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1 ***Egg size does not universally predict embryonic resources and hatchling***
2 ***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 *A. elongatus*, a large piscivorous species with large eggs. Our study
36 shows that systematically using egg area as a proxy of egg content between different species can
37 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 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 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 µm 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 as it captures both variation in the longest axis and in sphericity.

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

225 The parameters we measured in eggs are expected to covary (water weight with egg dry weight,
226 for example) both between and within species and we therefore used multivariate models wherever
227 possible (Appendix, Section D). To do so, we reformatted values of response variables into stacked
228 form and added a dummy factor “trait” with levels identifying the different egg parameters. All
229 models thus contained “trait” fixed effects by default to distinguish the egg parameters and to allow
230 for fitting trait-specific random effects and different residual variances per trait. We fitted models
231 using functions from library “nlme” (Pinheiro and Bates, 2000) in R software v. 3.5.3 (R Core Team,
232 2019).

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

240 When data on several related species are analysed, one needs to be aware that these did not
241 evolve independently for a large part of their history (Felsenstein, 1985). To account for this non-
242 independence in data from different species, phylogenetic comparative methods have been
243 developed (Garamszegi, 2014). These usually imply that species are treated as random effects.
244 However, our study was not originally designed as a comparative experiment and includes only five
245 species. It is generally recommended to avoid estimating variances of random effects with so few
246 levels (Bolker et al., 2009). When these have many levels, fixed effects tend to show increased rates
247 of type I errors in global tests (Lindsey, 1996). In agreement with this, Freckleton and Rees (2019)
248 found that with numbers of levels below ten, the rate of false positives and the power of tests of
249 covariates are hardly affected by whether species effects are modelled as fixed or random. Modelling
250 species effects as random also complicates inference on individual species differences (Hadfield et al.,
251 2009) and frustrates future use of parameter estimates in more extensive comparative studies. We
252 therefore decided to present results with fixed effect estimates of species effects and species
253 differences. We constructed fixed-effect models for species effects accounting for phylogenetic non-
254 independence by testing for species effects in the presence of genus (*Nothobranchius*) and subgenus
255 (*Megalebias*, piscivores) effects (Stearns, 1983; Martins and Hansen, 1996). In addition, we performed
256 phylogenetic mixed models (de Villemereuil and Nakagawa, 2014) with our datasets. When there are
257 inference differences between species fixed and random effect analyses, we note that in the text.

258 To test species-specific allometries in egg dry mass and water content related to egg size, we
259 used egg dry weight and water weight as log-transformed response variables in a bivariate model. Egg
260 water weight was fitted as egg wet weight minus dry weight, therefore we did not add total wet weight
261 as an explanatory variable (Appendix, Section E). The full model included “trait”, “species” and “log-
262 transformed egg area” with all the possible interactions as fixed effects to account for different
263 allometries between species and traits. The log-transformed egg area was zero-centered per species
264 prior the analysis to ensure that intercept difference terms capture the total differences between
265 groups while slopes estimate the scaling exponents (Appendix, Section D; Nakagawa et al., 2017). We

266 provide a simple rule here which can help interpreting estimated scaling exponents. If egg parameters
267 scale isometrically with egg volume and eggs are spheres, then they must scale in a hyperallometric
268 manner with egg area (with coefficient 3/2, Appendix, Section B). Hence, when exponents of egg area
269 are not estimated to be above 3/2, there are no hyperallometries for volume. Parental pair and
270 collection date were modelled as trait-specific random effects and residual variances were allowed to
271 differ between species and traits.

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

281 We assayed triglyceride content to test for relative species differences. Triglyceride content was
282 measured as diluted sample concentration in two replicates from the same egg. We then back-
283 calculated the total amount of egg triglycerides in the replicates using the sum of standard solvent
284 volume and egg volume taken as $V = \pi \frac{d_1 d_2 d_3}{6}$, assuming a spheroid egg shape. We used log-
285 transformed total amount of triglycerides from each replicate as a response and corrected for
286 different egg sizes by fitting log-transformed egg volume as an offset (Appendix, Section E). We fitted
287 fixed effect of “species” to test for intercept differences among species and interaction between
288 “species” and species-centered “log-transformed egg volume”. Full mixed-effects model that would
289 control for both pseudoreplication and species-specific residual variances did not converge. We
290 therefore fitted two alternative models: a mixed-effects model with sample ID as random effect; or a
291 general linear model with averaged values from the two subsamples accommodating species-specific

292 residual variance. The outcome of these two models was qualitatively similar and we report estimates
293 of species contrasts from the model with the averaged sub-sample values based on Tukey's post-hoc
294 test (function `glht()`, library "multcomp"; Hothorn et al., 2008).

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

304 To evaluate how well egg area predicts egg wet weight, dry weight, and hatchling size, we
305 calculated coefficients of determination (R^2) in mixed-effects models using function `r.squaredGLMM()`
306 (library "MuMIn"; Bartoń, 2018). This function allows to separate R^2 for the whole model ($R^2_{conditional}$)
307 from the fixed-effects part of the model ($R^2_{marginal}$) (Nakagawa and Schielzeth, 2013). We first fitted full
308 mixed-effects model with "species" in interaction with untransformed "egg area" as fixed effects
309 (Appendix, Section G), and pair and collection date random effects. Residual variances were allowed
310 to vary between species. We then fitted separate models per species with egg area fixed effects, and
311 pair and collection date random effects. We fitted the same models to the additional datasets from
312 2015 to compare predictive power of egg area among different studies.

313

314

315 **Results**

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

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

322

323 **Egg size**

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

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

331

332 **Dry egg mass and water content**

333 The allometric scaling exponents of egg dry and water weight on egg area differed between
334 species. The best model included a three-way interaction between species, trait and log-transformed
335 egg area (species:trait:log(egg area) interaction, $F_{4,532} = 2.67$, $p = 0.032$; species versus genus-subgenus
336 effects model comparison: $\chi^2_2 = 27.24$, $p < 0.001$). This means that the effects of log-egg area on log-
337 egg dry weight and log-water weight differed and that this difference depends on the species (Table

338 2, Fig. 2A). The fixed effect water weight allometric scaling exponent was significantly different from
339 *A. bellottii* and from dry weight only in eggs of *A. prognathus*. In *A. prognathus*, water weight appeared
340 to scale isometrically with egg area while egg area had no effect on egg dry weight (Fig. 2A).
341 *Austrolebias bellottii* and *A. nigripinnis* showed hypo-allometric, and *A. elongatus* hyper-allometric
342 effects of egg area on dry weight. None of the predicted exponents were above $3/2$ (Table 2), hence
343 there were no hyperallometries for the relationship with egg volume. The estimates for dry weight
344 effects in *A. prognathus* and *N. furzeri* were close to zero indicating very weak relationships between
345 egg size and dry weight. In the additional 2015 dataset, *A. bellottii* eggs' dry weight changed
346 hyperallometrically with egg area (log(egg area) effect \pm SE (standard error): 1.360 ± 0.085). Water
347 weight changed similarly to that (difference between log(egg area) effect on log(dry weight) vs.
348 log(water weight): -0.239 ± 0.128), hence both changed approximately isometrically with egg volume.

349 Overall, the annual killifish eggs contained between two and three times as much water as dry
350 matter. The estimated differences in species intercepts or of different traits on the same species
351 represent multiplicative total differences after back-transformation (Fig. 2, Table 1; Appendix, Section
352 D). Relative content of water in the eggs of *A. elongatus*, *A. prognathus*, together with *N. furzeri* was
353 higher than in the small *Austrolebias* species, *A. bellottii* and *A. nigripinnis* (Tables 1 and 2, Fig. 2B).
354 Eggs of *A. bellottii* from 2015 contained 2.66-times more water than dry matter (log-transformed
355 estimate: 0.979 ± 0.035), compared to 2.16 ratio in *A. bellottii* eggs collected in 2017. Egg dry weight
356 appeared to vary more than water weight (estimated SDs ratio between log(dry weight) and log(water
357 weight) was 1.294; CI [1.140, 1.467]) and eggs of *N. furzeri* were the most variable (SD (standard
358 deviation) ratio was 1.723; CI (confidence interval) [1.256, 2.362]). Phylogenetic mixed models found
359 significantly different species variances for dry and water weight, but random regressions of egg area
360 did not converge and these models could not provide species-specific estimates of allometric scaling.
361 When comparable, fixed effect and random effect estimates of species effects were highly correlated.

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

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CNS elemental analysis

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Carbon, nitrogen and sulphur contents varied among eggs of different species including species-specific allometric scaling exponents of egg area (Table 3). The compositional analysis with ilr-transformed data showed a significant three-way interaction (species:trait:log(egg area), $F_{8,360} = 2.20$, $p = 0.028$; species versus genus-subgenus effects models $\chi^2_{12} = 45.47$, $p < 0.001$) meaning that proportions of the three elements scaled differently with egg area and that these differences depended on the species. As the estimates from ilr-transformed data were hard to interpret, we refitted the full model with log-transformed total amounts of the elements (Table 3). Overall, carbon content scaling exponents of egg area varied among species, but nitrogen and sulphur scaling exponents were hardly different. The only exception was the scaling exponent estimate for sulphur in eggs of *A. nigripinnis* (Table 3). None of the slopes of egg area is significantly above 1.5, hence we can again conclude that there are no hyperallometries for egg volume.

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Egg elemental composition varied across different species in the fixed effect analysis. Note that the phylogenetic mixed model for total amounts found a significant species variance for allometry as well. 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), but this pattern of differences was not supported by the phylogenetic mixed model, which estimated a negative correlation between carbon content and the nitrogen to carbon ratio. Eggs of piscivores contained relatively more sulphur (to carbon) (Fig. 3B, Table 3), which was confirmed by the phylogenetic mixed model. The three elements

385 analysed together made up between 60 and 68 % of egg dry weight (carbon 54.1 %, nitrogen 8.7 %
386 and sulphur 0.4 %, on average; Table 1). The variance was particularly high for measurements of
387 sulphur in eggs of the small species (*A. bellottii*, *A. nigripinnis* and *N. furzeri*) (SD ratios were 6.330-
388 8.980).

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

389

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

390

391 **Triglyceride content analysis**

392 The relative amount of egg triglycerides varied within and among species (species, $F_{5,34} = 12.88$,
393 $p < 0.001$; Fig. 4). We found that, after accounting for different egg volumes, the amount of egg
394 triglycerides was highest in *A. elongatus* and lowest in *N. furzeri* which overlapped with the small
395 *Austrolebias* species, *A. bellottii* and *A. nigripinnis* (Fig. 4). We didn't observe a difference between
396 fresh eggs of *A. bellottii* and those collected from 6+ years old substrate (Fig. 4). We explored potential
397 allometric scaling exponent effects of egg volume on the amount of triglycerides and found a
398 significant interaction (species:log(egg volume) interaction, $F_{6,28} = 3.35$, $p = 0.013$), where *N. furzeri*
399 had a much larger positive value than the other species. However, this significant interaction and the
400 large estimate are due to four very small outlying concentrations among the smallest eggs, suggesting
401 that these eggs were incompletely composed and that we should not take this as evidence for
402 hyperallometry. In a phylogenetic mixed model, the species variance was significant, but specific
403 pairwise differences had large standard deviations and overlapped.

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

404

405 **Hatchling size and remaining yolk**

406 We successfully hatched 22% (92/427) of pre-hatching stage embryos. *Austrolebias bellottii*
407 hatchlings were larger than *A. nigripinnis* (Table 4). Within species, hatchling size did not depend on
408 egg area, but yolk sac area decreased with egg area (trait: centered log(area) interaction, $F_{1,172} = 4.73$,
409 $p = 0.031$, Fig. 5, Table 4). Yolk sac area also diminished with the number of days embryos spent in the
410 pre-hatching stage and we did not record any gain to the embryo by growing larger while waiting for
411 hatching (trait:daysPH interaction, $F_{1,172} = 4.61$, $p = 0.033$). In the additional 2015 dataset, there was
412 again an at most weak effect of egg area on hatchling size of *A. nigripinnis* (log(egg area) effect: 0.165
413 ± 0.091), hatchlings of *A. elongatus* showed a hypo-allometric increase in size with egg area (log(egg
414 area) effect: 0.381 ± 0.057) (Fig. 5). Yolk sac size had a higher variance than hatchling size in our 2017
415 dataset (estimated yolk sac size:hatchling size SDs ratio was 5.291; CI [4.304, 6.506]). No phylogenetic
416 mixed model was attempted on this dataset with three species.

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

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

419 **Egg size as predictor of other egg parameters**

420 Species differed markedly in how well egg area predicted the other egg parameters. On
421 average, egg area predicted egg wet weight best, then dry weight, followed by hatchling size. Overall,
422 it predicted best for eggs of *A. elongatus* (Table 5). Models with species-specific untransformed egg
423 area explained data better than an additive model with species and egg area effects for all the three
424 predicted parameters ($p < 0.001$). This suggests that egg area does not predict the other parameters
425 in the same way across different species. We then fitted egg area in separate species-specific models
426 and calculated two complementary measures of model fit: the coefficient of determination (R^2) for

427 the fixed effects only (R^2_{marginal}) and R^2 of the whole model including random effects ($R^2_{\text{conditional}}$). Both
428 fixed-effects and whole-model R^2 for species-specific models of egg wet weight ranged widely (Table
429 5). Egg area was a particularly good predictor of egg wet weight in *A. elongatus* ($R^2_{\text{marginal}} = 0.959$), but
430 performed less well in the other species (Table 5). When we applied the same model to the additional
431 *A. bellottii* dataset of wet weight from 2015, the very high value found ($R^2_{\text{marginal}} = 0.904$) contrasts
432 with *A. bellottii* from the 2017 experiment ($R^2_{\text{marginal}} = 0.210$). In egg dry weight models, R^2_{marginal} was
433 generally lower than for wet weight, again with particularly high values for *A. elongatus* from 2017
434 and *A. bellottii* from 2015 (Table 5). There was a small explanatory effect of egg area on hatchling size
435 in two species that hatched in the 2017 experiment (*A. bellottii* and *A. nigripinnis*) and in *A. nigripinnis*
436 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.
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438

439 Discussion

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

451

452 **Allometric scaling exponents of egg components on egg area differ between species while**

453 **patterns in exponents between traits measured on the same egg often remain similar.** The analysis

454 of allometries between traits in different species or groups is a recurring theme in morphometry

455 (Teissier and Huxley, 1936; Klingenberg, 2016; Nakagawa et al., 2017). We can always assume equal

456 allometries in different species, as a null model or a first working hypothesis. However, our analysis

457 demonstrates that even within a genus, allometric scaling exponents differ between species and also

458 within species between samples collected in different years. Even between two piscivorous species

459 from a single clade (Loureiro et al., 2018), different scaling exponents were found. What we also

460 observed was that traits measured on the same egg often shared the same or a similar allometric

461 pattern. For example, egg dry mass had the same allometric exponent with egg area as water weight

462 in each of the species except for *A. prognathus*. Amount of egg components is expected to scale

463 isometrically with egg volume rather than with egg area and volume might have been a better

464 measure of egg size. However, egg volume is difficult to obtain in a non-invasive manner with little

465 handling. In the Appendix (Section B), we explain that if we assume eggs to be approximately spherical,

466 parameter estimates of log-log regressions on egg area can be converted to the expected estimates

467 of log-log regressions on egg volume. Slope values in log-log regressions on egg area below 1.5 imply

468 that the response trait does not scale hyperallometrically with egg volume. For example, larger eggs

469 of *A. bellottii* and *A. nigripinnis* had lower dry weight than expected based on a simple linear increase

470 with egg area and also egg volume. The dry weight of *A. elongatus* eggs, however, increased more

471 than isometrically with area but approximately isometrically with egg volume. Considering that the

472 only slope values above 1.5 were most likely affected by outliers (in the triglyceride assay), we never

473 found any potential hyperallometric relationship with egg volume. Parental individuals might be

474 constrained in the amounts of resources they can allocate to each individual egg, such that they can

475 provide at best isometric amounts.

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

488

489 **Water content was higher in piscivores and *N. furzeri* than in the small *Austrolebias* species.**

490 Annual killifish eggs are exposed to conditions of water stress for several months during dry season
491 periods. Water contained in the eggs therefore represents a precious resource for the embryo. Annual
492 killifish evolved special egg structures to prevent excessive water loss such as microvilli on the
493 envelope or a chorion with extremely low water permeability (Machado and Podrabsky, 2007). Water
494 content of annual killifish eggs appears on the upper boundary of the 50-70% range reported for
495 freshwater teleosts (e.g. *Chondrostoma nasus* (Keckeis et al., 2000)). It is still much lower, however,
496 than the 90 % recorded in marine pelagophilous fish (Craik and Harvey, 1984), where it is responsible
497 for egg buoyancy (Lubzens et al., 2010). We found considerable interspecific variation in egg water
498 content and the studied species formed two groups. The large piscivorous *Austrolebias* and the African
499 *N. furzeri* had higher egg water contents than the two smaller *Austrolebias* species. This clustering is
500 surprising from the perspective of their contrasting ecological conditions during embryogenesis. The
501 African *N. furzeri* occurs in a subtropical semi-arid region and faces longer, colder dry periods in

502 comparison to the temperate South-American *Austrolebias* species. In *Austrolebias*, embryos have to
503 survive a dry season which is relatively humid but warmer. Possibly, piscivorous *Austrolebias* lay their
504 eggs near the fringes of the temporary ponds, where these might face drought regimes more similar
505 to the *N. furzeri* habitats. This remains an open question, however. In lab conditions, desiccation had
506 limited effects on embryonic survival and development in *Austrolebias bellottii* (Van Dooren and
507 Varela-Lasheras, 2018). As an alternative explanation, larger water content in eggs of some species
508 might stem from their higher sensitivity to desiccation and not necessarily represent specialization to
509 drier habitats.

510

511 **Elemental composition of eggs differed between species.** We performed an exploratory
512 analysis of the elemental composition of annual killifish eggs. We measured carbon content, an
513 element which is present in various molecules acting as sources of energy; nitrogen content which
514 provides a crude estimate of proteins; and sulphur content which is scarce in animal tissues but can
515 be found in proteins containing methionine or cysteine amino acids (Kamler, 2005). The elemental
516 composition of annual killifish eggs did not diverge from that of other fish such as zebrafish (46.0-54.0
517 % C, 9.7-11.1 % N) (Hachicho et al., 2015), Northern pike (50.7 % C, 11.5 % N) (Murry et al., 2008), or
518 common sole (49.9 % C, 9.5 % N) (Yúfera et al., 1999). The elemental composition was specific to the
519 different killifish species groups with the large eggs of piscivorous species containing relatively more
520 nitrogen and sulphur when compared to the small *Austrolebias* species. In the eggs of African *N.*
521 *furzeri*, we found relatively more nitrogen to carbon, again paralleling the large *Austrolebias* species.

522

523 **Egg triglycerides probably serve as an energetic reservoir for the final pre-hatching phase in**
524 **annual killifish.** It has been hypothesised that lipids from egg oil globules act as energy reservoir during
525 protracted pre-hatching phase (Brind et al., 1982). In other teleost species, it is usually spent shortly
526 after hatching (Kamler, 2008; Kohno et al., 1986; Yúfera et al., 1999). The globules contain a

527 considerable fraction (50-60%) of the total fish egg lipids (Wiegand, 1996). In annual killifish, the
528 proportion of total egg lipids stored in these oil globules can be particularly high, with up to 90%, and
529 the majority of these lipids are triglycerides (Brind et al., 1982). We therefore focused our analysis on
530 the initial amount of triglycerides in killifish eggs.

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

540

541 **Yolk sac reserves decrease in embryos faced with a protracted pre-hatching phase.** Another
542 energy source for fish that is mainly used during embryonic development are egg yolk lipoproteins
543 (primarily lipovitellin) (Brooks et al., 1997; Wiegand, 1996). Freshly hatched annual killifish still possess
544 a visible yolk sac, but an extended time of incubation (number of years) may lead to less viable
545 hatchlings with almost spent yolk (MV, personal observation). In the two species we incubated and
546 hatched (*A. bellottii* and *A. nigripinnis*), we monitored development regularly and, instead of hatching
547 individual embryos at the moment when they reached the pre-hatching stage, we triggered their
548 hatching at pre-specified date. We therefore ended up with hatchlings that had endured variable time
549 in the pre-hatching stage. The amount of remaining yolk at hatching consequently declined with time
550 spent in the pre-hatching stage. It seems that even during such a limited period (maximum 92 days),
551 embryos had consumed considerable proportion of their yolk reserves. We did not inspect diapause

552 III occurrence, so embryos may have been waiting in an active stage for a hatching cue (desiccation
553 and inundation). This scenario may well happen in natural conditions during the last part of the dry
554 season when humidity already increases. A larger yolk sac size would then increase persistence in the
555 pre-hatching stage, in addition to reserves in the lipid droplet. This is similar to an increased starvation
556 resistance during an initial post-hatching phase in other fish (Jardine and Litvak, 2003), amphibians or
557 lizards (Moore et al., 2015; Sinervo, 1990).

558

559 **Conclusion.** Egg parameter allometric scaling exponents and relationships between different
560 egg parameters are not conserved across annual killifish species. Scaling exponents also vary between
561 repeated samples on the same species but collected at different times in different conditions. Our
562 results therefore call for caution and more analyses of intra- and interspecific variation in egg content
563 allometries.

564

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

566 **A. Allometry with size**

567 An allometric scaling describes the relationship of a trait y with body size x (or with a proxy for
568 body size). Such relationships are usually characterized by a power law (Eqns. 1; Huxley and Teissier,
569 1936)

570
$$y = ax^b, \tag{Eqn. 1a}$$

571

572 which becomes after log-log transformation

573
$$\ln y = \ln a + b \ln x \tag{Eqn. 1b}$$

574

575 When parameter b (the scaling exponent) equals one then y is said to be isometric with body
576 size x . These two traits then scale linearly by a factor a (the factor of proportionality). When $b > 1$ we
577 speak of positive allometry or hyperallometry; $b < 1$ is called negative allometry or hypoallometry
578 (Shingleton, 2010). A log-log regression can be used to estimate b and $\ln a$. In its simplest least-squares
579 or ML (maximum likelihood) formulation, the regression assumes that the residual variance around
580 the regression line is independent of $\ln x$ (homoscedasticity).

581

582 **B. Different measures of body size**

583 In regression equations used to estimate scaling exponents and factors of proportionality, size
584 can be represented by a variable that scales with length of the individual in any life stage, the area of
585 a section, or with its volume. In the context of this study, we assume that eggs are well approximated
586 by considering them to be spheres with radius r . The area of an egg on a digital photograph then is
587 $A = \pi r^2$, 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 $\ln A = \frac{2}{3} \ln V -$

588 $\frac{2}{3} \ln \frac{4}{3\sqrt{\pi}}$. This last equation implies that the slope estimate in a log-log regression on area is two thirds
589 the scaling exponent of an allometry with volume as a measure of size. The estimate of the factor of
590 proportionality is also affected by this transformation and can be adjusted by adding 0.189789 to the
591 intercept of a log-log regression on area.

592

593 **C. Allometry between traits**

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

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

601

$$602 \quad y_1 = a_1 x^{b_1} \text{ and } y_2 = a_2 x^{b_2} \quad (\text{Eqn. 2a})$$

$$603 \quad \ln y_1 = \ln a_1 + b_1 \ln x \text{ and } \ln y_2 = \ln a_2 + b_2 \ln x \quad (\text{Eqn. 2b})$$

604

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

608

$$609 \quad y_2 = c y_1^d \quad (\text{Eqn. 3a})$$

610

611 or after log-log transformation

$$612 \quad \ln y_2 = \ln c + d \ln y_1 \quad (\text{Eqn. 3b})$$

613

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

$$615 \quad d = \frac{b_2}{b_1} \text{ and } c = \frac{a_2}{a_1 \frac{b_2}{b_1}} \quad (\text{Eqn. 4})$$

616

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

626

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

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

634 the other covariates modelled. This avoids that intercept terms suddenly get a different interpretation
635 when terms including body size are removed from the model, and ensures that intercept estimates in
636 all models compared represent average log-total amounts. These often need to be compared, while
637 estimating factors of proportionality can be less insightful when body sizes differ much between
638 groups. We can rewrite models for a trait y to accommodate this idea as

639

$$640 \quad \ln y = \ln a + b \ln x = \ln a + b \overline{\ln x} + b(\ln x - \overline{\ln x}) \quad (\text{Eqn. 5a})$$

641

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

643 and

644

$$645 \quad \ln y' = \ln a' + b' \overline{\ln x'} + b'(\ln x' - \overline{\ln x'}) \quad (\text{Eqn. 5b})$$

646

647 for observations y' and x' in the other group, for example. Log-log regressions with separate
648 estimated intercept and slope parameters for each of these groups will estimate the allometric scaling
649 exponents as the slopes on the group-centered log-body sizes. The factors of proportionality $\ln a$ and
650 $\ln a'$ are not separately estimated because intercept estimates will include terms which are
651 contributions of the average log-body size in the group scaled by the allometric scaling exponent.
652 Intercept estimates will also be affected by conversions as explained in section B.

653

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

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

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

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

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

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

684

685 **F. Measurement error**

686 Warton et al. (2006) discuss at length the consequences of measurement and equation error in
687 the analysis of bivariate allometries. They state that, in many instances, the two traits involved are
688 equally important and that therefore major axis (MA) or reduced major axis (RMA) are obvious least-
689 squares model-fitting choices. In a likelihood approach, one would then resort to likelihoods based on
690 multivariate distributions for each observation and the slope b becomes part of the specification of
691 the covariance between traits (Warton et al., 2006). Covariance regression models can then be used
692 to test for differences in slope b between groups. Voje et al. (2014) on the other hand, do opt for a
693 regression approach where body size is seen as an explanatory variable. In the models we fitted to
694 several traits, such a regression approach was implemented and no covariance regressions were fitted
695 for simplicity. When the main goal of inference is predicting a trait on the basis of body size, standard
696 regression models should be used (Warton et al., 2006). These are also most adequate in the absence
697 of measurement error in body size or they suffice when the goal of inference is to test whether body
698 size significantly affects another trait, i.e. whether b is non-zero or different from one in the case of
699 concentrations in models with an offset. In the presence of measurement error estimates of
700 regression slopes are affected, therefore when we need reliable estimates of b , measurement error
701 needs to be considered and taken into account. However, across a number studies inspected by
702 Warton et al. (2006) the effects seemed limited to a decrease in the estimated value of the slope b
703 which is below 8%. Next to other methods such as the method-of-moments regression (Warton et al.,
704 2006), Carroll et al. (2006) propose regression calibration and simex extrapolation as generally
705 applicable methods to derive regression slopes corrected for measurement error in body size which

706 still permit standard regression or mixed models can be fitted and the associated inference methods
707 used.

708

709 **G. Explaining variation in traits dependent on body size**

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

720

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734

735 **Conflict of interests**

736 We declare no conflict of interests.

737

738 **Supplementary Information**

739 Table S1. Age and total length of the spawning pairs in the 2017 experiment.

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

741 Data and R script accompanying this article will be available after acceptance under DOI
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743

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901

902 **Tables**

903 Table 1. **Overview of species mean values for the measured egg parameters.** The “Females” column reports number of individual spawning females whose
 904 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
 905 numbers shown in brackets give sample sizes (number of eggs analysed) for individual parameters. Standard deviation (SD) is provided for egg area along
 906 with the mean.

Species	Females	Egg d ₁ [mm]	Egg area [mm ²]	Water content [%]	N C S content [%]	Triglyceride concentration [mg/ml]	Incubated/ Hatched	Hatchling SL [mm]
<i>A. bellottii</i> (AB) (small species)	4	1.347±0.089 (928)	1.336±0.188 (928)	68.5±1.8 (99)	8.5±0.4 54.4±1.0 0.4±0.1 (39)	0.680±0.520 (10)	829/39	4.779±0.240 (39)
<i>A. nigripinnis</i> (AN) (small species)	4	1.299±0.076 (478)	1.214±0.148 (478)	68.5±1.6 (63)	8.6±0.4 54.9±0.9 0.4±0.1 (28)	0.465±0.260 (3)	415/51	4.113±0.147 (51)
<i>A. elongatus</i> (AE) (piscivore)	2 (5)	2.271±0.161 (116)	3.781±0.515 (116)	74.8±1.0 (14)	9.1±0.2 51.9±0.7 0.5±0.1 (12)	1.683±1.066 (6)	102/2	8.994±0.830 (2)
<i>A. prognathus</i> (AP) (piscivore)	1 (2)	2.468±0.165 (49)	4.420±0.589 (49)	73.4±1.7 (11)	8.8±0.1 52.4±0.7 0.5±0.0 (11)	1.293±1.600 (4)	38/0	-
<i>N. furzeri</i> (NF) (African small species)	7	1.254±0.059 (108)	1.154±0.105 (108)	73.5±2.3 (108)	8.9±0.4 54.3±1.4 0.4±0.1 (40)	0.387±0.441 (10)	-	-
<i>A. bellottii</i> (AB 2011) 6+ years in incubation	-	1.490±0.071 (7)	1.689±0.176 (7)	-	-	0.994±0.476 (7)	-	-
Additional datasets								
<i>A. bellottii</i> 2015 (AB 2015)	3	-	1.357 ± 0.370 (61)	72.4±7.1 (61)	-	-	-	-
<i>A. nigripinnis</i> 2015 (AN 2015)	1	-	1.594 ± 0.118 (28)	-	-	-	-/28	4.026±0.153 (28)
<i>A. elongatus</i> 2015 (AE 2015)	1	-	3.813 ± 0.524 (23)	-	-	-	-/23	7.018±0.450 (23)

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908

909 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
 910 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
 911 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
 912 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
 913 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
 914 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,
 915 with the same scaling exponent as egg dry weight. Parameter estimates are given with their standard errors (SE). Estimates significantly different from zero
 916 ($p < 0.05$) 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>	-0.116 (0.048)	0.012 (0.032)
Difference of <i>A. elongatus</i>	1.332 (0.063)	0.343 (0.043)
Difference of <i>A. prognathus</i>	1.579 (0.077)	0.222 (0.051)
Difference of <i>N. furzeri</i>	-0.678 (0.040)	0.277 (0.028)
log(egg area) (effect in <i>A. bellottii</i>)	0.628 (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>	0.823 (0.180)	0.038 (0.216)
Difference of log(egg area) in <i>A. prognathus</i>	-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)

917

918 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-
 919 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
 920 to species differences between log-total nitrogen and log-total carbon, and log-total sulphur and log-total carbon, respectively. Relative elemental
 921 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*
 922 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)
 923 (Fig. 3B). Allometric scaling of elements content with egg area differed among species, but was consistent for the different elements (except for sulphur
 924 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>	-0.110 (0.013)	0.006 (0.022)	-0.069 (0.056)
Difference of <i>A. elongatus</i> from <i>A. bellottii</i>	1.423 (0.018)	0.114 (0.027)	0.368 (0.061)
Difference of <i>A. prognathus</i> from <i>A. bellottii</i>	1.619 (0.013)	0.075 (0.020)	0.322 (0.043)
Difference of <i>N. furzeri</i> from <i>A. bellottii</i>	-0.617 (0.013)	0.053 (0.023)	-0.014 (0.060)
log(egg area) (effect in <i>A. bellottii</i>)	1.578 (0.081)	-0.016 (0.128)	0.613 (0.353)
Difference of log(egg area) in <i>A. nigripinnis</i>	-0.796 (0.204)	0.322 (0.336)	-2.680 (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>	-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)

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926

927 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
 928 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
 929 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
 930 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
 931 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>	-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)

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933

934 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
 935 (R^2_{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
 936 on hatchling size of *N. furzeri* (Vrtílek et al., 2017).

Species	Wet weight		Dry weight		Hatchling size	
	R^2_{marginal}	$R^2_{\text{conditional}}$	R^2_{marginal}	$R^2_{\text{conditional}}$	R^2_{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	-	-
Additional datasets						
<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

937

938 **Figure captions**

939 **Fig. 1. Species with larger body size lay larger eggs.** The plot shows the relationship between female
940 body size and egg size, measured as egg area, in the studied species of annual killifish. Full points
941 represent values collected for this study and empty points are for data from the two additional
942 datasets. Note that for data obtained from *Austrolebias bellottii* in 2015 size of the three females
943 was unknown. Species abbreviations: AB – *Austrolebias bellottii*, AN – *A. nigripinnis*, AE – *A.*
944 *elongatus*, AP – *A. prognathus*, NF – *Nothobranchius furzeri*.

945

946 **Fig. 2. Species-specific allometries between egg size and egg dry mass, and egg size and water**
947 **content.** Plot (A) shows species-specific slopes of the relationship between log-dry weight and the
948 species zero-centered log-egg area (full points, solid lines), and log-water weight and species zero-
949 centered log-egg area (empty points, dashed lines) (Table 2). Note that the lines for egg dry weight
950 and water weight are parallel in most of the species except *A. prognathus* (AP). The parallel lines
951 mean that water content scaled similarly to dry mass with egg size. Plot (B) then shows relative
952 amount of water and dry mass in the eggs of the different species. The distance between the log-dry
953 and log-water weight here corresponds to log-ratio of these two egg parameters (means are
954 denoted by horizontal lines and vertical lines give standard errors estimated from the model, Table
955 2). For their mean egg size (indicated as vertical dashed line at 0 in Figure 2A), the piscivores (*A.*
956 *elongatus* and *A. prognathus*) and *N. furzeri* have relatively higher proportion of water weight to dry
957 mass (wider distance between mean log-dry weight and mean log-water weight). Species
958 abbreviations: AB – *Austrolebias bellottii*, AN – *A. nigripinnis*, AE – *A. elongatus*, AP – *A. prognathus*,
959 NF – *Nothobranchius furzeri*.

960

961 **Fig. 3. Egg elemental composition differed among species.** The clouds of different coloured points
962 represent positions of the species in the three-dimensional space spanned by the three measured
963 elements in killifish eggs and the residual content (A – carbon, nitrogen and residual; B – carbon,
964 sulphur and residual; C – nitrogen, sulphur and residual). The position of each point (trivariate
965 coordinate for each egg) reflects the inter-dependence of the elemental content proportions as they
966 together must add up to 100 %. The original percentage values were transformed using isometric
967 log-ratio transformation, and then scaled and centered for visualization. Species abbreviations: AB –
968 *Austrolebias bellottii*, AN – *A. nigripinnis*, AE – *A. elongatus*, AP – *A. prognathus*, NF –
969 *Nothobranchius furzeri*.

970

971 Fig. 4. **Relative triglyceride content was higher in species with larger eggs.** Note that the extremely
972 long incubation (6+ years) did not reduce the level of triglycerides in the eggs of *Austrolebias bellottii*
973 from 2011 compared to the freshly laid eggs of the same species. Horizontal lines are for species
974 means and vertical lines for standard errors from the model. The lowercase letters on top of each
975 species data indicate differences in the relative amount of triglycerides (based on a model that
976 included species zero-centered log-egg volume as an offset). Each point represents log-transformed
977 amount of triglyceride amount in an egg. Species abbreviations: AB – *Austrolebias bellottii*, AN – *A.*
978 *nigripinnis*, AE – *A. elongatus*, AP – *A. prognathus*, NF – *Nothobranchius furzeri*.

979

980 Fig. 5. **Hatchling size corresponds with egg size only in the large species.** Hatchlings coming from
981 larger eggs were bigger only in *A. elongatus* (piscivore) from 2015, but in the small *Austrolebias* (*A.*
982 *bellottii* and *A. nigripinnis*) egg area had no effect on hatchling size. Species abbreviations: AB –
983 *Austrolebias bellottii*, AN – *A. nigripinnis*, AE – *A. elongatus*.

Fig 1

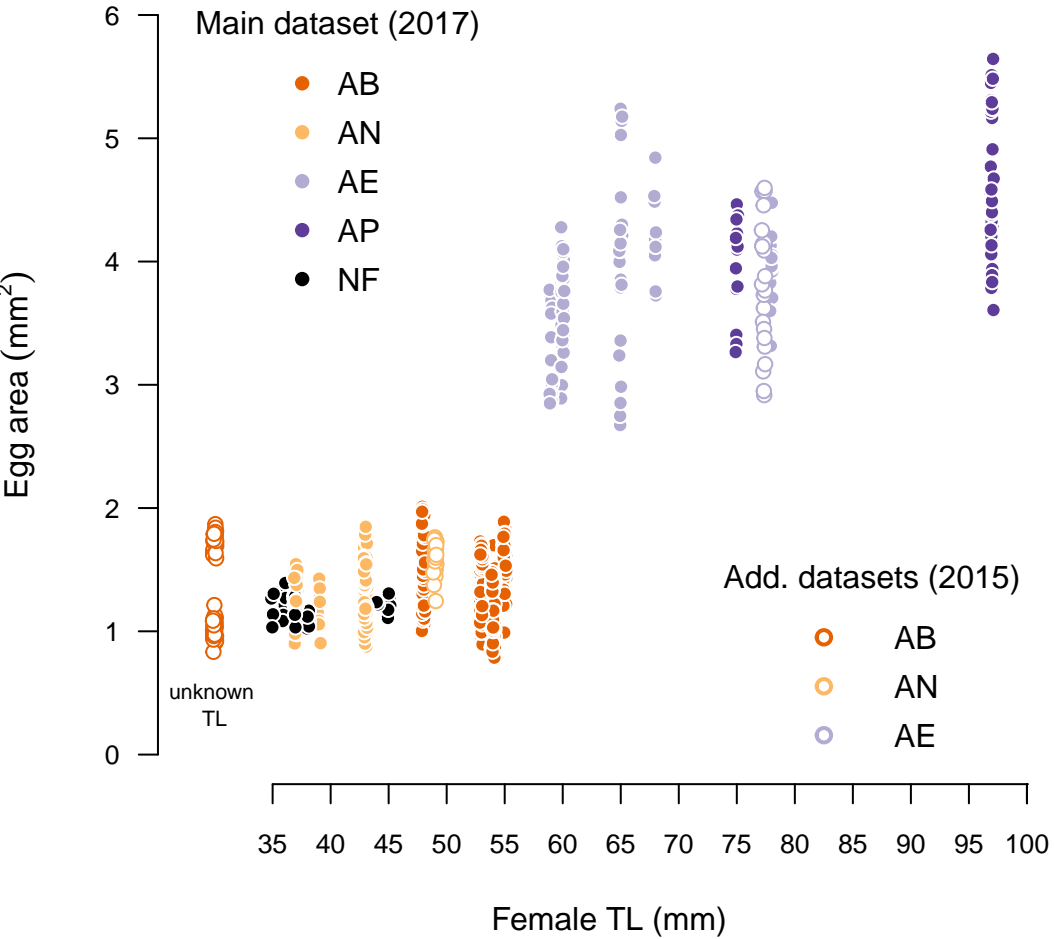


Fig 2A

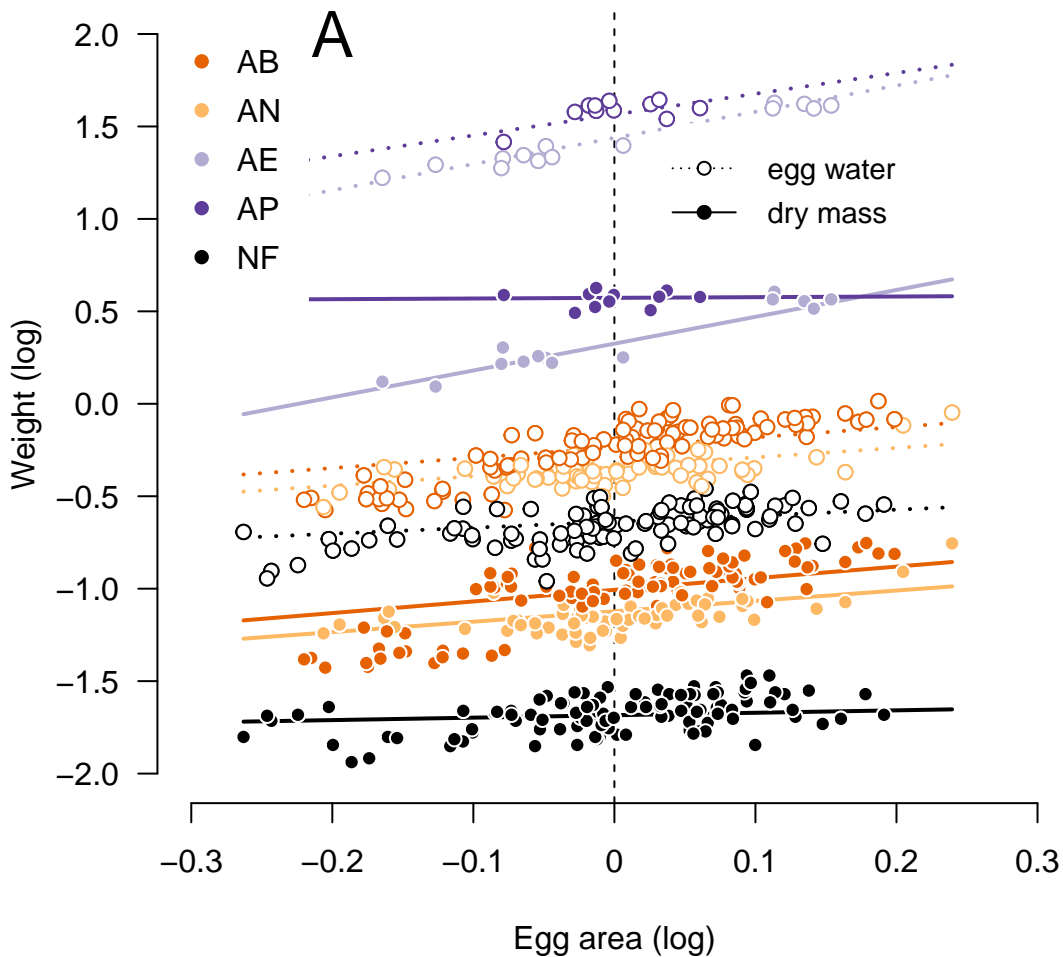


Fig 2B

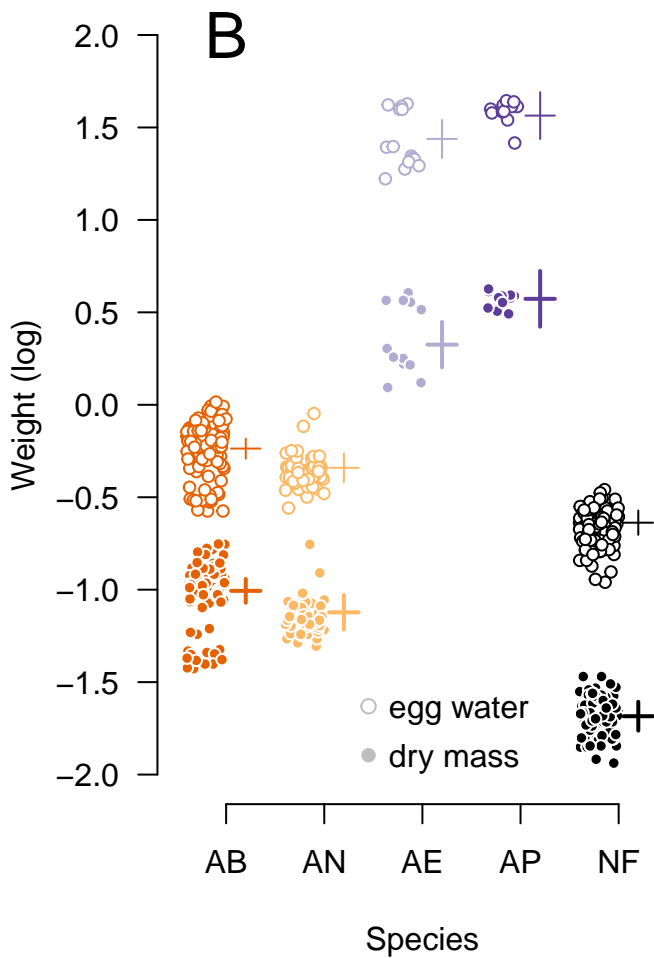


Fig 3A

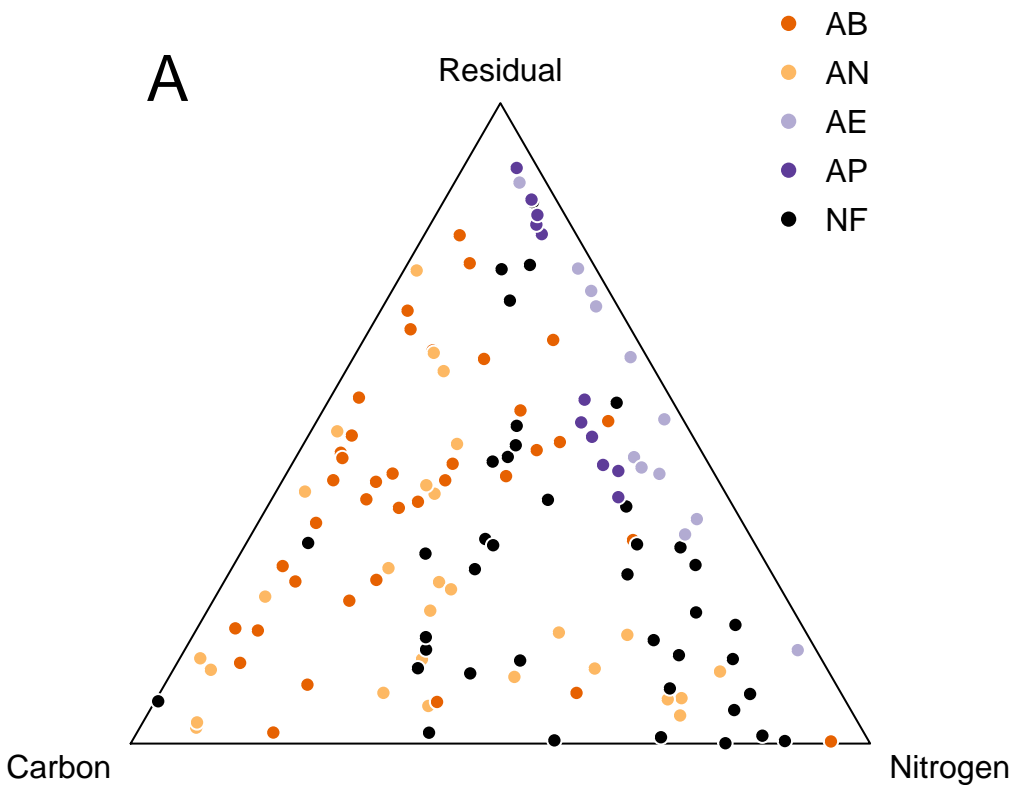


Fig 3B

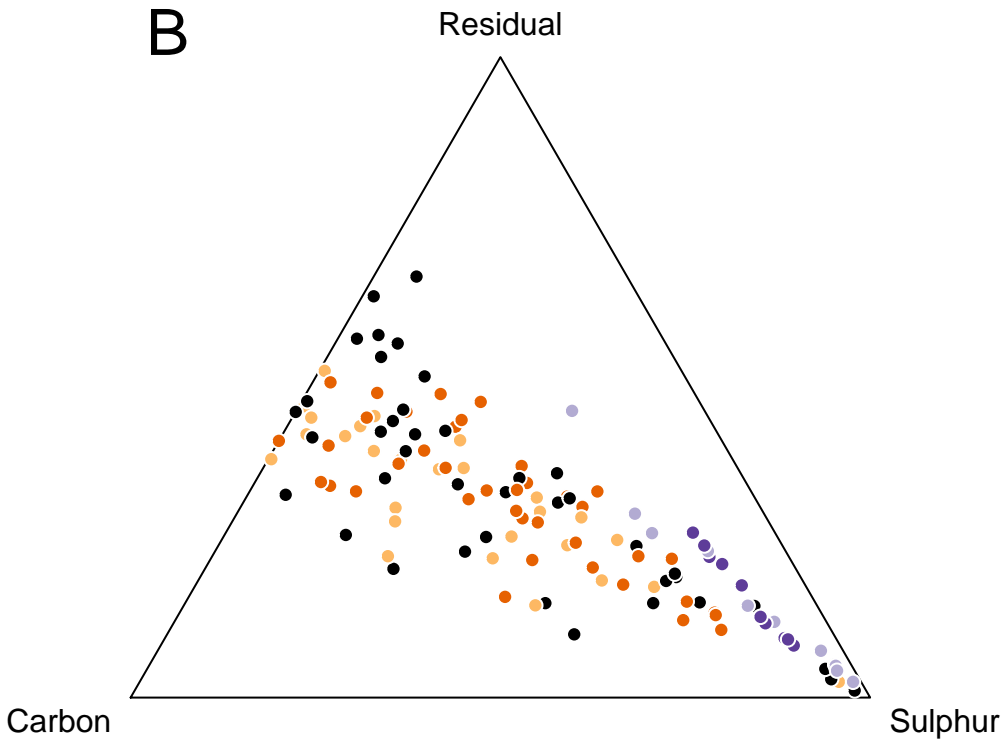


Fig 3C

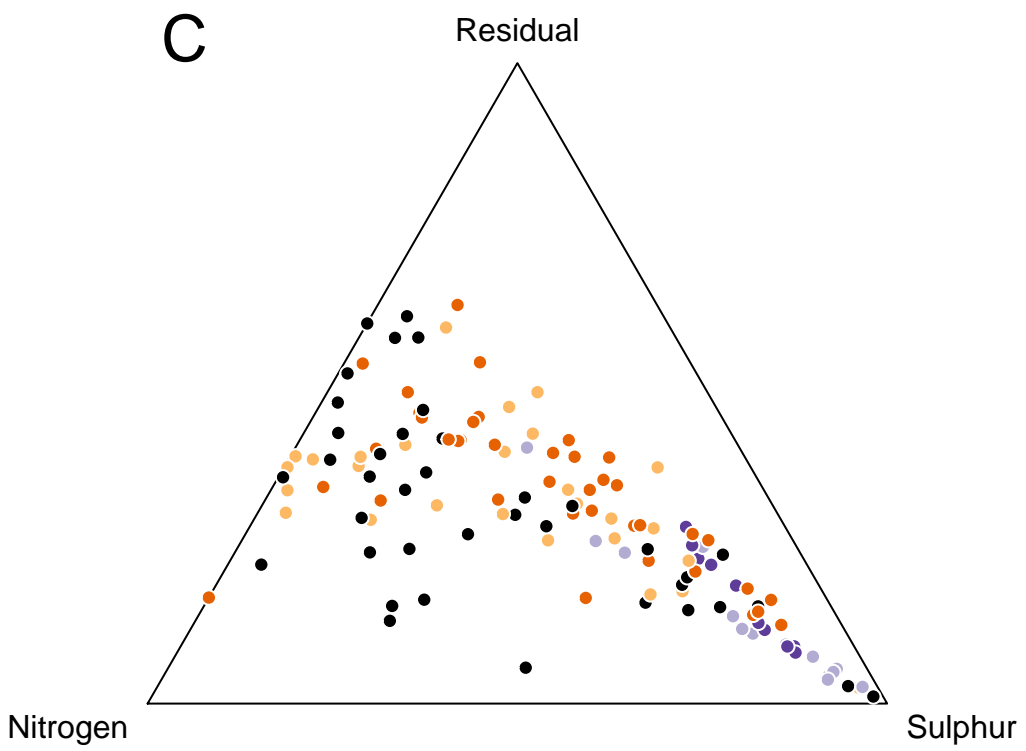


Fig 4

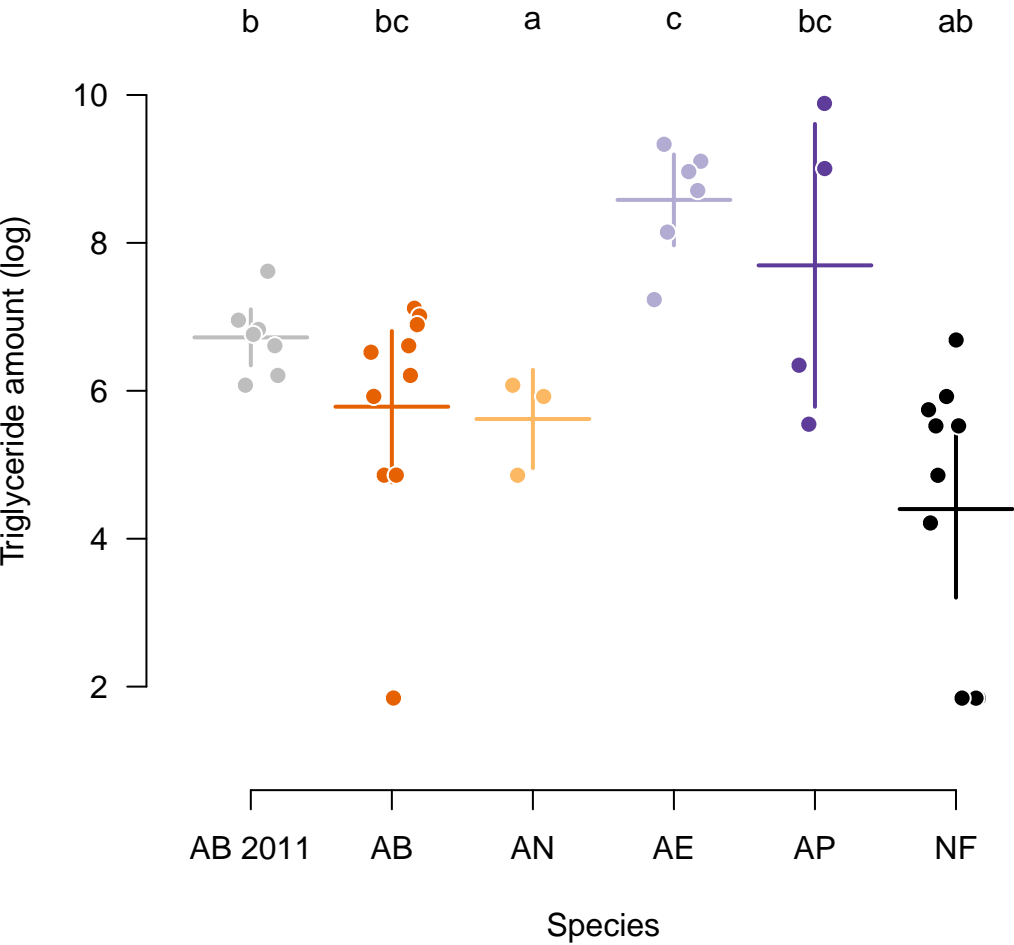
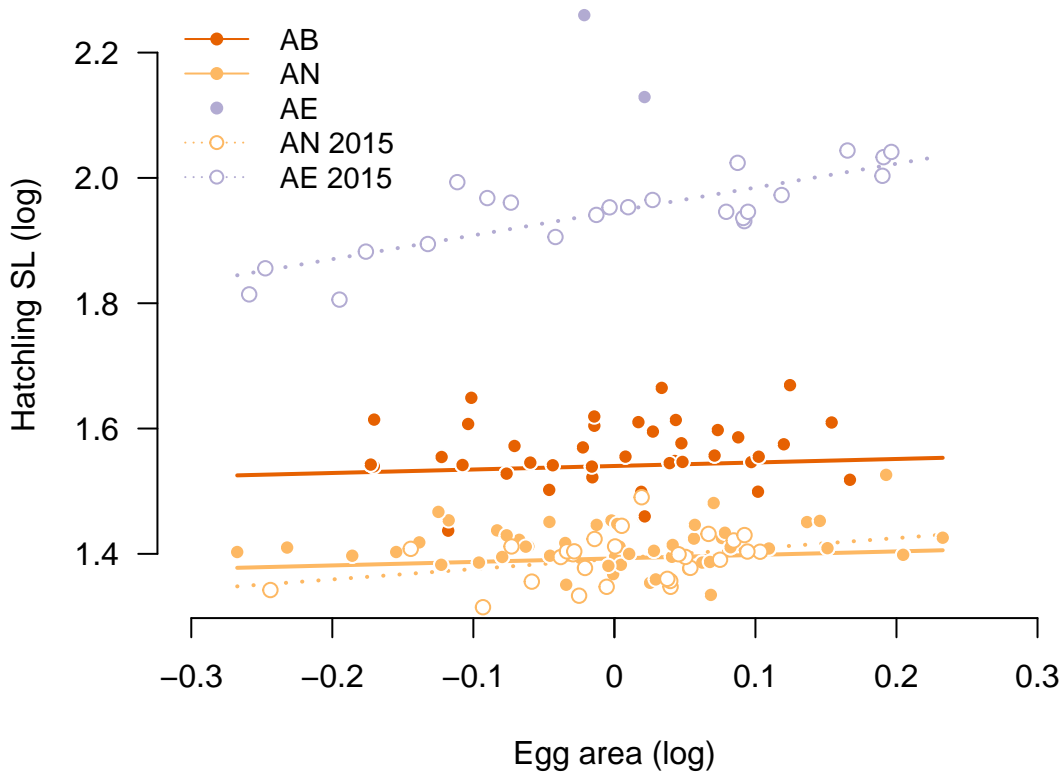


Fig 5



Conflict of interests

We declare no conflict of interests.



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