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Ontogenetic variation in the body stoichiometry of two fish species

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Abstract

One of the central questions of ecological stoichiometry theory is to what extent animal species maintain constant elemental composition in their bodies. Although several recent studies demonstrate intraspecific variation in animal elemental composition, relatively little is known about ontogenetic changes in vertebrates, especially during early life stages. We studied the intraspecific and interspecific ontogenetic variation in the body stoichiometry of two fish species in two different orders; fathead minnow (*Pimephales promelas*) and sheepshead minnow (*Cyprinodon variegatus*), reared under controlled laboratory conditions. During ontogeny, we measured the chemical composition of fish bodies, including carbon (C), nitrogen (N), phosphorus (P), calcium (Ca), and ribonucleic-acid (RNA) contents. We found that N and RNA contents were relatively high in early life stages and declined substantially during development. In contrast, body C and C:N ratios were relatively low in embryos, post-embryos and larvae, and increased remarkably thereafter. Concentrations and ratios of some elements (e.g., Ca, P, Ca:P) did not exhibit consistent ontogenetic trends, but fluctuated dynamically between consecutive developmental stages in both species. Specific growth rates correlated significantly with RNA contents in both species. Analyses of the relative importance of different P pools at each developmental stage revealed that RNA was a considerable P pool in post-embryos, while bone-associated P was the dominant body P pool in later stages. Our results suggest that the elemental composition of fish bodies changes considerably during ontogeny. Each ontogenetic stage has its own stoichiometric signature, but the timing, magnitude and direction of ontogenetic changes can vary substantially between taxa.

Keywords: nutrients, ecological stoichiometry, elemental homeostasis, organismal 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).

Thus, alterations in fish biomass and community assemblage influence the availability of nutrients to primary producers (McIntyre et al. 2008; Boros et al. 2009). Occupying relatively high trophic positions and being rich in nutrients, fish represent a locus where N and P are concentrated (e.g. Sterner and Elser 2002; Tarvainen et al. 2002; Vanni et al. 2013), which is important because these nutrients play a key role in limiting primary production (Lewis and Wurtsbaugh 2008). Because of their rapid growth and high mortality rates, young-of-the-year fish can be especially important in these processes (Kraft 1992; Lorenzen 2000). Hence, elemental stoichiometry of fish, including both sequestration and release of nutrients, has been of great interest during the recent decades (Kitchell et al. 1975; Parmenter and Lamarra 1991; Vanni 2002; Vrede et al. 2011; Vanni et al. 2013).

Several studies demonstrate that the body stoichiometry of fish may vary with ecological and environmental conditions such as habitat, resources, food quality, trophic state, predation pressure and stress (Boros et al. 2012; El-Sabaawi et al. 2012 a,b; Benstead et al. 2014; Dalton and Flecker 2014; Sullam et al. 2015). Thus, significant intraspecific differences in elemental composition may exist among individuals of different populations. However, ontogeny also can play a role in explaining intraspecific differences in the elemental composition of fish. Recent studies report that intraspecific variability in body stoichiometry is greater than previously thought, and that body size, ontogeny and/or morphology can explain a significant part of the variation (Pilati and Vanni 2007; Vrede et al. 2011).

The mass and relative proportion of different tissues and biochemicals can change dynamically during organismal development, and this can lead to changes in whole-body elemental composition, because different tissue types and biochemicals contain elements in different quantities. Bones and scales are rich in calcium (Ca) and P, while nucleic acids also contain significant amounts of P (Rønsholdt 1995; Vrede et al. 2004; Hendrixson et al. 2007). Muscle tissue stores considerable amounts of N as protein (Pangle and Sutton 2005; Vrede et

al. 2011), while energy-rich lipids are the most important C storage pools in animal bodies (Sterner and Elser 2002; Fagan et al. 2011). During ontogeny, fish may exhibit different strategies and distinct periods of energy accumulation and somatic growth (Post and Parkinson 2001; Biro et al. 2005; Nakazawa 2011), and body stoichiometry of individuals reflects such alterations between life stages. For instance, Deegan (1986) showed that the body composition of young-of-the-year gulf menhaden (*Brevoortia patronus*) changed considerably during ontogeny owing to a shift in energy allocation away from protein growth to lipid storage.

The growth rate hypothesis (Elser et al. 1996, 2003) states that fast-growing animals (which often include those in early life stages) need high quantities of ribonucleic acid (RNA) to achieve and maintain their high specific growth rates, and RNA content of tissues constitutes the most important P-pool of body in early phases of ontogeny (Elser et al. 1996; Vrede et al. 2004). Subsequently, RNA content of tissues declines with decreasing growth rates as ontogeny proceeds (Gillooly et al. 2005); in vertebrates this is accompanied by a gradual ossification (P and Ca allocation) of skeleton (Hendrixson et al. 2007; Pilati and Vanni 2007). For example, Sterner and Elser (2002) and Vrede et al. (2011) pointed out that rapidly growing animals commonly have low C:P and N:P ratios because of the increased P allocation to RNA. However, decreasing C:P and N:P ratios with growth were also reported for later stages of ontogeny in vertebrates, owing to the increasing P allocation to developing skeleton (Pilati and Vanni 2007). This suggests a realignment of P pools in body during ontogeny. Yet, the timing and magnitude of these changes, and how they vary among species, are still largely unknown; for fish we know very little about changes during early life stages.

In this study, we explored ontogenetic changes in the body stoichiometry of two fish species in two different orders. We raised fish from embryos to adults under controlled environmental conditions, and assessed their chemical composition (C, N, P, Ca and RNA) at

several developmental stages. The justification of our experiment is two-fold. First, preceding studies on ontogenetic stoichiometric shifts have been conducted only on a limited number of species and ontogenetic stages; to our knowledge, no previous studies include data on early developmental stages (i.e., embryos and post-embryos) as well as adults of fish. Secondly, intra- and interspecific variation in ontogenetic stoichiometry has not yet been studied in experiments in which feeding and environmental conditions are controlled. Because of these gaps, the factors that contribute to variability in organismal stoichiometry are still poorly understood and warrant more detailed examinations. We had the following objectives:

(1) To characterize the ontogenetic changes in the body composition of two fish species that belong to different taxonomic orders and that are adapted to different environments.

(2) To explore whether the two fish species show the same strategies in allocating nutrients to energy storage and somatic growth during ontogeny, or if the two species exhibit divergent patterns even when environmental conditions are similar.

(3) In the light of the growth rate hypothesis, to identify the life stages when RNA is the dominant P pool in fish, and when during development the P stored in RNA becomes negligible compared to the pool in the developing skeletal system.

Materials and methods

Study species

We studied two fish species from the class of ray-finned fishes (Actinopterygii): fathead minnow (*Pimephales promelas*) and sheepshead minnow (*Cyprinodon variegatus*). Fathead minnow (hereafter FM), in the order Cypriniformes, is a widespread fish species across North America, inhabiting all types of freshwater ecosystems. Sheepshead minnow (SM) belongs to the order Cyprinodontiformes and lives in brackish/saltwater environments from the Mid-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

ZM100 centrifugal mill (Retsch GmbH, Germany). Carbon and N contents of samples were measured using a CE Elantech Flash 2000 CHN analyser (CE Elantech, USA), while P contents were analysed following ignition at 550 °C and subsequent HCl digestion to convert all P to soluble reactive P, which was assayed with a Lachat QC 8000 FIA autoanalyser (Lachat Instruments, USA). For Ca content analysis, dried and homogenized subsamples were combusted at 550 °C and the produced ash was dissolved in HCl solution. Subsequently, Ca contents were determined with a Perkin-Elmer Optima 7300 DV Optical Emission Spectrometer (Perkin-Elmer Inc., USA). Prior to RNA analyses, deep-frozen and intact samples were homogenized with a sonicator. RNA contents of homogenates were extracted with a Maxwell LEV simplyRNA Blood Kit and Maxwell 16 Nucleic Acid Extraction System (Promega Corporation, USA). The quantity of the extracted RNA was measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA) in the Center for Bioinformatics and Functional Genomics at Miami University.

Statistical analyses

As the first step in data analyses, we used generalized additive models (GAM) with cubic regression spline smoothers to illustrate the ontogenetic changes of our studied variables for both species (proportions of elements are expressed as percentage of dry mass, proportion of RNA is expressed as percentage of wet mass, and ratios are expressed in molar units). GAMs (Hastie and Tibshirani 1990) are ideal and commonly used for visualizing non-linear statistical relationships (e.g., Guisan et al. 2002; Buisson et al. 2008; Schmera et al. 2012), which frequently occur with ecological variables. We added 95% confidence bands to the GAM plots, in order to indicate the reliability of the predicted values of the models and to provide a visual aid for assessing differences between the two species.

Next, to explore differences between the ontogenetic stages and species, we fitted an analysis of variance (ANOVA) model for each response variable, using species and ontogenetic stage as the grouping variable (FM embryo, SM embryo, etc.). After ANOVAs, Tukey's post-hoc tests were applied to compare all developmental stages in a pairwise manner within a species (e.g., FM embryos *vs*. FM post-embryos) and between the two species (e.g., FM embryos *vs*. SM embryos).

To assess changes in the associations among Ca, RNA and P during the developmental process, we used analyses of covariance models (ANCOVA) with a nested factorial design (i.e., for RNA *vs*. P and Ca *vs*. P). In the ANCOVA models, the ontogenetic stage grouping (categorical) variable was nested within the species grouping variable. The slope regression coefficient of the ANCOVA models enabled us to evaluate the statistical relationship between the response variable (RNA and Ca) and the continuous explanatory variable (P) of the model. For the ANCOVA models, we used a contrast matrix to make the *a priori* planned pairwise comparisons of the slope regression parameters between the ontogenetic stages separately for the two species.

Relationships between body component variables and specific growth rate (SGR; Brown 1946) of fish were assessed with Pearson's correlation analysis. Calculation of specific growth rate was based on total body length increments and was calculated as follows:

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

where s is a given ontogenetic stage, L_s and L_{s-1} are the average total body lengths (mm) at the s and $s-1$ ontogenetic stages, A_s and A_{s-1} are the average age at days of the s and $s-1$ ontogenetic stages. Note that calculation of SGR was not possible for embryos, and that addition of an arbitrary constant (0.01) to the formula was necessary because the body length 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

highest in post-embryos and decreased with growth both in FM (post-embryo: 210.4; larval: 3.3; juvenile: 1.7; adult: 1.1) and SM, respectively (post-embryo: 213.3; larval: 2.3; juvenile: 1.7; adult: 1.0). Pearson's correlation analysis revealed significant and positive relationships between SGR−RNA and SGR−N:P in both species. However, we also found significant negative correlations between SGR−P, SGR−Ca and SGR−Ca:P, but only in SM (Table 3).

We observed significant differences in many variables between consecutive ontogenetic stages of the same species, as well as between the earlier and later phases of development (e.g., between embryos and adults of the same species) (Table 2). These results show that body composition of both FM and SM changed considerably with growth, but also that the timing, magnitude and direction of these changes were dissimilar in the two species in several cases. For instance, C, C:P and Ca:P ratios differed significantly between the embryonic and post-embryonic stages of FM, but did not differ among these stages for SM. In contrast, N and RNA were significantly different between the embryonic and post-embryonic stages of SM, but were similar to each other in FM. One notable difference between species was that SM embryos had much higher Ca and P than FM embryos (Fig. 2). Thus, for FM, P and Ca differed markedly between earliest stages and adults, while for SM, adults differed only from post-embryos with respect to these parameters. More generally, a lack of significant P and Ca differences between ontogenetic stages was more typical for SM than for FM. Furthermore, C:P and especially Ca:P ratios were also very similar between the different ontogenetic stages of SM, in contrast to FM (Table 2).

Comparisons of interspecific differences within the same ontogenetic stage revealed that the body compositions and ratios of elements were the most divergent in embryos, while those of post-embryos and adults were very similar (Fig. 2). FM embryos had ~25% higher C and N contents than SM embryos, and the difference between species was significant for both variables. However, interspecific differences in C and N contents were relatively minimal

compared to differences in RNA content, C:P ratio, and N:P ratio, all of which were 140−150% higher in FM embryos. On the other hand, SM embryos contained significantly higher amounts of P (almost 2-times that of FM embryos), but the largest difference was for Ca, as SM embryos contained 10-times more of this element per unit body mass. Accordingly, molar Ca:P ratios were ~6-times higher in SM embryos than in FM embryos. Interspecific differences in Ca, P and Ca:P were negligible during the post-embryonic and larval stages, but became significant again for juveniles. Note that total lengths of the two species were very similar throughout the experiment (Fig. 1), which facilitated making interspecific comparisons in the chemical composition during ontogeny.

We found mostly positive associations, or no association, between RNA−P and Ca−P within given stages (Fig. 3). RNA and P were significantly correlated ($P < 0.001$) in post-embryos of FM and SM, but there was no significant relationship between these two variables in other developmental stages. Regression coefficients (slope parameters) of FM and SM post-embryos differed significantly ($P < 0.001$) from the same parameter of their larvae, juveniles and adults. For the Ca−P relationship, we found significant regressions only in larval fishes ($P < 0.001$ in both species) and in FM adults. Even though the Ca−P regression coefficients appeared to vary between developmental stages (Fig. 3), the only significant difference occurred between larvae and juveniles ($P < 0.05$ for both species).

Discussion

In this study, we followed the ontogenetic changes in the elemental stoichiometry of two, ecologically contrasting fish species, and quantified RNA and Ca to assess the relative importance of different P pools during ontogeny. Based on the homeostasis component of ES theory (Sterner and Elser 2002), our null hypothesis was that elemental composition of an 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

studies in the size−element content relationship. We also note that the diet shift to which our fish were subjected, a switch from *Artemia* to flake food in juveniles, could have influenced body stoichiometry. In particular, this represented an increase in dietary C:N from 4.9 (in *Artemia*) to 6.6 (TetraMin flake food), and this was accompanied by increased body C:N in both fish species (Fig. 1, Fig. 2). Thus, ontogenetic changes in body stoichiometry may be partially attributable to changing dietary stoichiometry. However, zebrafish reared on a constant diet of *Artemia* also showed increasing body C:N as they developed (Pilati and Vanni 2007), showing that such changes in body stoichiometry can occur in absence of a change in diet stoichiometry. In general, we know little about how body stoichiometry of vertebrates varies with diet stoichiometry (Benstead et al. 2014), but it is likely that both ontogeny and diet influence body stoichiometry of vertebrates.

The general patterns of changes in RNA contents were similar in both FM and SM, suggesting that RNA production may be a strictly regulated and common trait, i.e., high RNA levels are needed during early ontogeny when specific growth rates are high. This finding is in accordance with the growth rate hypothesis (Elser et al. 1996, 2003). In our study, RNA appeared to be an important P pool in the post-embryonic stage of both species, and proved to be negligible before and after this life stage. Similar trends were described in earlier studies (e.g., Elser et al. 1996; Vrede et al. 2004), assuming that RNA-bound P determines the total body P content only in early life stages, while the beginning of bone formation along with declining RNA levels realign the P pools in the body. Accordingly, correlations between P and Ca became significant in the larvae of both species, indicating the increasing importance of bone-associated P by this developmental stage.

The vast majority of studies dealing with the elemental stoichiometry of fish report body nutrient values obtained from juvenile and/or adult specimens, and do not include larval or 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.

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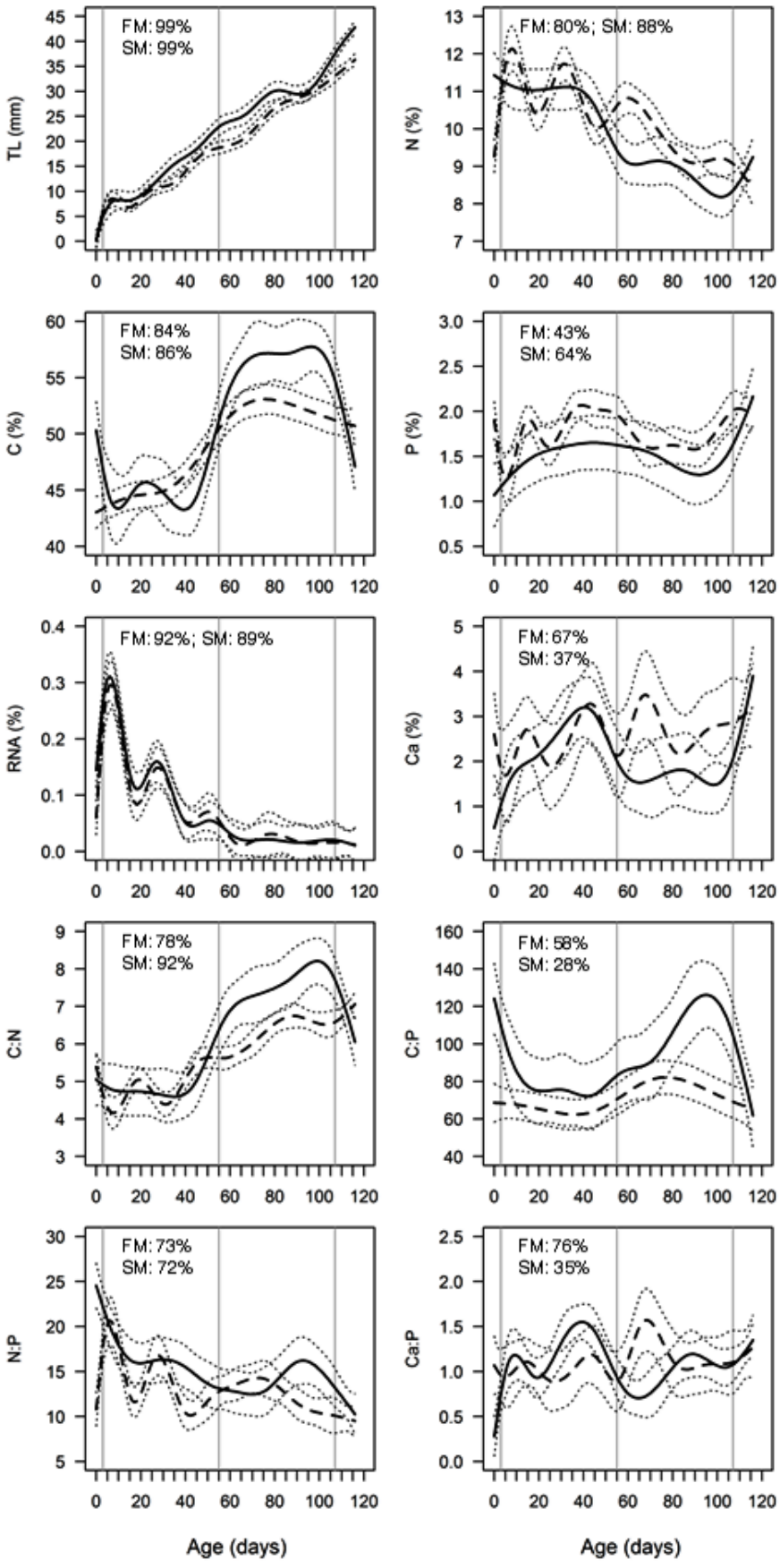
572 **Figure legends**

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

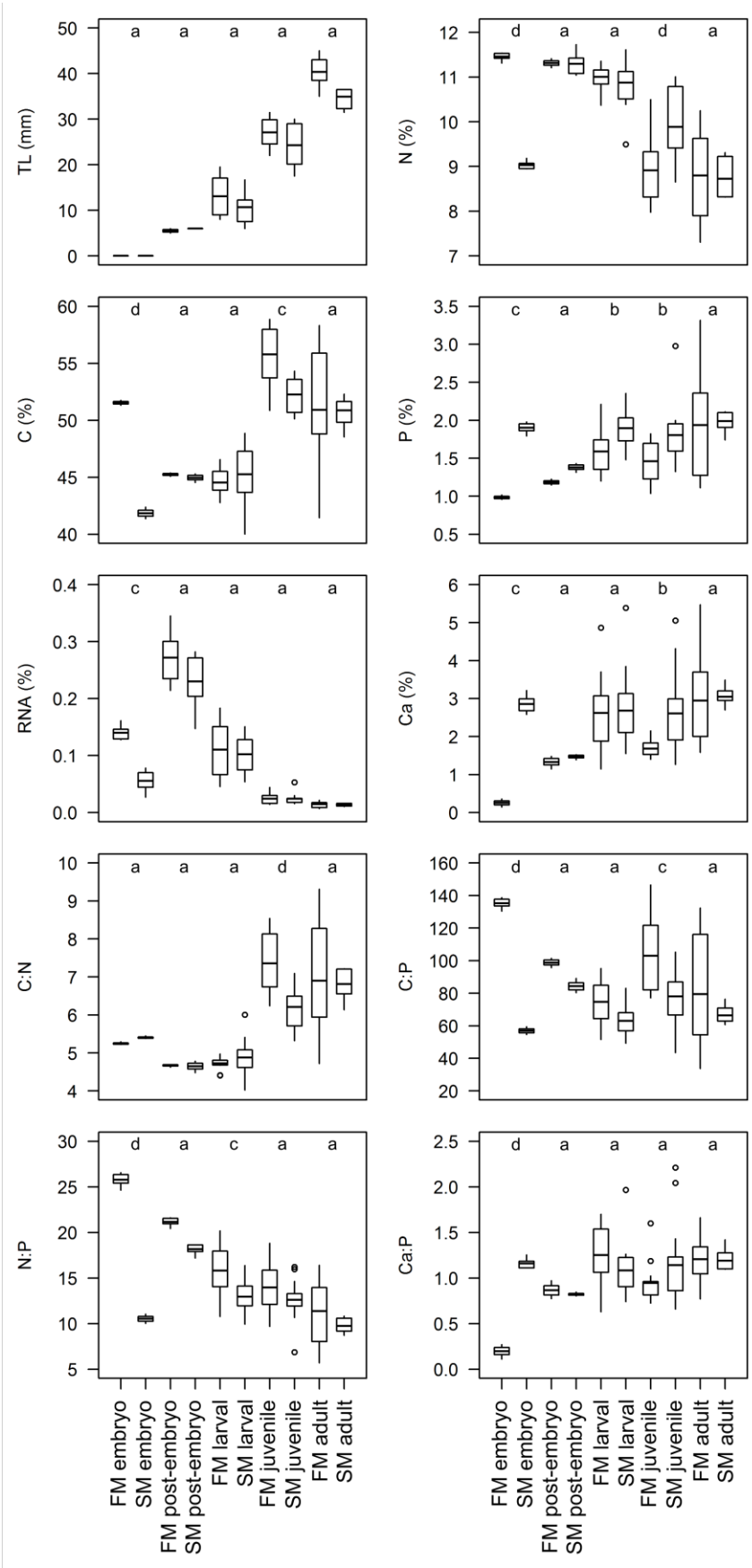
579 **Fig. 2** Box-plots showing the distribution of different variables, according to species and
580 ontogenetic stages (n = 36 per species; for details, see Table 1). Letters above the boxes
581 denote the similarity/difference of the same ontogenetic group of the two species (Tukey's
582 post-hoc 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)


TL (mm)
FM: 99%
SM: 99%
N (%)
FM: 80%; SM: 88%
C (%)
FM: 84%
SM: 86%
P (%)
FM: 43%
SM: 64%
RNA (%)
FM: 92%; SM: 89%
Ca (%)
FM: 67%
SM: 37%
C:N
FM: 78%
SM: 92%
C:P
FM: 58%
SM: 28%
N:P
FM: 73%
SM: 72%
Ca:P
FM: 76%
SM: 35%
Age (days)
Age (days)

586

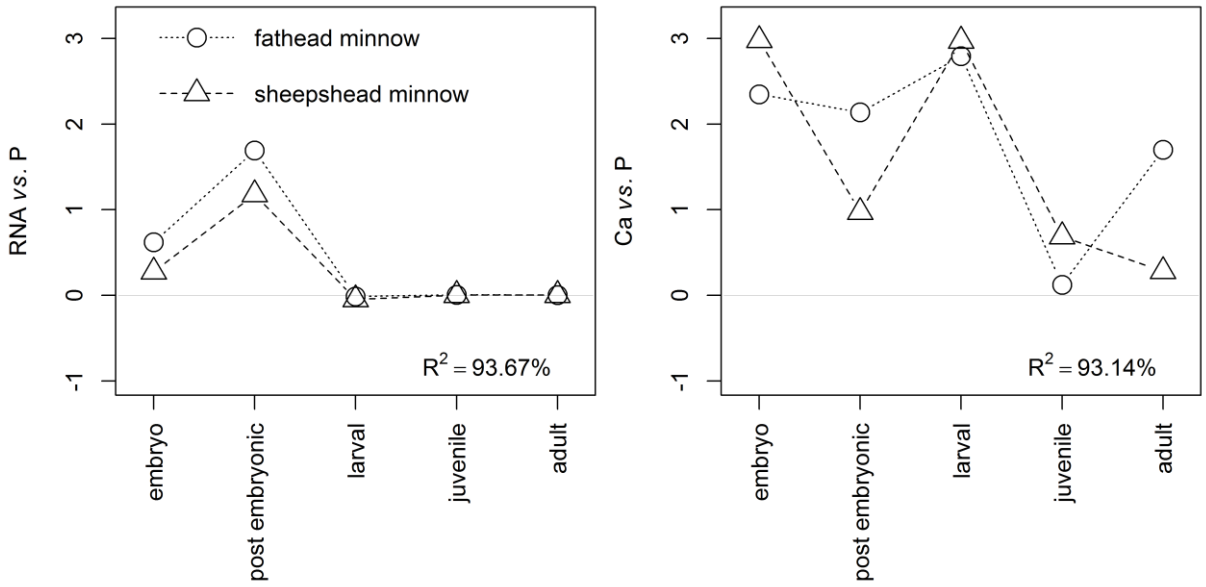
587 **Fig. 1**


TL (mm)
N (%)
C (%)
P (%)
RNA (%)
Ca (%)
C:N
C:P
N:P
Ca:P
FM embryo
SM embryo
FM post-embryo
SM post-embryo
FM larval
SM larval
FM juvenile
SM juvenile
FM adult
SM adult

588

589 **Fig. 2**

590



591

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

598 **Table 2** Tukey's pairwise comparisons from the ANOVA models, indicating several significant intraspecific
599 differences during ontogeny. Numbers in the table denote differences in the means of variables at each
600 ontogenetic state, while asterisks mark the significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). FM –
601 fathead minnow; SM – sheepshead minnow

602

	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

Table 3 Pearson's correlation tests of specific growth rates and body component variables. The "r" denotes the correlation coefficient, lower and upper 95% are the limits of the confidence intervals of the correlation coefficient, and "*P*" is the value of significance for the test with a null hypothesis of r = 0. Significant correlations are marked with an asterisk.

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