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# 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  
26 her offspring. It is therefore reasonable to employ egg size as a proxy for egg content when studying  
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  
37 can ignore relevant species-specific differences and mask within-species variability in egg content.

38

39           **Keywords:** *Austrolebias*, allocation strategy, allometry, egg composition, *Nothobranchius*  
40 *furzeri*

41

## 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  
55 size as a proxy for other egg parameters seems, therefore, a sufficient way to assess, or control for  
56 female allocation among individual offspring.

57           Some studies have shed doubt on the generality of strong positive correlations between egg  
58 size and egg content (Leblanc et al., 2014; Moran and McAlister, 2009; Murry et al., 2008), showing  
59 that egg dry mass proves to be a more reliable predictor of nutrient and energy content (Murry et al.,  
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

68 pond soil (Cellerino et al., 2016; Furness, 2016). Adaptation to this temporary habitat evolved multiple  
69 times from non-annual ancestors (Furness et al., 2015; Helmstetter et al., 2016). A common feature  
70 of annual killifish embryos is their ability to arrest development (by entering diapause) and potentially  
71 reduce energy expenditure at three discernible morphological stages (Podrabsky et al., 2010; Wourms,  
72 1972). Diapauses allow embryos of annual killifish to survive dry periods that may occasionally span  
73 even multiple years. Egg provisioning thus represents a crucial life-history process in annual killifish  
74 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  
88 relevance of egg area measured from digital photographs as a predictor for egg wet and dry weight  
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.

92

93

## 94 **Methods**

### 95 **Breeding pairs of *Austrolebias* species**

96 To collect eggs, we established breeding pairs of four *Austrolebias* species from captive  
97 populations maintained at the CEREEP station in Nemours-St. Pierre, France (approval no. B77-431-  
98 1). We used two small species – *A. bellottii* (population “Ingeniero Maschwitz”, 4 pairs) and *A.*  
99 *nigripinnis* (“La Guarderia”, 4 pairs); and two large (“piscivorous”) species – *A. elongatus* (“General  
100 Conesa”, 5 pairs) and *A. prognathus* (“Salamanca”, 2 pairs). Table S1 in the Supplementary Information  
101 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  $\mu$ S 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 Père et Fils, Arry, France).

109

### 110 **Breeding stock of *N. furzeri***

111 We wanted to compare egg content between two phylogenetically distinct genera of annual  
112 killifish - *Austrolebias* (South America) and *Nothobranchius* (Africa) (Furness et al., 2015). We therefore  
113 established a breeding stock of *N. furzeri* (population “MZCS414”) from the Institute of Vertebrate  
114 Biology CAS in Brno, Czech Republic at the CEREEP facility. We hatched *N. furzeri* according to the  
115 standard protocol (Polačik et al., 2016) using a 1:1 mixture of osmotic and tap water at 18 °C. We  
116 subsequently reared the fish at 27-28 °C and fed them with *Artemia salina* nauplii until the age of ten

117 days when they were weaned on *Tubifex* sp. We separated sexes when first marks of nuptial  
118 colouration of males appeared, at approximately three weeks, and paired the fish only for egg  
119 collection. *Nothobranchius furzeri* were hatched on July 4, 2017, shortly after the collection of eggs  
120 from *Austrolebias* began, so they reached full maturity (at an age of six weeks) when *Austrolebias* egg  
121 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  $\mu\text{m}$  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.

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

140

141           **Preparations for the analysis of egg content**

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

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

157

158           **Elemental content analysis**

159           The concentrations (weight percentage) of carbon, nitrogen and sulphur were determined by  
160 dry combustion with a CNS element analyser (Thermo Scientific Flash 2000) at the Global Change  
161 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 µl 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  
173 replicate using a microplate reader (BIO-RAD iMark). We used the reference standards to calculate  
174 triglyceride concentrations of the two replicates.

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  
188 of hatchling size.

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 l of peat boiled in 1 l 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 Misiones”) 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**

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

219 We assumed that the investigated egg parameters scaled allometrically with egg size and that  
220 these relationships can be described by constants of proportionality and scaling exponents (Appendix,  
221 Section A). We used egg area as a measure of size. In the Appendix, we explain how allometries can  
222 be analysed in a multi-species context and provide arguments for the modelling choices we made. We  
223 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).

232 We always started model selection with a full model and then tried to simplify it on the basis of  
233 log-likelihood ratio tests and F-tests on model log-likelihoods. We removed random effects first,  
234 followed by simplification of residual variance structures and then fixed effects (Zuur et al., 2009). We  
235 removed random effects by comparing models containing all residual variance terms and fixed effects  
236 fitted with restricted maximum likelihood (REML). When testing the importance of individual fixed  
237 effects, we refitted the models with maximum likelihood (ML). We then assessed fit of the selected  
238 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  
255 isometric log-ratio (ilr) (library “compositions”; van den Boogaart and Tolosana-Delgado, 2013). We  
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).

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

265 volume and egg volume taken as  $V = \pi \frac{d_1 d_2 d_3}{6}$ , assuming a spheroid egg shape. We used log-  
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  
279 interaction between “trait” (by default), “species” and species-centered “log-transformed egg area”  
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.

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

290 (Appendix, Section G), and pair and collection date random effects. Residual variances were allowed  
291 to vary between species. We then fitted separate models per species with egg area fixed effects, and  
292 pair and collection date random effects. We fitted the same models to the additional datasets from  
293 2015 to compare predictive power of egg area among different studies.

294

295

## 296 **Results**

297 We collected over six thousand eggs of five annual killifish species in 2017 of which we  
298 incubated 1384 and measured wet and dry weight of 295 eggs. We used 130 of these dried eggs for  
299 CNS elemental analysis and another 40 fresh eggs, which were weighed, for triglyceride content assay  
300 (including seven eggs collected from substrate incubated for 6+ years) (Table 1). The additional two  
301 datasets consisted of 61 wet and egg dry weights from a single species, and of 51 hatchling sizes from  
302 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**

305 Taking egg area determined from images as the measure of egg size, the collected eggs  
306 clustered into two groups according to adult sizes of the species they belonged to. Eggs were notably  
307 larger in the piscivores, *Austrolebias elongatus* and *A. prognathus*, compared to the small *Austrolebias*  
308 species, *A. bellottii* and *A. nigripinnis*, and to *Nothobranchius furzeri* (Fig. 1, Table 1). The size of *A.*  
309 *bellottii* eggs collected after 6+ years of incubation was larger compared to freshly laid eggs of the  
310 same species (Table 1). The eggs from the additional datasets were similar in size to eggs collected for  
311 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):  $-0.239 \pm 0.128$ ), hence both changed approximately isometrically with egg volume.

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

336 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  
338 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  
347 interpret, we re-fitted the full model with log-transformed total amounts of the elements (Table 3).  
348 Overall, carbon content scaling exponents of egg area varied among species, but nitrogen and sulphur  
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.

352 Egg elemental composition varied across different species. Species differences in the total  
353 amount of carbon can be largely explained by species effects on egg dry weight (compare the “log(dry  
354 weight)” column of Table 2 to the “log(total C)” in Table 3). The top half of the table indicates that the  
355 piscivores (*A. elongatus* and *A. prognathus*) and *N. furzeri* had relatively more nitrogen to carbon than  
356 *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  
358 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  
366 triglycerides was highest in *A. elongatus* and lowest in *N. furzeri* which overlapped with the small  
367 *Austrolebias* species, *A. bellottii* and *A. nigripinnis* (Fig. 4). We didn't observe a difference between  
368 fresh eggs of *A. bellottii* and those collected from 6+ years old substrate (Fig. 4). We explored potential  
369 allometric scaling exponent effects of egg volume on the amount of triglycerides and found a  
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  
373 these eggs were incompletely composed and that we should not take this as evidence for  
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  $\pm 0.091$ ), hatchlings of *A. elongatus* showed a hypo-allometric increase in size with egg area (log(egg  
385 area) effect:  $0.381 \pm 0.057$ ) (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  
394 predicted parameters ( $p < 0.001$ ). This suggests that egg area does not predict the other parameters  
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 ( $R^2_{\text{marginal}}$ ) and  $R^2$  of the whole model including random effects ( $R^2_{\text{conditional}}$ ). 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* ( $R^2_{\text{marginal}} = 0.959$ ), but

400 performed less well in the other species (Table 5). When we applied the same model to the additional  
401 *A. bellottii* dataset of wet weight from 2015, the very high value found ( $R^2_{\text{marginal}} = 0.904$ ) contrasts  
402 with *A. bellottii* from the 2017 experiment ( $R^2_{\text{marginal}} = 0.210$ ). In egg dry weight models,  $R^2_{\text{marginal}}$  was  
403 generally lower than for wet weight, again with particularly high values for *A. elongatus* from 2017  
404 and *A. bellottii* from 2015 (Table 5). There was a small explanatory effect of egg area on hatchling size  
405 in two species that hatched in the 2017 experiment (*A. bellottii* and *A. nigripinnis*) and in *A. nigripinnis*  
406 from 2015. The exception was again *A. elongatus* from 2015 with a high  $R^2_{\text{marginal}}$  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  
413 composition of eggs in five annual killifish species using data obtained from individual eggs. We tested  
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

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

424 of allometries between traits in different species or groups is a recurring theme in morphometry  
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  
437 of log-log regressions on egg volume. Slope values in log-log regressions on egg area below 1.5 imply  
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.

446 Egg area did not affect the other egg parameters in some species. We recorded weak-to-zero  
447 relationships between egg area and dry and water weight in *A. prognathus* and *N. furzeri*. The egg  
448 area relationship with hatchling size was also absent on the intra-specific level (for both *A. bellottii*  
449 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  
463 envelope or a chorion with extremely low water permeability (Machado and Podrabsky, 2007). Water  
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 (Craig 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

476 limited effects on embryonic survival and development in *Austrolebias bellottii* (Van Dooren and  
477 Varela-Lasheras, 2018). As an alternative explanation, larger water content in eggs of some species  
478 might stem from their higher sensitivity to desiccation and not necessarily represent specialization to  
479 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  
488 common sole (49.9 % C, 9.5 % N) (Yúfera et al., 1999). The elemental composition was specific to the  
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**  
494 **annual killifish.** It has been hypothesised that lipids from egg oil globules act as energy reservoir during  
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  
512 energy source for fish that is mainly used during embryonic development are egg yolk lipoproteins  
513 (primarily lipovitellin) (Brooks et al., 1997; Wiegand, 1996). Freshly hatched annual killifish still possess  
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

526 resistance during an initial post-hatching phase in other fish (Jardine and Litvak, 2003), amphibians or  
527 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.

534



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

536 **A. Allometry with size**

537 An allometric scaling describes the relationship of a trait  $y$  with body size  $x$  (or with a proxy for  
538 body size). Such relationships are usually characterized by a power law (Eqns. 1; Huxley and Teissier,  
539 1936)

540 
$$y = ax^b, \quad (\text{Eqn. 1a})$$

541

542 which becomes after log-log transformation

543 
$$\ln y = \ln a + b \ln x \quad (\text{Eqn. 1b})$$

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  
547 speak of positive allometry or hyperallometry;  $b < 1$  is called negative allometry or hypoallometry  
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**

553 In regression equations used to estimate scaling exponents and factors of proportionality, size  
554 can be represented by a variable that scales with length of the individual in any life stage, the area of  
555 a section, or with its volume. In the context of this study, we assume that eggs are well approximated  
556 by considering them to be spheres with radius  $r$ . The area of an egg on a digital photograph then is  
557  $A = \pi r^2$ , 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  $\ln A = \frac{2}{3}\ln V -$

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**

564 There are two schools in the analysis of multivariate allometries (Klingenberg, 2016). Either size  
565 corresponds to a specific measurement and can be an explanatory variable ("Gould–Mosimann"  
566 school), or it is the main axis of variation of the joint distribution of traits. This principal component is  
567 then seen as representing size variation ("Huxley–Jolicoeur" school) (Klingenberg, 2016). We follow  
568 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

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

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

574

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

578

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

580

581 or after log-log transformation

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

583

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

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

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  
593 for differences in parameters  $a$  and  $b$  and to assess isometries with size and between traits, modelling  
594 all traits jointly and with body size as a covariate seems preferable, such that parameter differences  
595 can be tested by constraining equality among subsets of parameters.

596

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

598 Following up on Gelman and Hill (2007), Nakagawa et al. (2017) discuss issues that arise when  
599 traits are regressed on body size in the presence of other covariates. Rather than being just an  
600 explanatory variable or a mediating trait, body size  $x$  can be a response affected by causal effects  
601 potentially shared with the other traits  $y$  which are regressed on it. While Shipley (2004) proposes a  
602 test to falsify potential effect orderings, (Nakagawa et al., 2017) propose not to regress on body size  
603 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

$$610 \quad \ln y = \ln a + b \ln x = \ln a + b \overline{\ln x} + b(\ln x - \overline{\ln x}) \quad (\text{Eqn. 5a})$$

611

612 for data belonging to the first group and with  $\overline{\ln x}$  being average log-body size in that group,  
613 and

614

$$615 \quad \ln y' = \ln a' + b' \overline{\ln x'} + b'(\ln x' - \overline{\ln x'}) \quad (\text{Eqn. 5b})$$

616

617 for observations  $y'$  and  $x'$  in the other group, for example. Log-log regressions with separate  
618 estimated intercept and slope parameters for each of these groups will estimate the allometric scaling  
619 exponents as the slopes on the group-centered log-body sizes. The factors of proportionality  $\ln a$  and  
620  $\ln a'$  are not separately estimated because intercept estimates will include terms which are  
621 contributions of the average log-body size in the group scaled by the allometric scaling exponent.  
622 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**

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

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

632 A second issue arises when a response variable  $y$  is calculated directly from body size, i.e. when  
633 the calculation of the response involves body size or its proxy. This becomes a regression of a variable  
634 on itself, amply discussed and criticised by Nee et al. (2005). We then need to resort to models that  
635 don't have explicit body size terms, hence intercepts again include a term depending on average size  
636 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  
647 but it seems better to say that estimated intercept terms include a term depending on average size  
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

652 approach avoids the use of ratios, but a proper model for compositional data is replaced with a model  
653 compositional 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  
663 regression approach where body size is seen as an explanatory variable. In the models we fitted to  
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  
668 size significantly affects another trait, i.e. whether  $b$  is non-zero or different from one in the case of  
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

676 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**

680 To calculate coefficients of determination or generalized coefficients of determination, regression  
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.

690

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701

## 702 **Conflict of interests**

703 We declare no conflict of interests.

704

## 705 **Supplementary Information**

706 Supplementary information for this article is available at publisher's website.

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- 849

850 **Tables**

851 Table 1. **Overview of species mean values for the measured egg parameters.** The “Females” column reports number of individual spawning females whose  
 852 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  
 853 numbers for egg parameters shown in brackets give sample sizes. Standard deviation (SD) is provided for egg area along with the mean.

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

854

855

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  
857 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  
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  
863 ( $p < 0.05$ ) are in bold.

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

864



865 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 log-  
866 transformed 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  
867 to species differences between log-total nitrogen and log-total carbon, and log-total sulphur and log-total carbon, respectively. Relative elemental  
868 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*  
869 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)  
870 (Fig. 3B). Allometric scaling of elements content with egg area differed among species, but was consistent for the different elements (except for sulphur  
871 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 <i>A. nigripinnis</i> from <i>A. bellottii</i>	<b>-0.110</b> (0.013)	0.006 (0.022)	-0.069 (0.056)
Difference of <i>A. elongatus</i> from <i>A. bellottii</i>	<b>1.423</b> (0.018)	<b>0.114</b> (0.027)	<b>0.368</b> (0.061)
Difference of <i>A. prognathus</i> from <i>A. bellottii</i>	<b>1.619</b> (0.013)	<b>0.075</b> (0.020)	<b>0.322</b> (0.043)
Difference of <i>N. furzeri</i> from <i>A. bellottii</i>	<b>-0.617</b> (0.013)	<b>0.053</b> (0.023)	-0.014 (0.060)
log(egg area) (effect in <i>A. bellottii</i> )	<b>1.578</b> (0.081)	-0.016 (0.128)	0.613 (0.353)
Difference of log(egg area) in <i>A. nigripinnis</i>	<b>-0.796</b> (0.204)	0.322 (0.336)	<b>-2.680</b> (0.823)
Difference of log(egg area) in <i>A. elongatus</i>	0.027 (0.184)	0.207 (0.270)	-0.045 (0.601)
Difference of log(egg area) in <i>A. prognathus</i>	<b>-1.393</b> (0.270)	-0.008 (0.403)	-0.418 (0.554)
Difference of log(egg area) in <i>N. furzeri</i>	<b>-0.565</b> (0.179)	0.221 (0.325)	0.884 (0.817)

872

873

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  
 876 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  
 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)
<i>A. bellottii</i> (reference)	1.540 (0.025)	3.437 (0.136)
Difference of <i>A. nigripinnis</i> from <i>A. bellottii</i>	<b>-0.148 (0.009)</b>	-0.091 (0.050)
log(egg area)	0.056 (0.045)	<b>-0.533 (0.245)</b>
Days in pre-hatching stage	0.001 (0.001)	<b>-0.004 (0.002)</b>

879

880

881 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 ( $R^2_{\text{marginal}}$ ) and the whole model including random effects ( $R^2_{\text{conditional}}$ ). 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).

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

884

885 **Figure captions**

886 **Fig. 1. Species with larger body size lay larger eggs.** The plot shows the relationship between female  
887 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  
889 obtained from *Austrolebias bellottii* in 2015 size of the three females was unknown. Species  
890 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.*  
924 *nigripinnis*, AE – *A. elongatus*, AP – *A. prognathus*, NF – *Nothobranchius furzeri*.

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

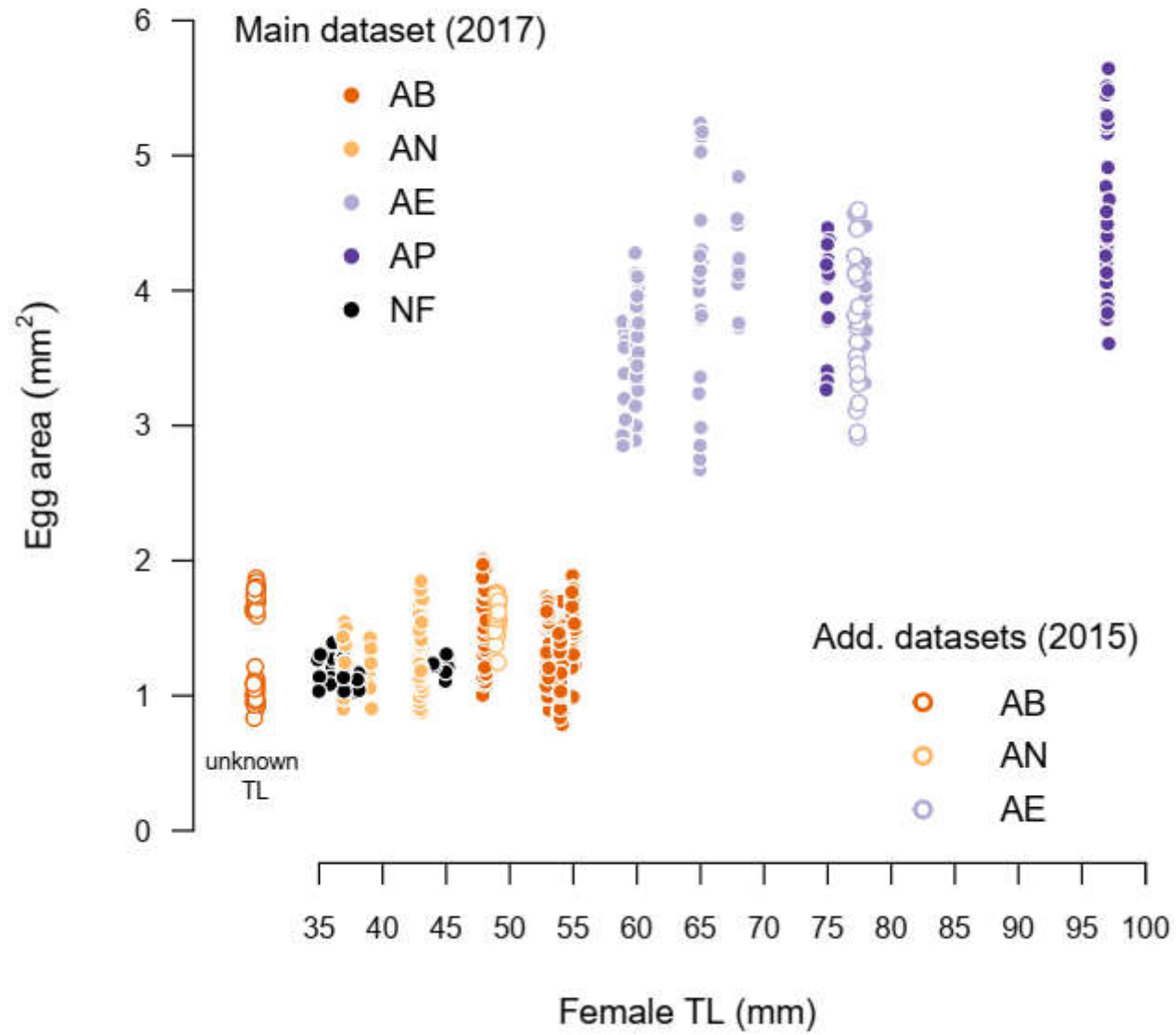


Fig. 2

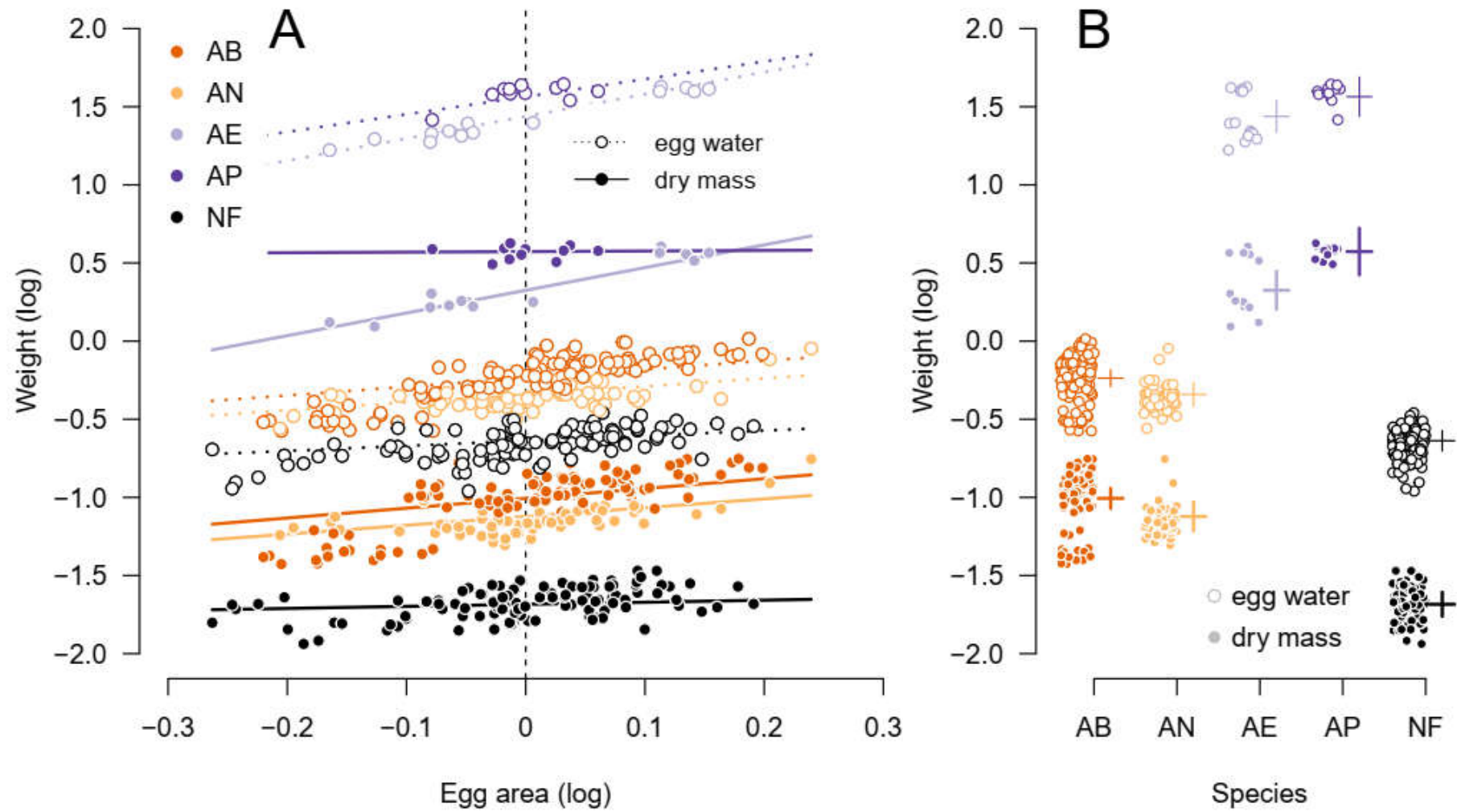


Fig. 3

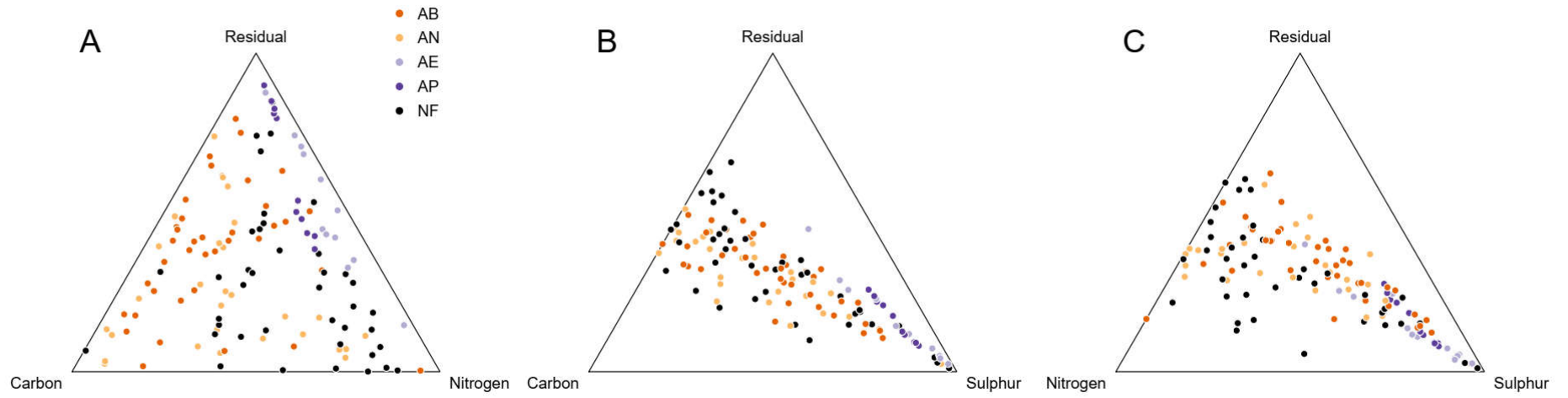




Fig. 4

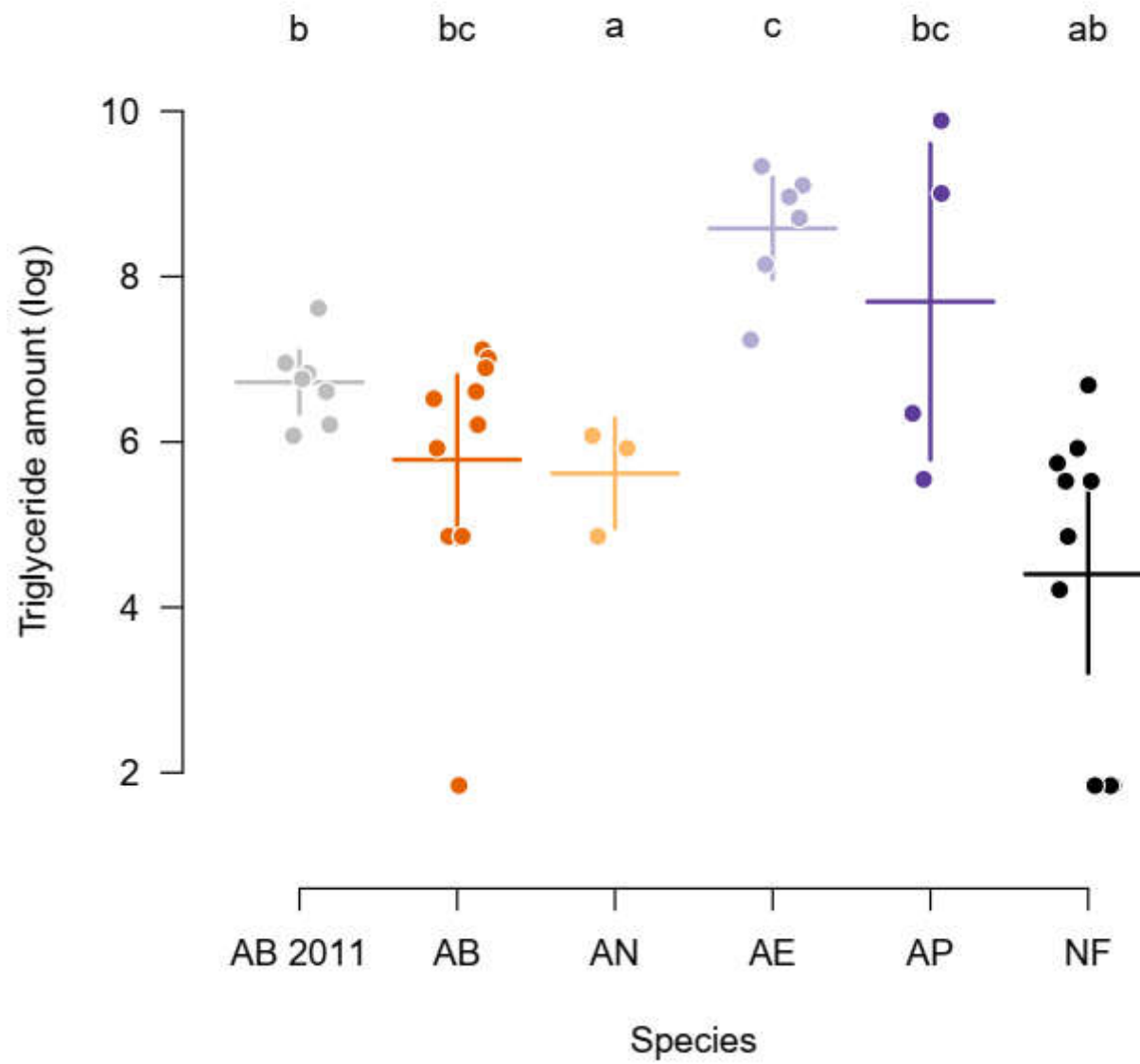


Fig. 5

