant species-specific differences and mask within-species variability in egg content. 37

38

Keywords: Austrolebias, allocation strategy, allometry, egg composition, Nothobranchius
 furzeri

42 Introduction

43 Egg size is an ecologically and evolutionary important trait in egg-laying animal species 44 (Bernardo, 1996; Fox and Czesak, 2000; Krist, 2011). In particular in species that provide little or no 45 parental care, the egg and the resources it contains are the main route of transmission of non-genetic 46 effects between a mother and its offspring. Parameters describing an egg thus do not only affect 47 individual offspring performance, but also reflect female's allocation strategy that eventually impacts 48 her fitness (Moore et al., 2015; Morrongiello et al., 2012; Smith and Fretwell, 1974). Egg size usually 49 provides a reasonable proxy for the other traits potentially important for offspring survival, 50 development and growth; like the amounts of water, nutrients, or energy contained in the egg (Berg 51 et al., 2001; Salze et al., 2005). Hence, we often observe long-term cascading effects of egg size on 52 early-life offspring performance (Segers and Taborsky, 2011; Self et al., 2018; Semlitsch and Gibbons, 53 1990). Measuring egg size is straightforward and can be performed in a non-invasive manner, usually 54 recorded as diameter or wet mass (Brooks et al., 1997; Krist, 2011; Räsänen et al., 2005). Using egg size as a proxy for other egg parameters seems, therefore, a sufficient way to assess, or control for 55 56 female allocation among individual offspring.

57 Some studies have shed doubt on the generality of strong positive correlations between egg size and egg content (Leblanc et al., 2014; Moran and McAlister, 2009; Murry et al., 2008), showing 58 that egg dry mass proves to be a more reliable predictor of nutrient and energy content (Murry et al., 59 60 2008). While we might measure maternal investment better by the means of egg dry weight, the 61 problem is that destroying the egg by drying prevents any follow-up monitoring at the individual 62 offspring level. Most egg parameters actually cannot be measured without destroying the embryo. 63 Egg size therefore remains the first option to test for effects of maternal investment on offspring life 64 history. To make valid predictions without sacrificing all eggs, we need good data on the relationship 65 of egg size with other egg parameters from multiple species.

66 Annual killifish (Cyprinodontiformes) are small freshwater fish adapted to seasonally desiccating 67 ponds where populations overcome dry periods in embryonic stages, thus creating egg banks in the

pond soil (Cellerino et al., 2016; Furness, 2016). Adaptation to this temporary habitat evolved multiple times from non-annual ancestors (Furness et al., 2015; Helmstetter et al., 2016). A common feature of annual killifish embryos is their ability to arrest development (by entering diapause) and potentially reduce energy expenditure at three discernible morphological stages (Podrabsky et al., 2010; Wourms, 1972). Diapauses allow embryos of annual killifish to survive dry periods that may occasionally span even multiple years. Egg provisioning thus represents a crucial life-history process in annual killifish that may affect embryonic survival or amount of energetic reserves available after hatching.

75 Bigger annual killifish species indeed produce disproportionately larger eggs compared to same-76 sized non-annual species (Eckerström-Liedholm et al., 2017). In the African annual killifish 77 Nothobranchius furzeri (Nothobranchiidae), egg size determines hatchling size irrespective of the time 78 spent in incubation (Vrtílek et al., 2017). This suggests that some resources in the egg that scale with 79 egg size affect hatchling size and that these are not consumed during diapause. There is not much 80 data from other annual killifish species, but Moshgani and Van Dooren (2011) found substantial 81 within-species variation in egg size of the South-American annual Austrolebias nigripinnis (Rivulidae). 82 They did not, however, relate egg size to amounts of resources or to hatchling size.

83 Here, we test for differences in allometries of egg content parameters with egg size in 84 ecologically and phylogenetically divergent species of annual killifish from South America (genus 85 Austrolebias) and Africa (species N. furzeri). We combine analyses of different egg composition 86 parameters – wet and dry egg mass; carbon, nitrogen and sulphur content; the amount of triglycerides 87 - to quantify resources such as water, nutrients and energy in individual eggs. We aimed to test the relevance of egg area measured from digital photographs as a predictor for egg wet and dry weight 88 89 within and across annual killifish species. In addition to that, we incubated a subset of the collected 90 clutches to assess the effect of egg area on hatchling traits. We evaluate the generality of our 91 conclusions by using two additional independently collected datasets.

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93

94 Methods

95 Breeding pairs of Austrolebias species

To collect eggs, we established breeding pairs of four *Austrolebias* species from captive populations maintained at the CEREEP station in Nemours-St. Pierre, France (approval no. B77-431-1). We used two small species – *A. bellottii* (population "Ingeniero Maschwitz", 4 pairs) and *A. nigripinnis* ("La Guarderia", 4 pairs); and two large ("piscivorous") species – *A. elongatus* ("General Conesa", 5 pairs) and *A. prognathus* ("Salamanca", 2 pairs). Table S1 in the Supplementary Information lists the ages and body sizes of the parental fish and includes details on their source populations.

102 The breeding pairs were kept in tanks of 21 litres for the small species or of 54 l for the piscivores 103 in a dedicated climate room at the CEREEP facility. To prepare tank water, we mixed reverse osmosis 104 and conditioned (JBL NitratEx, PhosEx, SilicatEx resin-filtered and Sera Toxivec 0.5 ml/l) tap water in a 105 1:1 ratio resulting in 200-300 μS conductivity. We replaced 1/3 of the water per tank on a weekly basis. 106 The room temperature was set to 23 °C during the light period of the day (8AM-10PM, 14 h) and to 107 18 °C when dark (10PM-8AM, 10 h), making water temperature vary between 17-23 °C. Fish were fed 108 daily with live *Tubifex* sp. (Grebil Pére et Fils, Arry, France).

109

110 Breeding stock of *N. furzeri*

We wanted to compare egg content between two phylogenetically distinct genera of annual killifish - *Austrolebias* (South America) and *Nothobranchius* (Africa) (Furness et al., 2015). We therefore established a breeding stock of *N. furzeri* (population "MZCS414") from the Institute of Vertebrate Biology CAS in Brno, Czech Republic at the CEREEP facility. We hatched *N. furzeri* according to the standard protocol (Polačik et al., 2016) using a 1:1 mixture of osmotic and tap water at 18 °C. We subsequently reared the fish at 27-28 °C and fed them with *Artemia salina* nauplii until the age of ten days when they were weaned on *Tubifex* sp. We separated sexes when first marks of nuptial colouration of males appeared, at approximately three weeks, and paired the fish only for egg collection. *Nothobranchius furzeri* were hatched on July 4, 2017, shortly after the collection of eggs from *Austrolebias* began, so they reached full maturity (at an age of six weeks) when *Austrolebias* egg collection finished. The breeding stock of *N. furzeri* consisted of seven pairs.

122

123 Egg collection and measurements

124 We collected Austrolebias eggs using two- or five-litre plastic spawning containers (depending 125 on the species). The containers were filled with a 0.5 cm layer of 300-400 μ m diameter glass beads 126 and 5 cm of peat granules (2-4-mm diameter fraction; HS aqua Torogran, Ulestraten, Netherlands) to 127 allow fish to dive into the substrate during spawning. Each tank had a single spawning container which 128 we replaced every other evening. Retrieved containers were left overnight in an adjacent climate 129 room at 23 °C. We collected eggs the following day using set of sieves of different mesh sizes as 130 described in (Moshgani and Van Dooren, 2011). We paired Nothobranchius to spawn for two hours 131 (9-11AM) in two-litre containers with one cm layer of the glass beads. We collected eggs six hours 132 later by sieving the spawning substrate.

We photographed fertilized killifish eggs and only measured eggs with a clear perivitelline space. Eggs were gently cleaned with a paintbrush and we photographed (Nikon COOLPIX 4500) ten random eggs per pair at each collection date under a dissecting microscope (Olympus SZH10) at 30x magnification. We measured two egg dimensions: the longest egg axis (d_1) and the perpendicular axis (d_2) in pixels (ImageJ ver.1.50i) and subsequently rescaled to lengths in millimetre using a 0.01-mm calibration slide. From these two lengths, egg area was calculated as $\frac{\pi}{4} d_1 d_2$. We use mainly egg area as the measure of egg size in this study.

141 Preparations for the analysis of egg content

We collected freshly laid eggs from Austrolebias species for the analysis of egg content at four 142 143 occasions among the egg collections for incubation (see "Incubation of embryos and hatching" below). After photographing, we stored each egg in a separate 1-ml Eppendorf tube with UV-sterilized water 144 (mixed as for incubation) at 23 °C in the incubator. Next day, we removed moisture from the eggs by 145 146 rolling them over a filter paper and measured their wet weight to the nearest 0.001 mg (Mettler Toledo XPE26 balance). The eggs for analysis of carbon, nitrogen and sulphur content (see "Elemental 147 content analysis" below) were subsequently oven-dried for 24 h at 70 °C, weighed for dry weight and 148 149 stored at 4 °C. The eggs for the triglyceride assay (see "Triglyceride assay" below) were each put fresh 150 in a dry Eppendorf tube and frozen at -20 °C before the analysis.

We took the opportunity to get a sample of eggs from extremely old clutches of *A. bellottii* spawned in February 2011 (6+ years of incubation) and compared their triglyceride content to that of freshly laid eggs of the same species. These clutches had been stored in damp mixed coco-coir and peat in zip-locked bags. We briefly dried the substrate to make the eggs visible, put the retrieved eggs into UV-sterilized water and then treated them in the same way as those above for the triglyceride assay.

157

158 Elemental content analysis

The concentrations (weight percentage) of carbon, nitrogen and sulphur were determined by dry combustion with a CNS element analyser (Thermo Scientific Flash 2000) at the Global Change Research Institute CAS, Brno, Czech Republic. Benzoic acid was used as a reference material.

162

163 Triglyceride assay

164 We assessed egg triglyceride reserves using a commercial Triglyceride Colorimetry Assay kit (ref. 165 10010303; Cayman Chemical, Ann Arbor, MI, USA) following the protocol for tissue homogenates. We 166 left frozen eggs to thaw at room temperature and put each one in a 1.5-ml Eppendorf tube with 0.2 167 ml of 5X salt solution (Standard Diluent). The tube content was homogenized using a disperser (IKA 168 T10 basic ULTRA-TURRAX) for 30 s and then centrifuged for 10 min (Eppendorf 5430, 10,000 r/min, 4 169 °C) to separate egg envelope and content. We put the supernatant into a clean Eppendorf tube and 170 mixed briefly on a vortex (Fisherbrand L-46). We pipetted 10 μ l aliquot in a prepared 96-well plate, 171 added 150 µl of diluted Enzyme Mixture and incubated the plate at room temperature for 10 min. We 172 made two replicates per sample. We measured the absorbance at 540 nm wavelength three times per replicate using a microplate reader (BIO-RAD iMark). We used the reference standards to calculate 173 triglyceride concentrations of the two replicates. 174

175

176

Incubation of embryos and hatching

177 To test for long-term effects of egg size on embryonic development, we incubated a sample of 178 the photographed eggs and hatched those having completed their development after a pre-179 determined duration. We stored eggs individually in 24-well plates filled with UV-sterilized mixed 180 water (1:1 ratio of aged tap and reverse osmosis water) at 23 °C in a Memmert IPP500 incubator. We 181 inspected development and survival of incubated embryos weekly. Inspections were more frequent, 182 however, during the first two weeks when embryonic mortality was highest. At each inspection, we 183 removed dead embryos and recorded the date of those reaching the pre-hatching developmental 184 stage, i.e. the "golden-eye" stage (Stage 5 in Varela-Lasheras and Van Dooren (2014), St. 43 in 185 Podrabsky et al. (2017)). We kept the pre-hatching stage embryos in their wells until the scheduled 186 hatching date. A minor fraction of the pre-hatching stage embryos (7 %, 29 of 427), however, hatched 187 spontaneously prior to the planned hatching date. These individuals were not used in further analyses of hatchling size. 188

189 We scheduled hatching four months after the date of last spawning and maximum time spent 190 in the pre-hatching stage was 92 days. To initiate hatching, we gently wiped egg dry by rolling over a 191 filter paper and put it back in a dry well. We stimulated hatching by rewetting the embryos 3 h later 192 using a 6:1 mixture of 23 °C osmotic water and peat extract (1 | of peat boiled in 1 | of osmotic water). 193 We inspected hatching success eight hours after rewetting, anaesthetized the hatched fish with clove 194 oil and photographed them from a lateral view under a dissecting microscope (magnification 15x for 195 small species and 10x for piscivores). We measured standard length (SL) of each hatchling and the 196 area of its remaining yolk. We repeated the hatching procedure on the following day with the 197 previously unhatched embryos.

198

199 Independent datasets of Austrolebias egg size, wet and egg dry weight, and hatchling size

200 We used two independent datasets of *Austrolebias* eggs collected in 2015 to generalize our 201 findings. The first dataset consisted of eggs of *Austrolebias bellottii* (population "Ingeniero 202 Maschwitz", 3 pairs, 61 eggs), where egg area was measured together with wet and egg dry weight. 203 The eggs were collected from spawning boxes containing moss *Sphagnum magellanicum* facilitating 204 rapid egg retrieval. We measured egg area, egg wet and dry weight as described above.

205 The other independent dataset was on egg size-hatchling size relationship from a pair of A. 206 elongatus ("Vivorata") and of A. nigripinnis ("San Javier Missiones") kept in a greenhouse. We 207 collected eggs from spawning boxes with a mix of coco-coir and peat during February and March 2015. 208 The substrate was gently dried until eggs could be seen and collected. Again, we measured egg size as 209 above and transferred eggs to 24-well plates with UV-sterilized water at two different temperatures 210 (22 °C and 25 °C) for incubation. Three months later (May 28, 2015), embryos in the pre-hatching stage 211 were air dried for four hours and rewetted to initiate hatching, so we could measure standard length 212 (SL) of the hatchlings.

213

214 Statistical analysis

We used the data for two different but related purposes: (1) we determined which explanatory variables contributed significantly to variability in egg parameters with a particular focus on species differences in allometric scaling exponents of egg size; and (2) we assessed how well egg size, measured as egg area on digital images, predicted egg wet weight, dry weight and hatchling size.

We assumed that the investigated egg parameters scaled allometrically with egg size and that these relationships can be described by constants of proportionality and scaling exponents (Appendix, Section A). We used egg area as a measure of size. In the Appendix, we explain how allometries can be analysed in a multi-species context and provide arguments for the modelling choices we made. We ignore effects of measurement error in egg size in our analysis (Appendix, Section F).

224 The parameters we measured in eggs are expected to covary (water weight with egg dry weight, 225 for example) both between and within species and we therefore used multivariate models wherever 226 possible (Appendix, Section D). To do so, we reformatted values of response variables into stacked 227 form and added a dummy factor "trait" with levels identifying the different egg parameters. All 228 models thus contained "trait" fixed effects by default to distinguish the egg parameters and to allow 229 for fitting trait-specific random effects and different residual variances per trait. We fitted models 230 using functions from library "nlme" (Pinheiro and Bates, 2000) in R software v. 3.5.3 (R Core Team, 231 2019).

We always started model selection with a full model and then tried to simplify it on the basis of log-likelihood ratio tests and F-tests on model log-likelihoods. We removed random effects first, followed by simplification of residual variance structures and then fixed effects (Zuur et al., 2009). We removed random effects by comparing models containing all residual variance terms and fixed effects fitted with restricted maximum likelihood (REML). When testing the importance of individual fixed effects, we refitted the models with maximum likelihood (ML). We then assessed fit of the selected models by inspecting the distributions of residuals.

239 To test species-specific allometries in egg dry mass and water content related to egg size, we 240 used egg dry weight and water weight as log-transformed response variables in a bivariate model. Egg 241 water weight was fitted as egg wet weight minus dry weight, therefore we did not add total wet weight 242 as an explanatory variable (Appendix, Section E). The full model included "trait", "species" and "log-243 transformed egg area" with all the possible interactions as fixed effects to account for different 244 allometries between species and traits. The log-transformed egg area was zero-centered per species 245 prior the analysis to ensure that intercept difference terms capture the total differences between 246 groups while slopes estimate the scaling exponents (Appendix, Section D; Nakagawa et al., 2017). We 247 provide a simple rule here which can help interpreting estimated scaling exponents. If egg parameters 248 scale isometrically with egg volume and eggs are spheres, then they must scale in a hyperallometric 249 manner with egg area (with coefficient 3/2, Appendix, Section B). Hence, when exponents of egg area 250 are not estimated to be above 3/2, there are no hyperallometries for volume. Parental pair and 251 collection date were modelled as trait-specific random effects and residual variances were allowed to 252 differ between species and traits.

253 Carbon, nitrogen, and sulphur (CNS) are components of egg dry weight with raw data values 254 given as percentages. We carried out compositional analysis with CNS percentages transformed using isometric log-ratio (ilr) (library "compositions"; van den Boogaart and Tolosana-Delgado, 2013). We 255 256 fitted them as responses in a trivariate general linear model because mixed-effects model did not 257 converge. The full model was of the same structure as the bivariate model above for egg dry weight 258 and water weight. Individual model parameters in the compositional analysis were, however, difficult 259 to interpret (van den Boogaart and Tolosana-Delgado, 2013). We therefore report parameter 260 estimates from the best model fitted with log-transformed total amounts of individual elements 261 calculated from CNS percentages and dry weights (Appendix, Section E).

We assayed triglyceride content to test for relative species differences. Triglyceride content was measured as diluted sample concentration in two replicates from the same egg. We then backcalculated the total amount of egg triglycerides in the replicates using the sum of standard solvent

volume and egg volume taken as $V = \pi \frac{d_1 d_2 d_2}{6}$, assuming a spheroid egg shape. We used log-265 266 transformed total amount of triglycerides from each replicate as a response and corrected for 267 different egg sizes by fitting log-transformed egg volume as an offset (Appendix, Section E). We fitted 268 fixed effect of "species" to test for intercept differences among species and interaction between 269 "species" and species-centered "log-transformed egg volume". Full mixed-effects model that would 270 control for both pseudoreplication and species-specific residual variances did not converge. We 271 therefore fitted two alternative models: a mixed-effects model with sample ID as random effect; or a 272 general linear model with averaged values from the two subsamples accommodating species-specific 273 residual variance. The outcome of these two models was qualitatively similar and we report estimates 274 of species contrasts from the model with the averaged sub-sample values based on Tukey's post-hoc 275 test (function glht(), library "multcomp"; Hothorn et al., 2008).

276 We tested for long-lasting effects of egg size on traits at hatching: hatchling size and remaining 277 yolk reserves. Hatchling size and yolk sac area were log-transformed and analysed using a bivariate 278 mixed-effects model as above for egg dry weight and water content. The full model included a triple interaction between "trait" (by default), "species" and species-centered "log-transformed egg area" 279 280 to account for different allometries in species and traits (Appendix, Section C). In addition to that, we 281 fitted an interaction between "trait" and the number of days spent in the pre-hatching stage. This was 282 to account for the variation in pre-hatching stage duration among embryos. Full mixed-effects model 283 containing random effects of pair and collection date did not converge. We thus fitted general linear 284 models with trait-specific residual variances.

To evaluate how well egg area predicts egg wet weight, dry weight, and hatchling size, we calculated coefficients of determination (R^2) in mixed-effects models using function r.squaredGLMM() (library "MuMIn"; Bartoń, 2018). This function allows to separate R^2 for the whole model ($R^2_{conditional}$) from the fixed-effects part of the model ($R^2_{marginal}$) (Nakagawa and Schielzeth, 2013). We first fitted full mixed-effects model with "species" in interaction with untransformed "egg area" as fixed effects

(Appendix, Section G), and pair and collection date random effects. Residual variances were allowed
to vary between species. We then fitted separate models per species with egg area fixed effects, and
pair and collection date random effects. We fitted the same models to the additional datasets from
2015 to compare predictive power of egg area among different studies.

294

295

296 **Results**

We collected over six thousand eggs of five annual killifish species in 2017 of which we incubated 1384 and measured wet and dry weight of 295 eggs. We used 130 of these dried eggs for CNS elemental analysis and another 40 fresh eggs, which were weighed, for triglyceride content assay (including seven eggs collected from substrate incubated for 6+ years) (Table 1). The additional two datasets consisted of 61 wet and egg dry weights from a single species, and of 51 hatchling sizes from another two species measured in 2015 (see Table 1 for more details).

Table 1. Overview of species mean values for the measured egg parameters.

303

304 Egg size

Taking egg area determined from images as the measure of egg size, the collected eggs clustered into two groups according to adult sizes of the species they belonged to. Eggs were notably larger in the piscivores, *Austrolebias elongatus* and *A. prognathus*, compared to the small *Austrolebias* species, *A. bellottii* and *A. nigripinnis*, and to *Nothobranchius furzeri* (Fig. 1, Table 1). The size of *A. bellottii* eggs collected after 6+ years of incubation was larger compared to freshly laid eggs of the same species (Table 1). The eggs from the additional datasets were similar in size to eggs collected for this study (Fig. 1).

Figure 1. Species with larger body size lay larger eggs.

312

313 Dry egg mass and water content

314 The allometric scaling exponents of egg dry and water weight on egg area differed between 315 species. The best model included a three-way interaction between species, trait and log-transformed 316 egg area (species:trait:log(egg area) interaction, $F_{4,532} = 2.67$, p = 0.032). This means that the effects of 317 log-egg area on log-egg dry weight and log-water weight differed and that this difference depends on 318 the species (Table 2, Fig. 2A). However, the water weight allometric scaling exponent was significantly 319 different from A. bellottii and from dry weight only in eggs of A. prognathus. In A. prognathus, water 320 weight appeared to scale isometrically with egg area while egg area had no effect on egg dry weight 321 (Fig. 2A). Austrolebias bellottii and A. nigripinnis showed hypo-allometric, and A. elongatus hyper-322 allometric effects of egg area on dry weight. None of the predicted exponents were above 3/2 (Table 323 2), hence there were no hyperallometries for the relationship with egg volume. The estimates for dry 324 weight effects in A. prognathus and N. furzeri were close to zero indicating very weak relationships 325 between egg size and dry weight. In the additional 2015 dataset, A. bellottii eggs' dry weight changed 326 hyperallometrically with egg area (log(egg area) effect \pm SE (standard error): 1.360 \pm 0.085). Water 327 weight changed similarly to that (difference between log(egg area) effect on log(dry weight) vs. 328 log(water weight): -0.239 ± 0.128), hence both changed approximately isometrically with egg volume.

Overall, the annual killifish eggs contained between two and three times as much water as dry matter. The estimated differences in species intercepts or of different traits on the same species represent multiplicative total differences after back-transformation (Fig. 2 Table 1; Appendix, Section D). Relative content of water in the eggs of *A. elongatus, A. prognathus*, together with *N. furzeri* was higher than in the small *Austrolebias* species, *A. bellottii* and *A. nigripinnis* (Tables 1 and 2, Fig. 2B). Eggs of *A. bellottii* from 2015 contained 2.66-times more water than dry matter (log-transformed estimate: 0.979 ± 0.035), compared to 2.16 ratio in *A. bellottii* eggs collected in 2017. Egg dry weight

- appeared to vary more than water weight (estimated SDs ratio between log(dry weight) and log(water
- 337 weight) was 1.294; CI [1.140, 1.467]) and eggs of *N. furzeri* were the most variable (SD (standard

deviation) ratio was 1.723; CI (confidence interval) [1.256, 2.362]).

Table 2. Species-specific scaling of egg dry mass and water content with egg area.

339

Figure 2. A) Species-specific slopes of the relationship between water weight and egg dry weight.B) Species differences in the amount of water to egg dry weight.

340

341 CNS elemental analysis

342 Carbon, nitrogen and sulphur contents varied among eggs of different species including species-343 specific allometric scaling exponents of egg area (Table 3). The compositional analysis with ilr-344 transformed data showed a significant three-way interaction (species:trait:log(egg area), $F_{8,360} = 2.20$, 345 p = 0.028) meaning that proportions of the three elements scaled differently with egg area and that 346 these differences depended on the species. As the estimates from ilr-transformed data were hard to interpret, we re-fitted the full model with log-transformed total amounts of the elements (Table 3). 347 Overall, carbon content scaling exponents of egg area varied among species, but nitrogen and sulphur 348 349 scaling exponents were hardly different. The only exception was the scaling exponent estimate for 350 sulphur in eggs of A. nigripinnis (Table 3). None of the slopes of egg area is significantly above 1.5, 351 hence we can again conclude that there are no hyperallometries for egg volume.

Egg elemental composition varied across different species. Species differences in the total amount of carbon can be largely explained by species effects on egg dry weight (compare the "log(dry weight)" column of Table 2 to the "log(total C)" in Table 3). The top half of the table indicates that the piscivores (*A. elongatus* and *A. prognathus*) and *N. furzeri* had relatively more nitrogen to carbon than *A. bellottii* (Fig. 3A). In addition to that, eggs of piscivores also contained relatively more sulphur (to

- 357 carbon) (Fig. 3B, Table 3). The three elements analysed together made up between 60 and 68 % of egg
- dry weight (carbon 54.1 %, nitrogen 8.7 % and sulphur 0.4 %, on average; Table 1). The variance was
- 359 particularly high for measurements of sulphur in eggs of the small species (A. bellottii, A. nigripinnis
- 360 and *N. furzeri*) (SD ratios were 6.330-8.980).

Table 3. Relative changes in the egg content of carbon, nitrogen and sulphur among the species.

361

Figure 3. Ternary plot of species differences in the proportion of carbon, sulphur and residual content.

362

363 Triglyceride content analysis

364 The relative amount of egg triglycerides varied within and among species (species, $F_{5,34} = 12.88$, 365 p < 0.001; Fig. 4). We found that, after accounting for different egg volumes, the amount of egg triglycerides was highest in A. elongatus and lowest in N. furzeri which overlapped with the small 366 367 Austrolebias species, A. bellottii and A. nigripinnis (Fig. 4). We didn't observe a difference between fresh eggs of A. bellottii and those collected from 6+ years old substrate (Fig. 4). We explored potential 368 allometric scaling exponent effects of egg volume on the amount of triglycerides and found a 369 370 significant species (species:log(egg volume) interaction, $F_{6,28} = 3.35$, p = 0.013) where N. furzeri had a 371 much larger positive value than the other species. However, this significant interaction and the large 372 estimate are due to four very small outlying concentrations among the smallest eggs, suggesting that these eggs were incompletely composed and that we should not take this as evidence for 373 374 hyperallometry.

Figure 4. Triglyceride concentration in the eggs of annual killifish species.

375

376 Hatchling size and remaining yolk

377 We successfully hatched 22% (92/427) of pre-hatching stage embryos. Austrolebias bellottii 378 hatchlings were larger than A. nigripinnis (Table 4). Within species, hatchling size did not depend on 379 egg area, but yolk sac area decreased with egg area (trait: centered log(area) interaction, $F_{1.172}$ = 4.73, 380 p = 0.031, Fig. 5, Table 4). Yolk sac area also diminished with the number of days embryos spent in the 381 pre-hatching stage and we did not record any gain to the embryo by growing larger while waiting for 382 hatching (trait:daysPH interaction, $F_{1,172}$ = 4.61, p = 0.033). In the additional 2015 dataset, there was 383 again an at most weak effect of egg area on hatchling size of A. nigripinnis (log(egg area) effect: 0.165 384 ± 0.091), hatchlings of A. elongatus showed a hypo-allometric increase in size with egg area (log(egg 385 area) effect: 0.381 ± 0.057) (Fig. 5). Yolk sac size had a higher variance than hatchling size in our 2017 386 dataset (estimated yolk sac size:hatchling size SDs ratio was 5.291; CI [4.304, 6.506]).

Table 4. Effect of egg size and duration of pre-hatching stage on hatchling size and yolk sac size.

387

Figure 5. Hatchling size increases with egg area among species but not within species.

388

389 Egg size as predictor of other egg parameters

390 Species differed markedly in how well egg area predicted the other egg parameters. On 391 average, egg area predicted egg wet weight best, then dry weight, followed by hatchling size. Overall, 392 it predicted best for eggs of A. elongatus (Table 5). Models with species-specific untransformed egg 393 area explained data better than an additive model with species and egg area effects for all the three predicted parameters (p < 0.001). This suggests that egg area does not predict the other parameters 394 395 in the same way across different species. We then fitted egg area in separate species-specific models 396 and calculated two complementary measures of model fit: the coefficient of determination (R^2) for 397 the fixed effects only $(R^2_{marginal})$ and R^2 of the whole model including random effects $(R^2_{conditional})$. Both 398 fixed-effects and whole-model R^2 for species-specific models of egg wet weight ranged widely (Table 399 5). Egg area was a particularly good predictor of egg wet weight in A. elongatus ($R^2_{marainal} = 0.959$), but

performed less well in the other species (Table 5). When we applied the same model to the additional *A. bellottii* dataset of wet weight from 2015, the very high value found ($R^2_{marginal} = 0.904$) contrasts with *A. bellottii* from the 2017 experiment ($R^2_{marginal} = 0.210$). In egg dry weight models, $R^2_{marginal}$ was generally lower than for wet weight, again with particularly high values for *A. elongatus* from 2017 and *A. bellottii* from 2015 (Table 5). There was a small explanatory effect of egg area on hatchling size in two species that hatched in the 2017 experiment (*A. bellottii* and *A. nigripinnis*) and in *A. nigripinnis* from 2015. The exception was again *A. elongatus* from 2015 with a high $R^2_{marginal}$ value (Table 5).

Table 5. Predictive power of egg area for egg wet weight, dry weight and hatchling size.

407

408

409 Discussion

410 Egg size can be a suitable measure of other egg parameters and offspring performance 411 (Barneche et al., 2018; Kamler, 2005; Krist, 2011). However, can we use egg size as a universal 412 predictor for other egg traits across species without any further adjustments? Here, we compared the composition of eggs in five annual killifish species using data obtained from individual eggs. We tested 413 414 for different allometric scaling exponents of egg composition parameters on egg area. We assessed 415 the explanatory power of egg area for variation in egg wet and dry weight and for hatchling size. We 416 found species-specific differences in the scaling exponents of egg dry mass and water content, in 417 elemental composition, species-specific allometries of triglyceride content and hatchling size among 418 the studied annual killifish species. Across species, egg area performed best as a predictor for egg wet 419 weight and within species, egg area predicted best other egg parameters in A. elongatus, a large 420 piscivore species with large eggs.

421

Allometric scaling exponents of egg components on egg area differ between species while
 patterns in exponents between traits measured on the same egg often remain similar. The analysis

of allometries between traits in different species or groups is a recurring theme in morphometry 424 425 (Teissier and Huxley, 1936; Klingenberg, 2016; Nakagawa et al., 2017). We can always assume equal 426 allometries in different species, as a null model or a first working hypothesis. However, our analysis 427 demonstrates that even within a genus, allometric scaling exponents differ between species and also 428 within species between samples collected in different years. Even between two piscivorous species 429 from a single clade (Loureiro et al., 2018), different scaling exponents were found. What we also 430 observed was that traits measured on the same egg often shared the same or a similar allometric 431 pattern. For example, egg dry mass had the same allometric exponent with egg area as water weight 432 in each of the species except for A. prognathus. Amount of egg components is expected to scale 433 isometrically with egg volume rather than with egg area and volume might have been a better 434 measure of egg size. However, egg volume is difficult to obtain in a non-invasive manner with little 435 handling. In the Appendix (Section B), we explain that if we assume eggs to be approximately spherical, 436 parameter estimates of log-log regressions on egg area can be converted to the expected estimates of log-log regressions on egg volume. Slope values in log-log regressions on egg area below 1.5 imply 437 438 that the response trait does not scale hyperallometrically with egg volume. For example, larger eggs 439 of A. bellottii and A. nigripinnis had lower dry weight than expected based on a simple linear increase 440 with egg area and also egg volume. The dry weight of A. elongatus eggs, however, increased more 441 than isometrically with area but approximately isometrically with egg volume. Considering that the 442 only slope values above 1.5 were most likely affected by outliers (in the triglyceride assay), we never 443 found any potential hyperallometric relationship with egg volume. Parental individuals might be 444 constrained in the amounts of resources they can allocate to each individual egg, such that they can 445 provide at best isometric amounts.

Egg area did not affect the other egg parameters in some species. We recorded weak-to-zero relationships between egg area and dry and water weight in *A. prognathus* and *N. furzeri*. The egg area relationship with hatchling size was also absent on the intra-specific level (for both *A. bellottii* and *A. nigripinnis*). This specific finding contrasts with a previous study of *N. furzeri* with remarkably 450 robust effect of egg size on hatchling size (Vrtílek et al., 2017) but also with A. elongatus 2015 data 451 from this study. Similarly, A. bellottii eggs from different years showed different allometric slopes. 452 Thus, notable sample or cohort effects are present in the analysis of annual killifish eggs, but the exact 453 source of this variability is unknown. Moshgani and Van Dooren (2011) found that egg size and 454 reproductive effort depended on the day when eggs were collected and attributed this to food quality 455 and environmental state variation. Similar effects might occur for egg composition as suggested by 456 the sample effects and the recurring presence of hypoallometries which could be caused by resource 457 limitations.

458

459 Water content was higher in piscivores and *N. furzeri* than in the small Austrolebias species. 460 Annual killifish eggs are exposed to conditions of water stress for several months during dry season 461 periods. Water contained in the eggs therefore represents a precious resource for the embryo. Annual 462 killifish evolved special egg structures to prevent excessive water loss such as microvilli on the envelope or a chorion with extremely low water permeability (Machado and Podrabsky, 2007). Water 463 464 content of annual killifish eggs appears on the upper boundary of the 50-70% range reported for 465 freshwater teleosts (e.g. Chondrostoma nasus (Keckeis et al., 2000)). It is still much lower, however, 466 than the 90 % recorded in marine pelagophilous fish (Craik and Harvey, 1984), where it is responsible 467 for egg buoyancy (Lubzens et al., 2010). We found considerable interspecific variation in egg water 468 content and the studied species formed two groups. The large piscivorous Austrolebias and the African 469 *N. furzeri* had higher egg water contents than the two smaller *Austrolebias* species. This clustering is 470 surprising from the perspective of their contrasting ecological conditions during embryogenesis. The 471 African N. furzeri occurs in a subtropical semi-arid region and faces longer, colder dry periods in 472 comparison to the temperate South-American Austrolebias species. In Austrolebias, embryos have to 473 survive a dry season which is relatively humid but warmer. Possibly, piscivorous Austrolebias lay their 474 eggs near the fringes of the temporary ponds, where these might face drought regimes more similar 475 to the N. furzeri habitats. This remains an open question, however. In lab conditions, desiccation had

limited effects on embryonic survival and development in *Austrolebias bellottii* (Van Dooren and
Varela-Lasheras, 2018). As an alternative explanation, larger water content in eggs of some species
might stem from their higher sensitivity to desiccation and not necessarily represent specialization to
drier habitats.

480

481 Elemental composition of eggs differed between species. We performed an exploratory 482 analysis of the elemental composition of annual killifish eggs. We measured carbon content, an 483 element which is present in various molecules acting as sources of energy; nitrogen content which 484 provides a crude estimate of proteins; and sulphur content which is scarce in animal tissues but can 485 be found in proteins containing methionine or cysteine amino acids (Kamler, 2005). The elemental 486 composition of annual killifish eggs did not diverge from that of other fish such as zebrafish (46.0-54.0 487 % C, 9.7-11.1 % N) (Hachicho et al., 2015), Northern pike (50.7 % C, 11.5 % N) (Murry et al., 2008), or common sole (49.9 % C, 9.5 % N) (Yúfera et al., 1999). The elemental composition was specific to the 488 489 different killifish species groups with the large eggs of piscivorous species containing relatively more 490 nitrogen and sulphur when compared to the small Austrolebias species. In the eggs of African N. 491 *furzeri*, we found relatively more nitrogen to carbon, again paralleling the large Austrolebias species.

492

493 Egg triglycerides probably serve as an energetic reservoir for the final pre-hatching phase in annual killifish. It has been hypothesised that lipids from egg oil globules act as energy reservoir during 494 495 protracted pre-hatching phase (Brind et al., 1982). In other teleost species, it is usually spent shortly 496 after hatching (Kamler, 2008; Kohno et al., 1986; Yúfera et al., 1999). The globules contain a 497 considerable fraction (50-60%) of the total fish egg lipids (Wiegand, 1996). In annual killifish, the 498 proportion of total egg lipids stored in these oil globules can be particularly high, with up to 90%, and 499 the majority of these lipids are triglycerides (Brind et al., 1982). We therefore focused our analysis on 500 the initial amount of triglycerides in killifish eggs.

501 Eggs of *A. elongatus* contained larger amounts of triglycerides compared to the small species. 502 The other piscivore, A. prognathus, overlapped with both A. elongatus and the small species. Eggs of 503 N. furzeri clustered with the small Austrolebias species. This suggests that the allocation of 504 triglycerides among annual killifish species might covary with egg size. We also analysed eggs of A. 505 *bellottii* that were incubated for over six years in damp substrate to compare their triglyceride reserves 506 to freshly laid eggs from the same species. No embryo of these long-stored eggs had reached pre-507 hatching stage and there was no apparent decline of triglycerides when compared to freshly laid eggs 508 of A. bellottii. Based on these findings, triglyceride levels seems to be retained until the pre-hatching 509 state in agreement with Brind et al. (1982).

510

511 Yolk sac reserves decrease in embryos faced with a protracted pre-hatching phase. Another energy source for fish that is mainly used during embryonic development are egg yolk lipoproteins 512 (primarily lipovitellin) (Brooks et al., 1997; Wiegand, 1996). Freshly hatched annual killifish still possess 513 514 a visible yolk sac, but an extended time of incubation (number of years) may lead to less viable 515 hatchlings with almost spent yolk (MV, personal observation). In the two species we incubated and 516 hatched (A. bellottii and A. nigripinnis), we monitored development regularly and, instead of hatching 517 individual embryos at the moment when they reached the pre-hatching stage, we triggered their 518 hatching at pre-specified date. We therefore ended up with hatchlings that had endured variable time 519 in the pre-hatching stage. The amount of remaining yolk at hatching consequently declined with time 520 spent in the pre-hatching stage. It seems that even during such a limited period (maximum 92 days), 521 embryos had consumed considerable proportion of their yolk reserves. We did not inspect diapause 522 III occurrence, so embryos may have been waiting in an active stage for a hatching cue (desiccation 523 and inundation). This scenario may well happen in natural conditions during the last part of the dry 524 season when humidity already increases. A larger yolk sac size would then increase persistence in the 525 pre-hatching stage, in addition to reserves in the lipid droplet. This is similar to an increased starvation

- resistance during an initial post-hatching phase in other fish (Jardine and Litvak, 2003), amphibians or
 lizards (Moore et al., 2015; Sinervo, 1990).
- 528
- 529 **Conclusion.** Egg parameter allometric scaling exponents and relationships between different 530 egg parameters are not conserved across annual killifish species. Scaling exponents also vary between 531 repeated samples on the same species but collected at different times in different conditions. Our 532 results therefore call for caution and more analyses of intra- and interspecific variation in egg content 533 allometries.

535 **Appendix.** Analysis of allometry among species.

536 A. Allometry with size

An allometric scaling describes the relationship of a trait y with body size x (or with a proxy for 537 body size). Such relationships are usually characterized by a power law (Eqns. 1; Huxley and Teissier, 538 539 1936) $y = ax^b$, (Eqn. 1a) 540 541 which becomes after log-log transformation 542 $\ln y = \ln a + b \ln x$ 543 (Eqn. 1b) 544 545 When parameter b (the scaling exponent) equals one then y is said to be isometric with body 546 size x. These two traits then scale linearly by a factor a (the factor of proportionality). When b > 1 we speak of positive allometry or hyperallometry; b < 1 is called negative allometry or hypoallometry 547 548 (Shingleton, 2010). A log-log regression can be used to estimate b and ln a. In its simplest least-squares 549 or ML (maximum likelihood) formulation, the regression assumes that the residual variance around 550 the regression line is independent of ln x (homoscedasticity).

551

552 B. Different measures of body size

In regression equations used to estimate scaling exponents and factors of proportionality, size can be represented by a variable that scales with length of the individual in any life stage, the area of a section, or with its volume. In the context of this study, we assume that eggs are well approximated by considering them to be spheres with radius *r*. The area of an egg on a digital photograph then is $A = \pi r^2$, whereas egg volume is $V = \frac{4}{3}\pi r^3$. The two are related by $V = \frac{4}{3\sqrt{\pi}}A^{3/2}$ or $lnA = \frac{2}{3}ln V -$ 558 $\frac{2}{3} ln \frac{4}{3\sqrt{\pi}}$. This last equation implies that the slope estimate in a log-log regression on area is two thirds 559 the scaling exponent of an allometry with volume as a measure of size. The estimate of the factor of 560 proportionality is also affected by this transformation and can be adjusted by adding 0.189789 to the 561 intercept of a log-log regression on area.

562

563 C. Allometry between traits

There are two schools in the analysis of multivariate allometries (Klingenberg, 2016). Either size corresponds to a specific measurement and can be an explanatory variable ("Gould–Mosimann" school), or it is the main axis of variation of the joint distribution of traits. This principal component is then seen as representing size variation ("Huxley–Jolicoeur" school) (Klingenberg, 2016). We follow the first approach.

569 When two traits y_1 and y_2 both relate to body size, then we can write out their respective 570 allometric relationships with body size *x* as

571

572
$$y_1 = a_1 x^{b_1}$$
 and $y_2 = a_2 x^{b_2}$ (Eqn. 2a)

573
$$\ln y_1 = \ln a_1 + b_1 \ln x$$
 and $\ln y_2 = \ln a_2 + b_2 \ln x$ (Eqn. 2b)

574

The parameters *a* and *b* can differ between traits and traits can each scale isometrically or allometrically with body size. We can also interpret the scaling between y_1 and y_2 by means of a power law:

578

579
$$y_2 = c y_1^{\ d}$$
 (Eqn. 3a)

581 or after log-log transformation

582
$$\ln y_2 = \ln c + d \ln y_1$$
 (Eqn. 3b)

583

584 We can combine Eqns. 2 and 3 to find that

585
$$d = \frac{b_2}{b_1} \text{ and } c = \frac{a_2}{a_1^{\frac{b_2}{b_1}}}$$
 (Eqn. 4)

586

587 This expression parallels the one by Jolicoeur (1963), which was based on principal component 588 analysis. Isometry with body size can differ between traits. We can also speak of isometry between a 589 pair of traits when d equals one. In this case the equality $b_1 = b_2$ must hold true. To test this equality, 590 there is no use in fitting models regressing one trait on another. Multivariate models that include both 591 b_1 and b_2 as parameters provide a test, and they model the allometric relationships per trait. When 592 both traits are isometric with body size, this equality condition is obviously satisfied as well. To test for differences in parameters a and b and to assess isometries with size and between traits, modelling 593 all traits jointly and with body size as a covariate seems preferable, such that parameter differences 594 can be tested by constraining equality among subsets of parameters. 595

596

597 D. Regressing on body size within groups (the problem)

Following up on Gelman and Hill (2007), Nakagawa et al. (2017) discuss issues that arise when traits are regressed on body size in the presence of other covariates. Rather than being just an explanatory variable or a mediating trait, body size *x* can be a response affected by causal effects potentially shared with the other traits *y* which are regressed on it. While Shipley (2004) proposes a test to falsify potential effect orderings, (Nakagawa et al., 2017) propose not to regress on body size or log-body size as is, but to use residuals with respect to group averages for the groups defined by

604 the other covariates modelled. This avoids that intercept terms suddenly get a different interpretation 605 when terms including body size are removed from the model, and ensures that intercept estimates in 606 all models compared represent average log-total amounts. These often need to be compared, while 607 estimating factors of proportionality can be less insightful when body sizes differ much between 608 groups. We can rewrite models for a trait y to accommodate this idea as 609 $\ln y = \ln a + b \ln x = \ln a + b \overline{\ln x} + b (\ln x - \overline{\ln x})$ 610 (Eqn. 5a) 611 for data belonging to the first group and with $\ln x$ being average log-body size in that group, 612 613 and 614 $\ln y' = \ln a' + b' \overline{\ln x'} + b' (\ln x' - \overline{\ln x'})$ 615 (Eqn. 5b) 616

for observations y' and x' in the other group, for example. Log-log regressions with separate estimated intercept and slope parameters for each of these groups will estimate the allometric scaling exponents as the slopes on the group-centered log-body sizes. The factors of proportionality $\ln a$ and $\ln a'$ are not separately estimated because intercept estimates will include terms which are contributions of the average log-body size in the group scaled by the allometric scaling exponent. Intercept estimates will also be affected by conversions as explained in section B.

623

624 E. When not to regress on centered log-body size

First of all, small sample sizes per group in the model or vastly different sample sizes can prevent
fitting the centered sizes well or reduce the estimation precision on other parameters in the model.

This makes it worthwhile to always fit models without regressions on body size as well, to check the stability of estimates of other parameters. In any case where we can't include sizes or centered sizes in models, we need to remain aware that the interpretation of an intercept becomes the one based on Eqns. 5: it is the factor of proportionality of an allometric relationship plus a term depending on the average size scaled by the allometric exponent.

A second issue arises when a response variable *y* is calculated directly from body size, i.e. when the calculation of the response involves body size or its proxy. This becomes a regression of a variable on itself, amply discussed and criticised by Nee et al. (2005). We then need to resort to models that don't have explicit body size terms, hence intercepts again include a term depending on average size scaled by the allometric exponent.

637 A third issue arises in the context of analysing concentrations or compositions. An analysis of a 638 response which is a ratio of a trait y and x by means of a model which includes a body size covariate, 639 can in fact be interpreted as a model that fits a body size term to test whether the dependence 640 between y and x deviates from isometry. A side effect of analysing a ratio is that it becomes less clear 641 what the pattern of residual variance in the ratio should be. A useful option seems to be using a log-642 log regression including log-body size as an offset term (i.e. specifying log-body size coefficient as 1 643 and thus assuming isometry). Then the response modelled is the trait y, for which variance properties 644 are better known or less constrained. In the case where we assume isometry (we analyse the 645 concentration y/x), there is no parameter b estimated. When data don't allow regressing on body size 646 terms to test for deviations from isometry, we can interpret all fitted terms as if we assume isometry but it seems better to say that estimated intercept terms include a term depending on average size 647 648 scaled by the allometric exponent minus one. For the analysis of compositions, dedicated methods 649 exist (van den Boogaart and Tolosana-Delgado, 2013). If model parameters are not easily 650 interpretable, we can switch to a multivariate analysis of total amounts to interpret parameters, while 651 testing contributions of covariates can be done according methods for compositional data. This

approach avoids the use of ratios, but a proper model for compositional data is replaced with a modelcompositional methods were invented to avoid.

654

655 F. Measurement error

656 Warton et al. (2006) discuss at length the consequences of measurement and equation error in 657 the analysis of bivariate allometries. They state that, in many instances, the two traits involved are 658 equally important and that therefore major axis (MA) or reduced major axis (RMA) are obvious least-659 squares model-fitting choices. In a likelihood approach, one would then resort to likelihoods based on 660 multivariate distributions for each observation and the slope b becomes part of the specification of 661 the covariance between traits (Warton et al., 2006). Covariance regression models can then be used 662 to test for differences in slope b between groups. Voje et al. (2014) on the other hand, do opt for a regression approach where body size is seen as an explanatory variable. In the models we fitted to 663 664 several traits, such a regression approach was implemented and no covariance regressions were fitted 665 for simplicity. When the main goal of inference is predicting a trait on the basis of body size, standard 666 regression models should be used (Warton et al., 2006). These are also most adequate in the absence 667 of measurement error in body size or they suffice when the goal of inference is to test whether body size significantly affects another trait, i.e. whether b is non-zero or different from one in the case of 668 669 concentrations in models with an offset. In the presence of measurement error estimates of 670 regression slopes are affected, therefore when we need reliable estimates of b, measurement error 671 needs to be considered and taken into account. However, across a number studies inspected by 672 Warton et al. (2006) the effects seemed limited to a decrease in the estimated value of the slope b 673 which is below 8%. Next to other methods such as the method-of-moments regression (Warton et al., 674 2006), Carroll et al. (2006) propose regression calibration and simex extrapolation as generally 675 applicable methods to derive regression slopes corrected for measurement error in body size which

still permit standard regression or mixed models can be fitted and the associated inference methods

677 used.

678

679 G. Explaining variation in traits dependent on body size

To calculate coefficients of determination or generalized coefficients of determination, regression 680 681 models are required that regress a response trait y on body size x. Here, we motivate our choice to do 682 this in linear regressions of y on x and not by means of log-log regressions. In log-log regressions, terms 683 including body size estimate allometric exponents. So, if we calculate coefficients of determination for 684 these regression terms, we don't assess the contribution of the parameters a, which are the primary 685 scaling parameters we are interested in as a description of the explanatory effects. Here, we are not 686 interested in the allometric exponents that control curvature of the allometry and we would also need 687 to interpret coefficients of determination for intercepts, which is counterintuitive. We therefore 688 analyse response of raw y on size x values. The non-linear effects then contribute to unexplained 689 variation of the linear regression.

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704	
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848

850 Tables

Table 1. **Overview of species mean values for the measured egg parameters.** The "Females" column reports number of individual spawning females whose eggs were analysed for composition or went into incubation (the number in brackets corresponds to females in the incubation dataset if the two differ). The numbers for egg parameters shown in brackets give sample sizes. Standard deviation (SD) is provided for egg area along with the mean.

	Fomaloc	Equation $\pm 5D \mathrm{[mm^2]}$	Water	NICLS contant [9/]	Triglyceride	Incubated/		
Species	remaies	Egg area ± SD [mm]	content [%]	NICIS content [%]	concentration [mg/ml]	Hatched		
Austrolebias bellottii (small species) (AB)	4	1.336 ± 0.188 (928)	68.5 (99)	8.5 54.4 0.4 (39)	6.8 (10)	829/39		
Austrolebias nigripinnis (small species) (AN)	4	1.214 ± 0.148 (478)	68.5 (63)	8.6 54.9 0.4 (28)	4.7 (3)	415/51		
Austrolebias elongatus (piscivore) (AE)	2 (5)	3.781 ± 0.515 (116)	74.8 (14)	9.1 51.9 0.5 (12)	16.8 (6)	102/2		
Austrolebias prognathus (piscivore) (AP)	1 (2)	4.420 ± 0.589 (49)	73.4 (11)	8.8 52.4 0.5 (11)	12.9 (4)	38/0		
Nothobranchius furzeri (small species - African) (NF)	7	1.154 ± 0.105 (108)	73.5 (108)	8.9 54.3 0.4 (40)	3.9 (10)	-		
Austrolebias bellottii 6+ years in incubation (AB	-	1.689 ± 0.176 (7)	-	-	9.9 (7)	-		
2011)								
Additional datasets								
Austrolebias bellottii 2015 (AB 2015)	3	1.357 ± 0.370 (61)	83.1 (61)	-	-	-		
Austrolebias nigripinnis 2015 (AN 2015)	1	1.594 ± 0.118 (28)	-	-	-	-/28		
Austrolebias elongatus 2015 (AE 2015)	1	3.813 ± 0.524 (23)	-	-	-	-/23		

854

856 Table 2. Species-specific allometries of egg dry mass and water content with egg area. We zero-centered the log-transformed egg area for each species prior to analysis. The "log(dry weight)" column corresponds to the mean effects of species on log-transformed egg dry weight, where a first set of species effects 857 858 represents differences from A. bellottii. The species differences for log-egg area effects (lower half of the first column) are differences in slopes of log-dry 859 weight on centered log-egg area from that of A. bellottii (Fig. 2A). The "log(water weight) vs log(dry weight)" column gives estimates of the mean multiplicative 860 species differences in log-water weight compared to A. bellottii. The reference estimate for A. bellottii here represents the log-ratio between egg water weight 861 and dry weight (Fig. 2B). Note that egg water weight approximately increased with egg area (lower half of the second column) across four out of five species, 862 with the same scaling exponent as egg dry weight. Parameter estimates are given with their standard errors (SE). Estimates significantly different from zero (p < 0.05) are in bold. 863

Fixed Effect	log(dry weight)	log(water weight) vs log(dry weight)
A. bellottii (reference)	-1.006 (0.033)	0.769 (0.021)
Difference of A. nigripinnis	-0.116 (0.048)	0.012 (0.032)
Difference of A. elongatus	1.332 (0.063)	0.343 (0.043)
Difference of A. prognathus	1.579 (0.077)	0.222 (0.051)
Difference of N. furzeri	- 0.678 (0.040)	0.277 (0.028)
log(egg area) (effect in A. bellottii)	0.628 (0.116)	-0.074 (0.134)
Difference of log(egg area) in A. nigripinnis	-0.066 (0.155)	0.022 (0.183)
Difference of log(egg area) in A. elongatus	0.823 (0.180)	0.038 (0.216)
Difference of log(egg area) in A. prognathus	-0.592 (0.321)	1.164 (0.401)
Difference of log(egg area) in <i>N. furzeri</i>	- 0.496 (0.156)	0.268 (0.183)

Table 3. **Relative changes in egg total amounts of carbon, nitrogen and sulphur between species**. The "log(total C)" column gives the estimate of mean logtransformed total egg carbon for each species using *A. bellottii* as a reference followed by slopes of centered log-egg area. The two other columns correspond to species differences between log-total nitrogen and log-total carbon, and log-total sulphur and log-total carbon, respectively. Relative elemental composition of eggs varied across different species. The top half of the table indicates that the piscivores' (*A. elongatus* and *A. prognathus*) and *N. furzeri* eggs had relatively more nitrogen to carbon than *A. bellottii* (Fig. 3A). In addition to that, eggs of piscivores also contained relatively more sulphur (to carbon) (Fig. 3B). Allometric scaling of elements content with egg area differed among species, but was consistent for the different elements (except for sulphur content in *A. nigripinnis*). The estimates are given with their standard errors (SE). Values significantly different from zero (*p* < 0.05) are in bold.

Fixed Effect	log(total C)	log(total N) vs log(total C)	log(total S) vs log(total C)
<i>A. bellottii</i> (reference)	-1.661 (0.009)	-1.861 (0.015)	-4.992 (0.040)
Difference of A. nigripinnis from A. bellottii	- 0.110 (0.013)	0.006 (0.022)	-0.069 (0.056)
Difference of A. elongatus from A. bellottii	1.423 (0.018)	0.114 (0.027)	0.368 (0.061)
Difference of A. prognathus from A. bellottii	1.619 (0.013)	0.075 (0.020)	0.322 (0.043)
Difference of N. furzeri from A. bellottii	- 0.617 (0.013)	0.053 (0.023)	-0.014 (0.060)
log(egg area) (effect in A. bellottii)	1.578 (0.081)	-0.016 (0.128)	0.613 (0.353)
Difference of log(egg area) in A. nigripinnis	-0.796 (0.204)	0.322 (0.336)	-2.680 (0.823)
Difference of log(egg area) in A. elongatus	0.027 (0.184)	0.207 (0.270)	-0.045 (0.601)
Difference of log(egg area) in A. prognathus	-1.393 (0.270)	-0.008 (0.403)	-0.418 (0.554)
Difference of log(egg area) in <i>N. furzeri</i>	- 0.565 (0.179)	0.221 (0.325)	0.884 (0.817)

874 Table 4. Effect of egg size and duration of pre-hatching stage on hatchling size and yolk sac size. The "log(hatchling size)" column reports parameter estimates 875 of species differences and scaling exponents of egg area (Fig. 5). The "log(yolk sac area) vs log(hatchling size)" column then gives the proportional changes for yolk sac area. The effect of log-transformed egg area appeared to be zero on hatchling size, but negative on yolk sac area in both of the species. Yolk sac 876 877 area declined with the number of days spent in the pre-hatching stage. Estimates are given with their standard errors (SE). Terms significantly different from 878 zero (p < 0.05) are in bold.

Fixed Effect	log(hatchling size)	log(yolk sac area) vs log(hatchling size)
A. bellottii (reference)	1.540 (0.025)	3.437 (0.136)
Difference of A. nigripinnis from A. bellottii	-0.148 (0.009)	-0.091 (0.050)
log(egg area)	0.056 (0.045)	-0.533 (0.245)
Days in pre-hatching stage	0.001 (0.001)	-0.004 (0.002)

879

Table 5. Predictive power of egg area on wet weight, dry weight and hatchling size. The R^2 estimate was conducted separately for fixed-effects model part

- 882 ($R^2_{marginal}$) and the whole model including random effects ($R^2_{conditional}$). We compare 2017 data with the additional 2015 dataset and also with a published study
- 883 on hatchling size of *N. furzeri* (Vrtílek et al., 2017).

	Wet weight		Dry weight		Hatchling size			
Species	R ² marginal	R ² conditional	R ² marginal	R ² conditional	R ² marginal	R ² conditional		
A. bellottii	0.210	0.915	0.136	0.898	0.016	0.016		
A. nigripinnis	0.290	0.805	0.231	0.777	0.028	0.028		
A. elongatus	0.959	0.959	0.907	0.927	-	-		
A. prognathus	0.355	0.355	0.004	0.004	-	-		
N. furzeri	0.124	0.747	0.058	0.338	-	-		
Additional datasets								
A. bellottii 2015	0.904	0.925	0.815	0.815	-	-		
A. nigripinnis 2015	-	-	-	-	0.104	0.104		
A. elongatus 2015	-	-	-	-	0.654	0.654		
N. furzeri 2016 (Vrtílek et al., 2017)	-	-	-	-	0.172	0.398		

885 Figure captions

Fig. 1. Species with larger body size lay larger eggs. The plot shows the relationship between female

body size and egg size in the studied species of annual killifish. Full points represent values collected

888 for this study and empty points are for data from the two additional datasets. Note that for data

obtained from Austrolebias bellottii in 2015 size of the three females was unknown. Species

abbreviations: AB – Austrolebias bellottii, AN – A. nigripinnis, AE – A. elongatus, AP – A. prognathus,

891 NF – Nothobranchius furzeri.

892

893 Fig. 2. Species-specific allometries between egg size and egg dry mass, and egg size and water 894 content. Plot (A) shows species-specific slopes of the relationship between log-dry weight and the 895 species zero-centered log-egg area (full points, solid lines), and log-water weight and species zero-896 centered log-egg area (empty points, dashed lines) (Table 2). Note that the lines for egg dry weight 897 and water weight are parallel in most of the species except A. prognathus (AP). The parallel lines 898 mean that water content scaled similarly to dry mass with egg size. Plot (B) then shows relative 899 amount of water and dry mass in the eggs of the different species. The distance between the log-dry 900 and log-water weight here corresponds to log-ratio of these two egg parameters (means are 901 denoted by horizontal lines and vertical lines give standard errors estimated from the model, Table 902 2). For their mean egg size (indicated as vertical dashed line at 0 in Figure 2A), the piscivores (A. 903 elongatus and A. prognathus) and N. furzeri have relatively higher proportion of water weight to dry 904 mass (wider distance between mean log-dry weight and mean log-water weight). Species 905 abbreviations: AB – Austrolebias bellottii, AN – A. nigripinnis, AE – A. elongatus, AP – A. prognathus, 906 NF – Nothobranchius furzeri.

907

908 Fig. 3. Egg elemental composition differed among species. The ternary plots show relationship 909 between the three measured elements in killifish eggs and the residual content (A – carbon, nitrogen 910 and residual; B – carbon, sulphur and residual; C – nitrogen, sulphur and residual). The position of 911 each point (trivariate coordinate for each egg) reflects the inter-dependence of the elemental 912 content proportions as they together must add up to 100 %. The original percentage values were 913 transformed using isometric log-ratio transformation, and then scaled and centered for visualization. 914 Species abbreviations: AB – Austrolebias bellottii, AN – A. nigripinnis, AE – A. elongatus, AP – A. 915 prognathus, NF – Nothobranchius furzeri.

916

- 917 Fig. 4. Relative triglyceride content was higher in species with larger eggs. Note that the extremely 918 long incubation (6+ years) did not reduce the level of triglycerides in the eggs of Austrolebias bellottii 919 from 2011 compared to the freshly laid eggs of the same species. Horizontal lines are for species 920 mean and vertical lines for standard errors from the model. The lowercase letters on top of each 921 species data indicate differences in the relative amount of triglycerides (based on a model that 922 included species zero-centered log-egg volume as an offset). Each point represents log-transformed 923 amount of triglyceride amount in an egg. Species abbreviations: AB – Austrolebias bellottii, AN – A. nigripinnis, AE – A. elongatus, AP – A. prognathus, NF – Nothobranchius furzeri. 924 925
- 926 Fig. 5. Hatchling size corresponds with egg size only in the large species. Hatchlings coming from
- 927 larger eggs were bigger only in *A. elongatus* (piscivore) from 2015, but in the small *Austrolebias* (*A.*
- 928 bellottii and A. nigripinnis) egg area had no effect on hatchling size. Species abbreviations: AB -
- 929 Austrolebias bellottii, AN A. nigripinnis, AE A. elongatus.

Fig. 1



5. I

Fig. 2



Fig. 3



Fig. 4



Fig. 5

