Ontogenetic variation in the body stoichiometry of two fish species

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1   **Abstract**

2   One of the central questions of ecological stoichiometry theory is to what extent animal

3   species maintain constant elemental composition in their bodies. Although several recent

4   studies demonstrate intraspecific variation in animal elemental composition, relatively little is

5   known about ontogenetic changes in vertebrates, especially during early life stages. We

6   studied the intraspecific and interspecific ontogenetic variation in the body stoichiometry of

7   two fish species in two different orders; fathead minnow (*Pimephales promelas*) and

8   sheepshead minnow (*Cyprinodon variegatus*), reared under controlled laboratory conditions.

9   During ontogeny, we measured the chemical composition of fish bodies, including carbon

10   (C), nitrogen (N), phosphorus (P), calcium (Ca), and ribonucleic-acid (RNA) contents. We

11   found that N and RNA contents were relatively high in early life stages and declined

12   substantially during development. In contrast, body C and C:N ratios were relatively low in

13   embryos, post-embryos and larvae, and increased remarkably thereafter. Concentrations and

14   ratios of some elements (e.g., Ca, P, Ca:P) did not exhibit consistent ontogenetic trends, but

15   fluctuated dynamically between consecutive developmental stages in both species. Specific

16   growth rates correlated significantly with RNA contents in both species. Analyses of the

17   relative importance of different P pools at each developmental stage revealed that RNA was a

18   considerable P pool in post-embryos, while bone-associated P was the dominant body P pool

19   in later stages. Our results suggest that the elemental composition of fish bodies changes

20   considerably during ontogeny. Each ontogenetic stage has its own stoichiometric signature,

21   but the timing, magnitude and direction of ontogenetic changes can vary substantially

22   between taxa.

23

24   Keywords: nutrients, ecological stoichiometry, elemental homeostasis, organismal

25   development, phosphorus pools

Introduction

Ecological stoichiometry (ES) theory provides a framework for predicting how different species vary in storing and recycling nutrients (Sterner and Elser 2002). ES expresses biological interactions in terms of the balance of energy (carbon; C) and nutrients such as nitrogen (N) and phosphorus (P) (Sterner and Elser 2002; El-Sabaawi et al. 2012a). One of the early tenets of ES theory was that heterotrophic organisms maintain relatively constant elemental composition in their bodies, in the face of variable food nutrient contents or ingestion rates. Moreover, the theory also assumes that to a large extent the nutrient stoichiometry of animals is a genetically determined trait, arising from evolutionary pressures on form and function (Sterner and Elser 2002). The assumption of species-specific and tightly constrained elemental homeostasis generates the conclusion that body nutrient concentrations within a particular species are relatively constant across populations and life stages. However, several recent analyses challenge the notion that animals are as homeostatic in their elemental composition as previously hypothesized (e.g., Pilati and Vanni 2007; Hood and Sterner 2010; Vrede et al. 2011; Boros et al. 2012; El-Sabaawi et al. 2012 a,b; Back and King 2013; Benstead et al. 2014). These studies mandate that we reconsider and refine widespread notions about taxon-specific constancy in elemental composition. As Hendrixson et al. (2007) state, "strict homeostasis is a simplifying assumption about a complex reality, where nutrient content varies with many factors". Nakazawa (2011) argued that assuming a constant body elemental composition is only an approximation and a simplification that has been used for model development and that ecological stoichiometry theory is still incomplete in this sense.

Fish have been frequently studied in the context of ecological stoichiometry, as their biomasses often constitute important nutrient pools in aquatic ecosystems (Kitchell et al. 1975; Sereda et al. 2008; Vanni et al. 2013), and they can support a substantial proportion of the demands of primary producers via nutrient recycling (Vanni 2002; McIntyre et al. 2008).

51 Thus, alterations in fish biomass and community assemblage influence the availability of

52 nutrients to primary producers (McIntyre et al. 2008; Boros et al. 2009). Occupying relatively

53 high trophic positions and being rich in nutrients, fish represent a locus where N and P are

54 concentrated (e.g. Sterner and Elser 2002; Tarvainen et al. 2002; Vanni et al. 2013), which is

55 important because these nutrients play a key role in limiting primary production (Lewis and

56 Wurtsbaugh 2008). Because of their rapid growth and high mortality rates, young-of-the-year

57 fish can be especially important in these processes (Kraft 1992; Lorenzen 2000). Hence,

58 elemental stoichiometry of fish, including both sequestration and release of nutrients, has

59 been of great interest during the recent decades (Kitchell et al. 1975; Parmenter and Lamarra

60 1991; Vanni 2002; Vrede et al. 2011; Vanni et al. 2013).

61   Several studies demonstrate that the body stoichiometry of fish may vary with ecological

62 and environmental conditions such as habitat, resources, food quality, trophic state, predation

63 pressure and stress (Boros et al. 2012; El-Sabaawi et al. 2012 a,b; Benstead et al. 2014;

64 Dalton and Flecker 2014; Sullam et al. 2015). Thus, significant intraspecific differences in

65 elemental composition may exist among individuals of different populations. However,

66 ontogeny also can play a role in explaining intraspecific differences in the elemental

67 composition of fish. Recent studies report that intraspecific variability in body stoichiometry

68 is greater than previously thought, and that body size, ontogeny and/or morphology can

69 explain a significant part of the variation (Pilati and Vanni 2007; Vrede et al. 2011).

70   The mass and relative proportion of different tissues and biochemicals can change

71 dynamically during organismal development, and this can lead to changes in whole-body

72 elemental composition, because different tissue types and biochemicals contain elements in

73 different quantities. Bones and scales are rich in calcium (Ca) and P, while nucleic acids also

74 contain significant amounts of P (Rønsholdt 1995; Vrede et al. 2004; Hendrixson et al. 2007).

75 Muscle tissue stores considerable amounts of N as protein (Pangle and Sutton 2005; Vrede et

76   al. 2011), while energy-rich lipids are the most important C storage pools in animal bodies

77   (Sterner and Elser 2002; Fagan et al. 2011). During ontogeny, fish may exhibit different

78   strategies and distinct periods of energy accumulation and somatic growth (Post and

79   Parkinson 2001; Biro et al. 2005; Nakazawa 2011), and body stoichiometry of individuals

80   reflects such alterations between life stages. For instance, Deegan (1986) showed that the

81   body composition of young-of-the-year gulf menhaden (*Brevoortia patronus*) changed

82   considerably during ontogeny owing to a shift in energy allocation away from protein growth

83   to lipid storage.

84   The growth rate hypothesis (Elser et al. 1996, 2003) states that fast-growing animals

85   (which often include those in early life stages) need high quantities of ribonucleic acid (RNA)

86   to achieve and maintain their high specific growth rates, and RNA content of tissues

87   constitutes the most important P-pool of body in early phases of ontogeny (Elser et al. 1996;

88   Vrede et al. 2004). Subsequently, RNA content of tissues declines with decreasing growth

89   rates as ontogeny proceeds (Gillooly et al. 2005); in vertebrates this is accompanied by a

90   gradual ossification (P and Ca allocation) of skeleton (Hendrixson et al. 2007; Pilati and

91   Vanni 2007). For example, Sterner and Elser (2002) and Vrede et al. (2011) pointed out that

92   rapidly growing animals commonly have low C:P and N:P ratios because of the increased P

93   allocation to RNA. However, decreasing C:P and N:P ratios with growth were also reported

94   for later stages of ontogeny in vertebrates, owing to the increasing P allocation to developing

95   skeleton (Pilati and Vanni 2007). This suggests a realignment of P pools in body during

96   ontogeny. Yet, the timing and magnitude of these changes, and how they vary among species,

97   are still largely unknown; for fish we know very little about changes during early life stages.

98   In this study, we explored ontogenetic changes in the body stoichiometry of two fish

99   species in two different orders. We raised fish from embryos to adults under controlled

100   environmental conditions, and assessed their chemical composition (C, N, P, Ca and RNA) at

101 several developmental stages. The justification of our experiment is two-fold. First, preceding

102 studies on ontogenetic stoichiometric shifts have been conducted only on a limited number of

103 species and ontogenetic stages; to our knowledge, no previous studies include data on early

104 developmental stages (i.e., embryos and post-embryos) as well as adults of fish. Secondly,

105 intra- and interspecific variation in ontogenetic stoichiometry has not yet been studied in

106 experiments in which feeding and environmental conditions are controlled. Because of these

107 gaps, the factors that contribute to variability in organismal stoichiometry are still poorly

108 understood and warrant more detailed examinations. We had the following objectives:

109 (1) To characterize the ontogenetic changes in the body composition of two fish species that

110 belong to different taxonomic orders and that are adapted to different environments.

111 (2) To explore whether the two fish species show the same strategies in allocating nutrients

112 to energy storage and somatic growth during ontogeny, or if the two species exhibit

113 divergent patterns even when environmental conditions are similar.

114 (3) In the light of the growth rate hypothesis, to identify the life stages when RNA is the

115 dominant P pool in fish, and when during development the P stored in RNA becomes

116 negligible compared to the pool in the developing skeletal system.

117

118 **Materials and methods**

119 **Study species**

120 We studied two fish species from the class of ray-finned fishes (Actinopterygii): fathead

121 minnow (*Pimephales promelas*) and sheepshead minnow (*Cyprinodon variegatus*). Fathead

122 minnow (hereafter FM), in the order Cypriniformes, is a widespread fish species across North

123 America, inhabiting all types of freshwater ecosystems. Sheepshead minnow (SM) belongs to

124 the order Cyprinodontiformes and lives in brackish/saltwater environments from the Mid-

125 Atlantic United States to South America. We chose these species because both are

126 omnivorous, are similar in size, have rapid growth to maturity under ideal temperature

127 (22−24 °C) and food supply, and are easily raised under laboratory conditions. Thus, it was

128 possible to conduct an experiment using the same food source and environmental conditions

129 for both species and to raise fish to adulthood in a reasonable time frame. However, they

130 belong to different taxonomic orders and live in different environments (freshwater *vs.*

131 saltwater), allowing us to compare two species with different evolutionary histories.

132

133 **Experimental design**

134 Fish were hatched and raised in the aquatic laboratories of the Animal Care Facility of

135 Miami University (Oxford, OH, USA). Light intensity and temperature were controlled in the

136 same manner in freshwater and saltwater rooms, with 12:12 day/night photoperiods and

137 constant 23 °C water temperature. We obtained fish embryos from breeding individuals

138 maintained in the facility and held them in aerated beakers, and then placed in 40 L aquariums

139 after hatching. Both FM and SM cultures were allocated into 3 different replicate groups held

140 in separate aquariums, to maintain a fish density that did not reduce growth. Animal handling

141 and experimental procedures were approved by Miami University's Institutional Animal Care

142 and Use Committee (Protocol No. 860).

143 The experiment lasted for ~4 months, April–August 2012. For comparison of body

144 composition, we divided the ontogeny of fish to the following categories: embryo, post-

145 embryo, larva, juvenile and adult. In the "dynamic energy budget" theory, Kooijman (2000)

146 divided the ontogeny of multicellular animals to three basic life stages: embryo (individuals

147 that do not feed or reproduce), juvenile (individuals that feed but do not reproduce), and adult.

148 We elaborated on this classification by including two additional life stages (post-embryonic

149 and larval) to characterize ontogeny at a finer scale. Designation of ontogenetic stages was

150 based on *a posteriori* growing characteristics, i.e., based on distinct size classes (Table 1), and

151   on an individual's ability to consume bigger food particles (i.e., a diet shift that corresponded

152   to the beginning of juvenile stage). In addition, fish > 30 mm were considered to be young

153   adults (Van Aerle et al. 2004). Post-embryonic and larval fish were fed two times per day

154   with brine shrimp larvae (*Artemia* sp.), while juveniles and adults consumed TetraMin flake

155   food designed for aquarium fishes, also two times per day. During sampling days, fish were

156   not fed in the morning to avoid the possible effects of consumed food on body chemistry

157   analyses.

158

159   **Sampling and sample analyses**

160   We sampled fish randomly from aquariums using a hand-net. Embryos were sampled one

161   day before hatching and post-embryos 1−3 days after hatching. All subsequent samplings

162   were performed at 10–12 -day intervals thereafter. Samples of embryos and post-embryos

163   were pooled (15−20 individuals per sample) because individuals in these developmental

164   stages were too small to produce enough material for all analyses. For larvae, juveniles and

165   adults, samples consisted of single individuals. For all samples, subsamples from whole-body

166   homogenates were taken for the various analyses. Carbon, N, P and Ca analyses require dried

167   samples, while RNA content can be measured only from wet tissues; thus, we had to use

168   different fish for elemental analyses and RNA measurements. During samplings, first we

169   randomly selected 3 fish per species for measuring elemental composition (1 fish per

170   aquarium) and then another 3 fish of similar size for RNA analyses.

171   We anesthetized and sacrificed fish using ice-slurry immersion (Blessing et al. 2010). After

172   death, length and body mass were recorded (except for embryos), and then fish for RNA

173   analyses were immediately immersed in liquid nitrogen and stored in a -80 °C freezer until

174   sample processing. Whole fish samples for C, N, P and Ca analyses were dried to a constant

175   weight at 60 °C and ground to a fine powder with a mortar and pestle, and with a Retsch

176 ZM100 centrifugal mill (Retsch GmbH, Germany). Carbon and N contents of samples were

177 measured using a CE Elantech Flash 2000 CHN analyser (CE Elantech, USA), while P

178 contents were analysed following ignition at 550 °C and subsequent HCl digestion to convert

179 all P to soluble reactive P, which was assayed with a Lachat QC 8000 FIA autoanalyser

180 (Lachat Instruments, USA). For Ca content analysis, dried and homogenized subsamples were

181 combusted at 550 °C and the produced ash was dissolved in HCl solution. Subsequently, Ca

182 contents were determined with a Perkin-Elmer Optima 7300 DV Optical Emission

183 Spectrometer (Perkin-Elmer Inc., USA). Prior to RNA analyses, deep-frozen and intact

184 samples were homogenized with a sonicator. RNA contents of homogenates were extracted

185 with a Maxwell LEV simplyRNA Blood Kit and Maxwell 16 Nucleic Acid Extraction System

186 (Promega Corporation, USA). The quantity of the extracted RNA was measured with a

187 NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA) in the Center for

188 Bioinformatics and Functional Genomics at Miami University.

189

190 **Statistical analyses**

191   As the first step in data analyses, we used generalized additive models (GAM) with cubic

192 regression spline smoothers to illustrate the ontogenetic changes of our studied variables for

193 both species (proportions of elements are expressed as percentage of dry mass, proportion of

194 RNA is expressed as percentage of wet mass, and ratios are expressed in molar units). GAMs

195 (Hastie and Tibshirani 1990) are ideal and commonly used for visualizing non-linear

196 statistical relationships (e.g., Guisan et al. 2002; Buisson et al. 2008; Schmera et al. 2012),

197 which frequently occur with ecological variables. We added 95% confidence bands to the

198 GAM plots, in order to indicate the reliability of the predicted values of the models and to

199 provide a visual aid for assessing differences between the two species.

200   Next, to explore differences between the ontogenetic stages and species, we fitted an

201   analysis of variance (ANOVA) model for each response variable, using species and

202   ontogenetic stage as the grouping variable (FM embryo, SM embryo, etc.). After ANOVAs,

203   Tukey's post-hoc tests were applied to compare all developmental stages in a pairwise

204   manner within a species (e.g., FM embryos *vs*. FM post-embryos) and between the two

205   species (e.g., FM embryos *vs*. SM embryos).

206   To assess changes in the associations among Ca, RNA and P during the developmental

207   process, we used analyses of covariance models (ANCOVA) with a nested factorial design

208   (i.e., for RNA *vs*. P and Ca *vs*. P). In the ANCOVA models, the ontogenetic stage grouping

209   (categorical) variable was nested within the species grouping variable. The slope regression

210   coefficient of the ANCOVA models enabled us to evaluate the statistical relationship between

211   the response variable (RNA and Ca) and the continuous explanatory variable (P) of the

212   model. For the ANCOVA models, we used a contrast matrix to make the *a priori* planned

213   pairwise comparisons of the slope regression parameters between the ontogenetic stages

214   separately for the two species.

215   Relationships between body component variables and specific growth rate (SGR; Brown

216   1946) of fish were assessed with Pearson's correlation analysis. Calculation of specific

217   growth rate was based on total body length increments and was calculated as follows:

$$SGR_s(\%) = \frac{(\ln(L_s + 0.01) - \ln(L_{s-1} + 0.01))}{A_s - A_{s-1}} \times 100$$

218   where *s* is a given ontogenetic stage, L_s and L_{s-1} are the average total body lengths (mm) at the

219   *s* and *s–1* ontogenetic stages, A_s and A_{s-1} are the average age at days of the *s* and *s–1*

220   ontogenetic stages. Note that calculation of SGR was not possible for embryos, and that

221   addition of an arbitrary constant (0.01) to the formula was necessary because the body length

222   of embryos was undefinable.

223   Decisions about statistical significance were set at $P = 0.05$ level. All statistical analyses

224   were performed in the R environment (R Core Team 2014). We used the "mgcv" package

225   (Wood 2006) for the GAMs, and the "multcomp" package (Hothorn et al. 2008) for the post-

226   hoc comparisons.

227

228   **Results**

229   The chemical compositions of both fish species changed considerably during ontogeny,

230   often non-linearly. Most parameters showed fluctuating patterns but often with overall

231   increasing or decreasing trends over time (Fig. 1). Nitrogen and RNA contents and N:P ratios

232   of fish bodies were high in early life stages and declined substantially during growth. In

233   contrast, C contents and C:N ratios were relatively low in embryos, post-embryos and larvae,

234   and increased markedly ~60 days after hatching, corresponding to the beginning of the

235   juvenile stage and a diet shift from *Artemia* larvae (molar C/N/P ratio: 94/19/1) to flake food

236   (C/N/P: 87/13/1). In contrast to the aforementioned relatively consistent temporal trends, P,

237   Ca and Ca:P fluctuated dynamically between consecutive developmental stages with no such

238   definite trends during ontogeny (Fig. 1, Fig. 2). In most cases, the means of these fluctuating

239   variables did not differ significantly between earlier and later stages of development (Table

240   2).

241   The two species showed somewhat different strategies in allocating biogenic elements to

242   their bodies, especially in their embryonic phase (Fig. 1, Fig. 2). In particular, for many

243   elemental contents (N, P, Ca), fluctuations between developmental stages were much more

244   pronounced in SM than in FM. However, we also observed many commonalities between

245   species in the long-term trends (over 120 days) (Fig. 1). For example, RNA content peaked

246   3−5 days after hatching and declined markedly thereafter in both species, and RNA contents

247   were very similar between species (Fig. 2). Accordingly, specific growth rates were the

248   highest in post-embryos and decreased with growth both in FM (post-embryo: 210.4; larval:

249   3.3; juvenile: 1.7; adult: 1.1) and SM, respectively (post-embryo: 213.3; larval: 2.3; juvenile:

250   1.7; adult: 1.0). Pearson's correlation analysis revealed significant and positive relationships

251   between SGR−RNA and SGR−N:P in both species. However, we also found significant

252   negative correlations between SGR−P, SGR−Ca and SGR−Ca:P, but only in SM (Table 3).

253   We observed significant differences in many variables between consecutive ontogenetic

254   stages of the same species, as well as between the earlier and later phases of development

255   (e.g., between embryos and adults of the same species) (Table 2). These results show that

256   body composition of both FM and SM changed considerably with growth, but also that the

257   timing, magnitude and direction of these changes were dissimilar in the two species in several

258   cases. For instance, C, C:P and Ca:P ratios differed significantly between the embryonic and

259   post-embryonic stages of FM, but did not differ among these stages for SM. In contrast, N

260   and RNA were significantly different between the embryonic and post-embryonic stages of

261   SM, but were similar to each other in FM. One notable difference between species was that

262   SM embryos had much higher Ca and P than FM embryos (Fig. 2). Thus, for FM, P and Ca

263   differed markedly between earliest stages and adults, while for SM, adults differed only from

264   post-embryos with respect to these parameters. More generally, a lack of significant P and Ca

265   differences between ontogenetic stages was more typical for SM than for FM. Furthermore,

266   C:P and especially Ca:P ratios were also very similar between the different ontogenetic stages

267   of SM, in contrast to FM (Table 2).

268   Comparisons of interspecific differences within the same ontogenetic stage revealed that

269   the body compositions and ratios of elements were the most divergent in embryos, while

270   those of post-embryos and adults were very similar (Fig. 2). FM embryos had ~25% higher C

271   and N contents than SM embryos, and the difference between species was significant for both

272   variables. However, interspecific differences in C and N contents were relatively minimal

273 compared to differences in RNA content, C:P ratio, and N:P ratio, all of which were

274 140−150% higher in FM embryos. On the other hand, SM embryos contained significantly

275 higher amounts of P (almost 2-times that of FM embryos), but the largest difference was for

276 Ca, as SM embryos contained 10-times more of this element per unit body mass.

277 Accordingly, molar Ca:P ratios were ~6-times higher in SM embryos than in FM embryos.

278 Interspecific differences in Ca, P and Ca:P were negligible during the post-embryonic and

279 larval stages, but became significant again for juveniles. Note that total lengths of the two

280 species were very similar throughout the experiment (Fig. 1), which facilitated making

281 interspecific comparisons in the chemical composition during ontogeny.

282   We found mostly positive associations, or no association, between RNA−P and Ca−P

283 within given stages (Fig. 3). RNA and P were significantly correlated ($P < 0.001$) in post-

284 embryos of FM and SM, but there was no significant relationship between these two variables

285 in other developmental stages. Regression coefficients (slope parameters) of FM and SM

286 post-embryos differed significantly ($P < 0.001$) from the same parameter of their larvae,

287 juveniles and adults. For the Ca−P relationship, we found significant regressions only in larval

288 fishes ($P < 0.001$ in both species) and in FM adults. Even though the Ca−P regression

289 coefficients appeared to vary between developmental stages (Fig. 3), the only significant

290 difference occurred between larvae and juveniles ($P < 0.05$ for both species).

291

292 **Discussion**

293   In this study, we followed the ontogenetic changes in the elemental stoichiometry of two,

294 ecologically contrasting fish species, and quantified RNA and Ca to assess the relative

295 importance of different P pools during ontogeny. Based on the homeostasis component of ES

296 theory (Sterner and Elser 2002), our null hypothesis was that elemental composition of an

297 individual is constant throughout its lifespan. We recognize that this is a simplification of ES

298 theory (Hendrixson et al. 2007; Nakazawa 2011). However, this null hypothesis provided a

299 "baseline" against which we could analyse the extent of deviations from it. Our results

300 showed that elemental composition of fish bodies varied significantly among developmental

301 stages, indicating that the fixed, species-specific elemental homeostasis does not apply to fish

302 when rapidly growing individuals and early stages of ontogeny are included. In fact,

303 intraspecific differences during ontogeny were often as great as variation among the two

304 species. The observed ontogenetic plasticity and interspecific differences suggest different

305 constraints and potentially differential elemental limitation among species, especially in the

306 early developmental stages.

307 The most remarkable interspecific difference was found between embryos. The much

308 higher (10-fold) Ca levels of SM, compared to FM, may imply that SM start bone formation

309 in the earliest phase of ontogeny, in contrast to the FM individuals, which may have primitive

310 skeleton in this developmental stage. However, the decline in Ca from the embryonic to post-

311 embryonic stages of SM suggests that this is not likely to be the explanation. Another possible

312 explanation is that SM lives in saltwater, and higher concentrations of minerals (such as Ca)

313 in the body could facilitate maintaining the osmotic balance in the embryos that have

314 undeveloped osmoregulation.

315 The scarcity of studies that include all developmental stages of fish (or other animals)

316 reared under controlled conditions renders it difficult to compare our results explicitly with

317 previously published studies. Nevertheless, we can make some comparisons with other

318 studies. Pilati and Vanni (2007) studied the ontogenetic changes in the body stoichiometry of

319 gizzard shad (*Dorosoma cepedianum*) in a lake and zebrafish (*Danio rerio*) reared under

320 controlled laboratory conditions. Even though Pilati and Vanni (2007) did not include

321 embryos and post-embryos in their study, comparison with our results reveals several

322 common traits across fish species. Specifically, they also found that body C increased, while

323   body N decreased in both gizzard shad and zebrafish, for fish beyond larval stages, similar to

324   both fish species in our study. Increased body C probably indicates increased lipid storage

325   after the larval stage (Fagan et al. 2011). The consistent declines in body N contents do not

326   necessarily imply a loss of muscle mass as ontogeny proceeds, but rather may indicate that C

327   content dominates body elemental composition and any changes in the proportion of C may

328   drive the relative proportions of most other elements, including N. In other words, there may

329   be a "dilution effect" of body C on other elements. Moreover, Pilati and Vanni (2007)

330   reported increasing body P and Ca contents from the larval stage until the beginning of the

331   early juvenile stage, and then relatively constant proportion of these elements in larger fish.

332   We found similar trends in the changes of P and Ca in our study, and presume that variable

333   but generally increasing levels of these elements throughout the observed period of ontogeny

334   indicated that skeletons were developing and ossifying continuously. However, the

335   aforementioned stoichiometric dilution could result in temporal fluctuations in body P and Ca,

336   and a weakening of the relationship between age and elemental concentrations.

337   The observed positive correlation between N:P ratios and growth rates in both FM and SM

338   is consistent with the findings of Davis and Boyd (1978), Tanner et al. (2000) and Pilati and

339   Vanni (2007) for various fish species. Another relevant study by Vrede et al. (2011) yielded

340   slightly different results, as they pointed out that size effect was significant on the whole-body

341   C, P, C:N, C:P and N:P of Eurasian perch (*Perca fluviatilis*), while N contents did not change

342   considerably with growth as it was observed in case of FM and SM. However, Vrede et al.

343   (2011) did not include fish from the earliest phases of ontogeny in their analyses (their

344   smallest fish were > 50 mm), thus the comparison with our results must be done with caution.

345   Nevertheless, several similarities can be demonstrated for ontogenetic trends in C, P and C:N.

346   Sterner and George (2000) studied the changes in the body composition of cyprinids, and

347   reported significant negative correlations between body size and P and N contents of fish, and

348 also significant but positive correlation between body length and C content of fish. Sterner

349 and George (2000) used fish > 20 mm in their analyses, and no embryos, post-embryos or

350 larvae. If we restrict observations only to fish > 20 mm in our study, we can see similarities

351 with Sterner and George (2000) in C and N trends, but conflicting trends in P. Furthermore,

352 Sterner and George (2000) found increasing N:P ratios with size in cyprinid minnows, a

353 pattern opposite that found by Davis and Boyd (1978), Pilati and Vanni (2007), and our study.

354   Increasing N:P and decreasing C:N ratio could indicate increased N allocation to muscle

355 tissue (Pangle and Sutton 2005; Vrede et al. 2011). In contrast, increasing C:N and C:P ratios

356 could be the consequences of increased lipid storage in fish. Decreasing C:P and N:P ratios

357 along with increasing % Ca values may indicate intensive bone formation (Hendrixson et al.

358 2007; Pilati and Vanni 2007). Our results suggest that bone formation and the concomitant P

359 and Ca allocation, and muscle formation, both contributed to ontogenetic changes in body

360 N:P. This contrasts somewhat with Pilati and Vanni's (2007) findings for gizzard shad

361 residing in a eutrophic lake, where changes in body P largely drove body N:P dynamics.

362 However, our findings are similar to those for zebra fish grown in the lab (Vanni and Pilati

363 2007), which showed declining body N and increasing body P during ontogeny.

364   Comparisons of the scant number of studies on ontogenetic variation in fish body

365 stoichiometry reveal some commonalities, but also considerable and apparent variation

366 among species. Opposing trends could arise from differences in the size ranges of fish used,

367 and/or from actual interspecific differences in the dynamics of lipid storage, muscle

368 development and bone formation during ontogeny. It should be noted that in our study, the

369 food supply of fish was optimal, which could result in relatively high lipid storage and

370 consequently a relatively strong dilution of elements in lower proportions (e.g., P). In nature,

371 the availability of food resources can be highly variable, perhaps leading to different degrees

372 of stoichiometric dilution. This could represent an important source of variation among

373   studies in the size−element content relationship. We also note that the diet shift to which our

374   fish were subjected, a switch from *Artemia* to flake food in juveniles, could have influenced

375   body stoichiometry. In particular, this represented an increase in dietary C:N from 4.9 (in

376   *Artemia*) to 6.6 (TetraMin flake food), and this was accompanied by increased body C:N in

377   both fish species (Fig. 1, Fig. 2). Thus, ontogenetic changes in body stoichiometry may be

378   partially attributable to changing dietary stoichiometry. However, zebrafish reared on a

379   constant diet of *Artemia* also showed increasing body C:N as they developed (Pilati and

380   Vanni 2007), showing that such changes in body stoichiometry can occur in absence of a

381   change in diet stoichiometry. In general, we know little about how body stoichiometry of

382   vertebrates varies with diet stoichiometry (Benstead et al. 2014), but it is likely that both

383   ontogeny and diet influence body stoichiometry of vertebrates.

384   The general patterns of changes in RNA contents were similar in both FM and SM,

385   suggesting that RNA production may be a strictly regulated and common trait, i.e., high RNA

386   levels are needed during early ontogeny when specific growth rates are high. This finding is

387   in accordance with the growth rate hypothesis (Elser et al. 1996, 2003). In our study, RNA

388   appeared to be an important P pool in the post-embryonic stage of both species, and proved to

389   be negligible before and after this life stage. Similar trends were described in earlier studies

390   (e.g., Elser et al. 1996; Vrede et al. 2004), assuming that RNA-bound P determines the total

391   body P content only in early life stages, while the beginning of bone formation along with

392   declining RNA levels realign the P pools in the body. Accordingly, correlations between P

393   and Ca became significant in the larvae of both species, indicating the increasing importance

394   of bone-associated P by this developmental stage.

395   The vast majority of studies dealing with the elemental stoichiometry of fish report body

396   nutrient values obtained from juvenile and/or adult specimens, and do not include larval or

397   embryonic stages. This bias has potential implications when assessing the role of fish as

398 nutrient sinks or sources, because younger fish cohorts often dominate population numbers or

399 feeding rates. Given that the body composition of young individuals differs significantly from

400 that of adults, the quantity of nutrients sequestered by growing fish, or recycled from

401 decomposing carcasses may strongly depend on fish population age structure. Rapidly

402 growing larvae could represent a nutrient sink, and sink strength may be especially high for P

403 given the ontogenetic increase in this element (Kraft et al. 1992; Pilati and Vanni 2007).

404 Natural fish mortality, which ranges between $10\% - 67\%$ per year in populations (Chidami

405 and Amyot 2008, and references therein), is inversely proportional to body length in younger

406 fish (Lorenzen 2000), suggesting that embryos, post-embryos or larvae are the most exposed

407 to mortality. Decaying fish carcasses may not function as permanent nutrient sinks in many

408 cases (Parmenter and Lamarra 1991; Boros et al. 2015), and ontogenetic variation in

409 elemental composition could influence the rates and ratios by which carcasses release

410 nutrients. Thus, consideration of age-specific nutrient contents could have important

411 implications not only for ES theory, but also for determining the importance of different fish

412 cohorts in internal nutrient loading.

413 Time scale is a potentially important factor when considering the importance of elemental

414 homeostasis and the applicability of ES theory. For instance, it is acceptable to assume

415 constant body composition in studies focusing on nutrient excretion on a given day, because

416 body nutrient requirements and storage are not likely to vary significantly within such a short

417 time interval. However, over longer periods and for studies with age-structured populations,

418 ontogenetic changes must be taken into consideration. One key question then is: At what time

419 scale does it become important to incorporate ontogenetic changes in body stoichiometry, to

420 accurately predict nutrient cycling by animals? In terms of mediating nutrient excretion,

421 changes in body stoichiometry are probably more important for larval fish than for older fish,

422 because body composition and specific growth rate change more rapidly for larvae than for

423   older individuals (Pilati and Vanni 2007). However, much more theoretical and empirical

424   work is needed to resolve this question.

425   In summary, our results provide evidence that elemental stoichiometry of fish is not simply

426   species-specific. Rather, each ontogenetic stage may have its own stoichiometric signature,

427   which is determined to a great extent by evolved physiological and morphological traits. Fish

428   are excellent vertebrate models for these kinds of studies, but we also need to learn more

429   about ontogenetic variation in animals in general. Thus, we encourage further studies that

430   more extensively explore intraspecific and interspecific variation in body stoichiometry,

431   including all ontogenetic stages of a wide range of aquatic and terrestrial taxa.

438

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572   **Figure legends**

573   **Fig. 1** Generalized additive models illustrating the intra- and interspecific variations in body

574   composition during ontogeny (n = 36 per species; for details, see Table 1). Percentage values

575   in the upper left corner show the explained variances (r^2 values, i.e., the goodness of model

576   fit). Vertical lines indicate the end of post-embryonic, larval and juvenile stages.

577   Solid line: fathead minnow; dashed line: sheepshead minnow; dotted line: limits of 95%

578   confidence intervals

579   **Fig. 2** Box-plots showing the distribution of different variables, according to species and

580   ontogenetic stages (n = 36 per species; for details, see Table 1). Letters above the boxes

581   denote the similarity/difference of the same ontogenetic group of the two species (Tukey's

582   post-hoc test; a – no significant difference; b – $P < 0.05$; c – $P < 0.01$; d – $P < 0.001$)

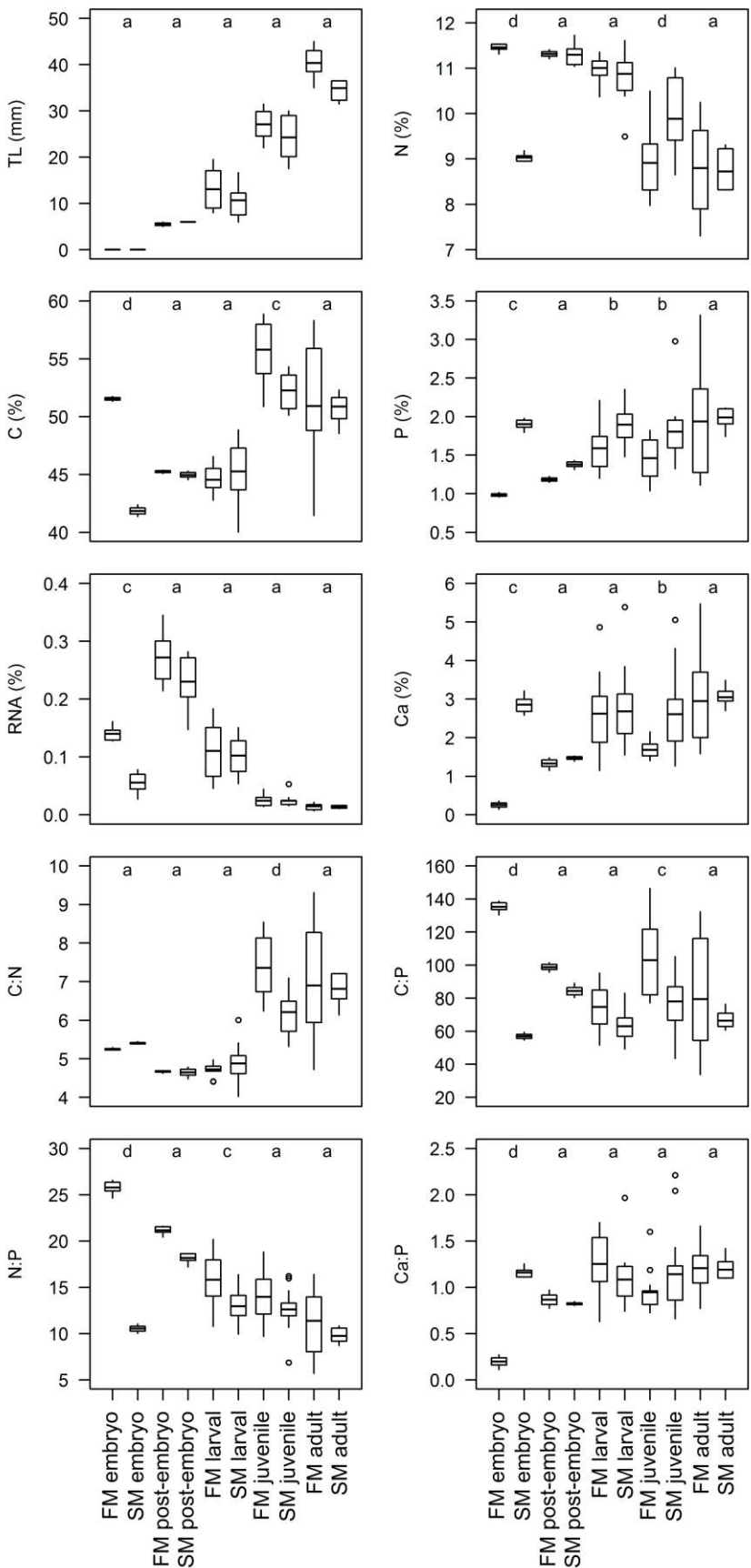583   **Fig. 3** Regression coefficients estimated for each ontogenetic stage from the ANCOVA

584   models (response *vs*. covariant). R^2 values indicate the general variances explained by the

585   models (i.e., the goodness of fit)
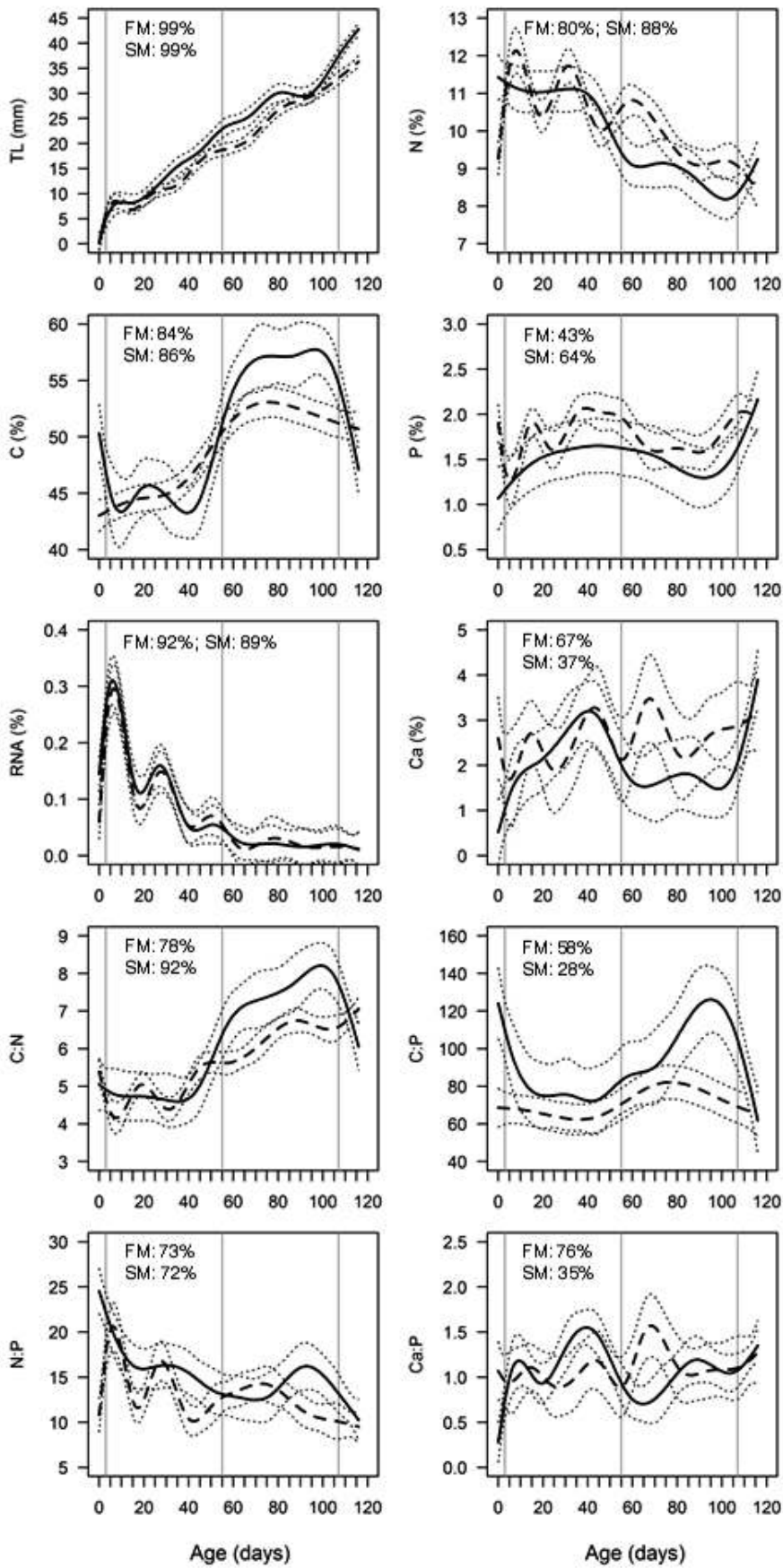
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587   **Fig. 1**

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589   **Fig. 2**

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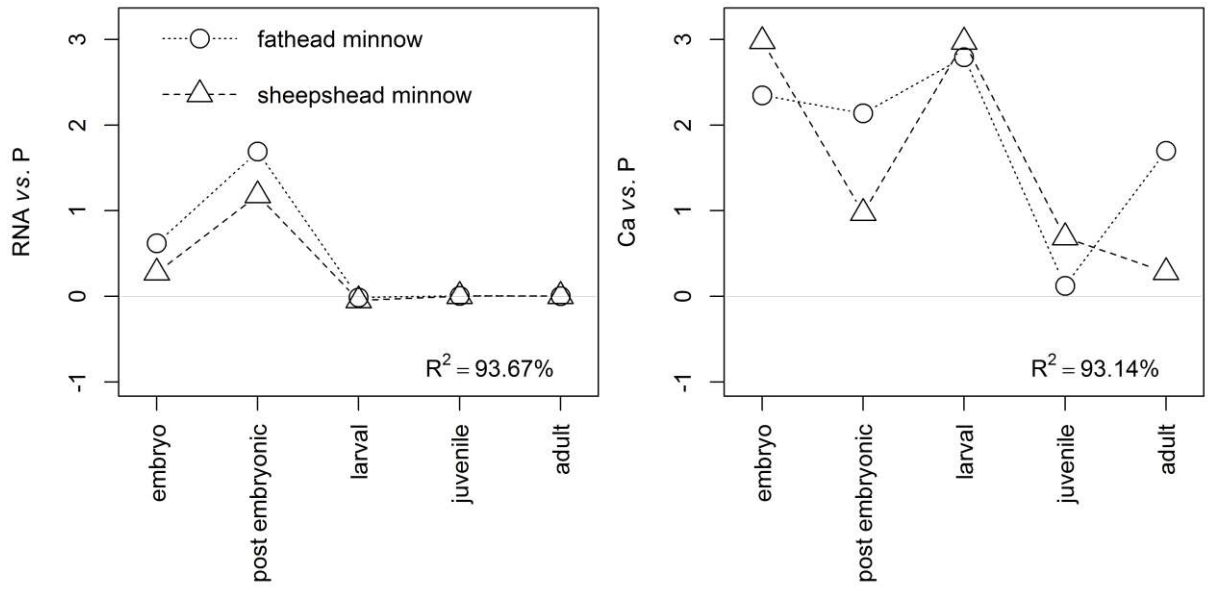
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592   **Fig. 3**

593

594 **Table 1** Total body lengths, sample sizes and age of fathead (FM) and sheepshead (SM) minnows at different

595 developmental stages. Mean ± SD denotes the arithmetic average and standard deviation; range is showed as an

596 interval between the minimum and maximum values. Note that body length of embryos was not measurable.

597

		Embryo	Post-embryo	Larval	Juvenile	Adult
FM total length (mm)	mean ± SD	-	5.5±0.5	13.0±4.4	27.1±3.3	40.3±3.4
	range	-	5.0−6.0	8.0−19.5	22.0−30.0	35.5−45.0
SM total length (mm)	mean ± SD	-	6.0±0.0	10.7±3.7	24.2±4.7	34.9±2.3
	range	-	6.0−6.0	7.0−16.7	17.5−30.0	31.5−36.5
Number of samples/species		3	3	12	12	6
Days elapsed after hatching		0	1−3	4−55	56−107	108−120

Table 2 Tukey's pairwise comparisons from the ANOVA models, indicating several significant intraspecific

differences during ontogeny. Numbers in the table denote differences in the means of variables at each

ontogenetic state, while asterisks mark the significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). FM –

fathead minnow; SM – sheepshead minnow

		FM				SM			
C		Post-embryo	Larval	Juvenile	Adult	Post-embryo	Larval	Juvenile	Adult
	Embryo	6.30 **	7.00 ***	4.23 *	0.63	3.11	3.40	10.40 ***	9.00 ***
	Post-embryo		0.70	10.53 ***	5.67 **		0.29	7.29 ***	5.90 **
	Larval			11.23 ***	6.37 ***			6.99 ***	5.61 ***
	Juvenile				4.86 ***				1.40
N		Post-embryo	Larval	Juvenile	Adult	Post-embryo	Larval	Juvenile	Adult
	Embryo	0.14	0.45	2.54 ***	2.66 ***	2.27 ***	1.85 ***	0.86 *	0.31
	Post-embryo		0.31	2.40 ***	2.52 ***		0.42	1.41 **	2.57 ***
	Larval			2.09 ***	2.21 ***			0.99 ***	2.15 ***
	Juvenile				0.12				1.16 ***
P		Post-embryo	Larval	Juvenile	Adult	Post-embryo	Larval	Juvenile	Adult
	Embryo	0.20	0.60 *	0.48	0.95 ***	0.52	0.01	0.10	0.09
	Post-embryo		0.40	0.28	0.75 **		0.52 *	0.42	0.61 *
	Larval			0.13	0.35 *			0.09	0.01
	Juvenile				0.48 **				0.19
RNA		Post-embryo	Larval	Juvenile	Adult	Post-embryo	Larval	Juvenile	Adult
	Embryo	0.13 ***	0.03	0.12 ***	0.13 ***	0.17 ***	0.05 *	0.03	0.04
	Post-embryo		0.16 ***	0.25 ***	0.26 ***		0.13 ***	0.21 ***	0.22 ***
	Larval			0.09 ***	0.10 ***			0.08 ***	0.09 ***
	Juvenile				0.01				0.01

Ca

	Post-embryo	Larval	Juvenile	Adult	Post-embryo	Larval	Juvenile	Adult
Embryo	1.08	2.37 ***	1.43 *	2.70 ***	1.39	0.17	0.25	0.19
Post-embryo		1.29 *	0.36	1.62 **		1.22 *	1.14	1.58 *
Larval			0.94 *	0.33			0.08	0.36
Juvenile				1.26 **				0.44

C:N

	Post-embryo	Larval	Juvenile	Adult	Post-embryo	Larval	Juvenile	Adult
Embryo	0.58	0.52	2.11 ***	1.65 ***	0.76	0.53	0.80	1.41 **
Post-embryo		0.06	2.69 ***	2.23 ***		0.23	1.56 **	2.17 ***
Larval			2.63 ***	2.18 ***			1.33 ***	1.94 ***
Juvenile				0.45				0.61

C:P

	Post-embryo	Larval	Juvenile	Adult	Post-embryo	Larval	Juvenile	Adult
Embryo	36.54 *	60.60 ***	32.27 *	55.88 ***	27.41	6.00	21.13	9.41
Post-embryo		24.06	4.27	19.34		21.41	6.27	18.00
Larval			28.33 ***	4.71			15.13 *	3.41
Juvenile				23.62 **				11.73

N:P

	Post-embryo	Larval	Juvenile	Adult	Post-embryo	Larval	Juvenile	Adult
Embryo	4.62 *	9.97 ***	11.83 ***	14.41 ***	7.63 ***	2.42	2.06	0.79
Post-embryo		5.36 ***	7.21 ***	9.79 ***		5.21 **	5.56 ***	8.41 ***
Larval			1.85	4.44 ***			0.36	3.21 **
Juvenile				2.58 *				2.85 *

Ca:P

	Post-embryo	Larval	Juvenile	Adult	Post-embryo	Larval	Juvenile	Adult
Embryo	0.67 *	1.05 ***	0.75 ***	1.01 ***	0.34	0.08	0.02	0.03
Post-embryo		0.38	0.08	0.34		0.26	0.32	0.37
Larval			0.31 *	0.05			0.06	0.11
Juvenile				0.26				0.05

603

604 **Table 3** Pearson's correlation tests of specific growth rates and body component variables. The "r" denotes the

605 correlation coefficient, lower and upper 95% are the limits of the confidence intervals of the correlation

606 coefficient, and "*P*" is the value of significance for the test with a null hypothesis of r = 0. Significant

607 correlations are marked with an asterisk.

608

Fathead minnow

	r	lower 95%	upper 95%	*P*
TL	-0.82	-0.99	0.68	0.18
C	-0.59	-0.99	0.86	0.41
N	0.78	-0.73	0.99	0.22
P	-0.83	-0.99	0.66	0.17
RNA*	0.98	0.35	0.99	0.02
Ca	-0.71	-0.99	0.79	0.29
C:N	-0.71	-0.99	0.79	0.29
C:P	0.41	-0.91	0.98	0.59
N:P*	0.96	0.04	0.99	0.03
Ca:P	-0.65	-0.99	0.83	0.35

Sheepshead minnow

	r	lower 95%	upper 95%	*P*
TL	-0.75	-0.99	0.75	0.25
C	-0.66	-0.99	0.82	0.34
N	0.74	-0.76	0.99	0.26
P*	-0.97	-0.99	-0.22	0.02
RNA*	0.96	-0.06	0.99	0.04
Ca*	-0.98	-0.99	-0.43	0.01
C:N	-0.73	-0.99	0.78	0.27
C:P	0.75	-0.76	0.99	0.25
N:P*	0.96	-0.03	0.99	0.04
Ca:P*	-0.99	-0.99	-0.64	0.01

609