ABSTRACT

ONTOGENETIC VARIATION IN THE NUTRIENT STOICHIOMETRY OF JUVENILE FISH

by Kelsea Nicole Downs

Most stoichiometric models and empirical studies to date do not take into account the ontogenetic shifts that may occur in the stoichiometry of animal bodies. To gain a better understanding of when and where ontogenetic shifts are occurring we studied the interspecific ontogenetic variation in body stoichiometry of ten different fish species from six different families. We measured the chemical composition of fish bodies including Carbon (C), Nitrogen (N), phosphorus (P), calcium (Ca) and ribonucleic acid (RNA) from embryo stage and over the course of early development. We found evidence that fish elemental stoichiometry varies over development and that there are differences across species. The results of our study based on exploring body elemental changes through time support the findings of other studies indicating that fish do not follow strict species-specific elemental homeostasis during ontogeny.
ONTOGENETIC VARIATION IN THE NUTRIENT STOICHIOMETRY OF
JUVENILE FISH

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Introduction:

Ecological stoichiometry theory provides a framework to examine the relative balance of elements such as carbon (C), nitrogen (N) and phosphorus (P) that are found in, and recycled by, animals (Sterner and Elser 2002). Early stoichiometric models assumed that animals are largely homeostatic in their body elemental composition and that within a species, body nutrient concentrations remain relatively constant across different populations and stages of development (Sterner and Elser 2002). However, recent studies have shown intraspecific variation in body stoichiometry as a function of habitat, growth rates, reproduction, body size, and morphology (Sterner et al 2002, Hendrixson et al 2007, McIntyre and Flecker 2010, Vrede et al 2004, Vrede et al 2011). Yet, most stoichiometric models and empirical studies do not take into account the ontogenetic shifts that may occur in the stoichiometry of animal bodies (Pilati and Vanni 2007, Boros et al 2015, Showalter et al 2016, Leal et al 2017). To gain a better understanding of when and where ontogenetic shifts are occurring, the central theme of my thesis is to determine the variation in the nutrient stoichiometry (C, N, and P) over early development in several common species of fish.

In some aquatic ecosystems, fish consume, store, transport, and release significant amounts of carbon (C), nitrogen (N) and phosphorus (P), thereby modulating direct and indirect effects on the ecosystems in which they live (Vanni 2002, Vanni et al. 2013), thus making them excellent organisms to study in the context of ecological stoichiometry. In lakes and streams fish biomass constitutes important nutrient pools and supports primary producers through nutrient recycling (Vanni 2002, McIntyre et al. 2008). The role fish play in nutrient cycling may depend on the body nutrient stoichiometry of the fish. For example, fish with a high concentration of P in their bodies may excrete relatively little P, because they need to store most of the P they consume. Expressed as ratios, this implies that, all else being equal, a fish with a low N:P ratio in its body (i.e., high P content) would excrete at relatively high N:P (i.e., excrete little P) (Sterner and Elser 2002).

The nutrient contents of animal bodies are a function of the relative proportions of specific anatomical features and biochemical compounds. Muscle tissue stores large amounts of N as protein (Vrede et al 2011), while much C is stored as energy (lipids) (Sterner and Elser 2002). For invertebrates (insects, mollusks, crustaceans), the biggest pool of P in bodies is RNA, a P-rich molecule. All else being equal, animal species with high growth rates have high RNA...
contents (allowing for rapid protein synthesis) and thus high overall body P concentrations (Sterner and Elser 2002). However, for vertebrates such as fish, bone and scales are important in determining the balance of nutrients, because these structures are very P-rich (bone is basically calcium phosphate) (Davis and Boyd 1978, Hendrixson et al. 2007, Pilati and Vanni 2007).

During development, the stoichiometry of an individual fish's body may change greatly as the proportions of different tissues and biochemical compounds change. Newborn fish (larvae) have undeveloped bones that have not yet ossified, so they likely have very little P in their bodies. As they grow, young fish may thus need to greatly increase their body P contents to grow bones. Very little data exist on the ontogenetic (developmental) changes in stoichiometry as fish grow from embryos to adults. A study of two fish species, gizzard shad and zebra fish (Pilati and Vanni 2007) showed that, as individuals of both species develop, the P concentration in their bodies doubles or triples, from ~1% of dry mass in newborn larvae to ~2% (zebra fish) or 3% (gizzard shad) in just a few months. After this rapid increase, concentrations remain relatively stable into adulthood (Pilati and Vanni 2007). Data on calcium (Ca) concentrations and the Ca:P ratio suggest that the increase in body P is due to mainly to bone development (Pilati and Vanni 2007).

In vertebrates such as fish, C:P and N:P ratios may decrease with body size because the relative allocation to P-rich bone is expected to increase with size (Vanni 2002). However, the growth rate hypothesis predicts the opposite because in most animals, including fish, young individuals have higher specific growth rates than older individuals. Thus, the growth rate hypothesis predicts that young individuals with fast growth rates will have high body P (low C:P) whereas older individuals with slower growth rates will have higher C:P (Vrede et al 2004, Elser et al 1996, Back and King 2013). Thus, it is possible that some of the increase in body P (decrease in C:P or N:P) that accompany skeletal development may be offset by decreasing RNA content. Understanding these trends in nutrient pools is important because shifts in fish body elemental composition can influence the storage and recycling of potentially limiting nutrients and hence ecosystem productivity (Vrede et al 2004, Elser et al. 2007, Schindler 2011).

While several studies have looked at stoichiometric differences among fish species (Goodyear and Boyd 1972, Davis and Boyd 1978, Sterner and George 2000, Vanni 2002, Hendrixson et al 2006, McIntyre and Flecker 2010), ontogenetic changes have been examined only in six species (Pilati and Vanni 2007, Boros et al 2015, Showalter et al 2016, Leal et al...
2017); thus there are still wide gaps in the knowledge of elemental allocation strategies. Our study is the first to examine several fish species using a high-frequency sampling scheme (weekly) to quantify changes during their most rapid developmental period, starting with the embryonic phase. This project explores ontogenetic changes in the body stoichiometry of ten different fish species representing fish from six different families and five different orders (Table 1). Specifically, we had the following objectives:

1. To investigate the ontogenetic changes in the body composition of ten common fish species belonging to six different families and five different taxonomic orders.
2. To explore interspecific variation in the relationship between body composition and age as well as the interaction between age and species.
3. To understand P allocation strategies of these ten fish species, specifically to determine ontogenetic variation in the allocation of P to RNA and bones.

Hypotheses and Predictions:

*Elemental changes during development*

To address our objectives, we will test two hypotheses. My first hypothesis is that changes in stoichiometry during development will be driven by growth and the high demand for the P needed for RNA and skeletal construction. We predict that all species of fish will grow and develop bones and scales, which will result in increased P and Ca levels (Figure 1). P and Ca concentration in their bodies will be low initially, i.e., during the embryonic stage (no bones or scales), will increase rapidly as the fish grow, and then will reach a relatively stable state once fish have developed bones and scales (Figure 1). Since the demand for P will be universal among species, we expect to see the same general pattern among species and families, but this needs to be tested as currently there are published data on ontogenetic stoichiometry for only four six species (Pilati and Vanni 2007, Boros et al 2015, Showalter et al 2016, Leal et al 2017).

Increasing body P and Ca should correlate with decreasing trends in body C and N (Figure 1). We predict that across all species, embryos will have the highest C due to high lipid content. As the fish develop C will be utilized for growth with less allocation of C to tissue/fat storage; therefore, body C will decline during development. We also predict that body N will start out high and decline with increasing body size (Figure 1). Most of what we know about body N is based on studies of adult fish, several studies have shown that N and P trend in opposite directions and N generally decreases with body size as per mass larger animals have more
invested into P rich bone (Sterner and Elser 2002, Vanni 1996 and Davis and Boyd 1978) (Figure 1). Also, during ontogeny fish will be growing bones thus having increased P, this could create a dilution effect by P. However, little is know about N dynamics in developing fish.

Changing C, N, P and Ca pools will drive trends in elemental ratios. We predict decreasing C:N, N:P and C:P (Figure 1) with increasing trends in Ca:P (Figure 1). Lastly, we predict that RNA body content will increase dramatically from the embryo stage to newly hatched larvae, and then decline with decreasing growth rates as fish age (Figure 1). During development, there is a high demand for the P needed for RNA and skeletal construction. Changes in body compositions should match changing elemental needs during development. Body nutrient contents should reflect these demands with high sequestration rates of P and calcium into tissues, including bones and scales.

*Elemental changes across species*

Our second hypothesis is that within the general ontogenetic pattern described above (e.g., increasing body P concentrations and the corresponding decrease in N:P; Fig. 1), there will be significant variation in stoichiometry among families, and less variation among species within a family. We predict, based on studies of adult fish species, that families with more pronounced bone, spines and scales (e.g., sunfish) will show a more pronounced increase in body P and a decrease in N:P, compared to families with less bony material (e.g., trout) (Vanni et al 2002, Vrede et al 2004, Pilati and Vanni 2007, Hendrixson et al 2007). In contrast, we predict little variation among species within a family. While it is known that adults of some fish families differ in body stoichiometry, essentially nothing is known about the interspecific variation in the stoichiometry of fish as they undergo ontogenetic changes. Because they share a common evolutionary history, fishes within a family have the same generalized body plan, while different families may have different body plans. These anatomical differences should be reflected in nutrient stoichiometry. Taxonomic identity at the family level has been shown to have important effects on the phosphorus content of adult fish, based on bone and scale structure. Bony and spiny fish in families such as Percidae (perch, walleye), Centrarchidae (sunfish, bass) and Loricariidae (armored catfish) tend to have significantly higher P content than fish in families such as Salmonidae (trout, salmon) and Cyprinidae (minnows) (Sterner and George 2000, Vanni et al. 2002, Hendrixson et al. 2007, McIntyre and Flecker 2010). Body nitrogen content also can vary based on family and is related to protein and muscle structure (Sterner and George 2000).
Aspredinidae ("banjo catfish") and Anguillidae (eels) have significantly lower body N content than Umbridae (mudminnows) and Ictaluridae (North American catfish) families (McIntyre and Flecker 2010). However, while we know something about the differences in the nutrient stoichiometry of adult fish, little is known about the correlation between interspecific variation in stoichiometry as fish undergo ontogenetic changes.

**Materials and Methods:**

We studied ten fish species representing six different families and five different orders from the class of ray-finned fishes (Actinopterygii) (Table 1). Several species bluegill (Lepomis macrochirus), largemouth bass (Micropterus salmoides), muskellunge (Esox masquinongy), walleye (Sander vitreus), channel catfish (Ictalurus punctatus) yellow perch (Perca flavescens) and fathead minnows (Pimephales promelas) are native to Ohio and are commonly found and stocked in streams and reservoirs throughout Ohio and North America, while other species studied brown trout (Salmo trutta), rainbow trout (Oncorhynchus mykiss) and saugeye (Sander vitreus x Sander canadensis) (a hybrid of walleye and sauger) are not native to Ohio but are stocked annually in streams and reservoirs across the state and thus have important ecological and economical impacts. Three of the families studied (Cyprinidae, Salmonidae, and Ictaluridae) had “soft-rays” and “softer” tissue while the other three families (Centrarchidae, Esocidae and Percidae) are more “bony” fish with spiny rays. For three families (Centrarchidae, Percidae, and Salmonidae), 2-3 species per family were studied so that we could evaluate variation both within and among families (Table 1). In addition, for two percid species, sampling was conducted at two hatcheries to evaluate intraspecific variation (Table 1).

Fish were spawned, hatched and reared in hatcheries operated by the Ohio Department of Natural Resources-Division of Wildlife. This allowed us a unique opportunity because we knew exactly when the fish spawned and was able to collect eggs and sample larvae weekly from the time fish hatched until they reached the juvenile stage. We recognize that the hatchery ponds are not intact ecosystems, where fish stoichiometry may be somewhat different. However, the hatchery ponds and tanks allowed for a controlled environment to collect comparative information on variation among species and families without external environmental factors. In addition, it is often difficult to sample and identify larval fish in lakes and rivers. Finally, the similarities in body P and C:P between gizzard shad from a lake and zebra fish raised in the lab suggest that general patterns are consistent among habitats (Pilati and Vanni 2007). It is
important to note that unlike a natural setting fish in the hatchery had consistent food availability over the duration of our experiment and elemental analysis of the food revealed similar levels of C, N and P across species (Supplemental Table 1).

Fish were collected weekly. Samples were analyzed for whole-organism tissue C, N, P, and Ca while whole-organism RNA samples were collected biweekly. Collection began at the embryo stage, and for each sampling event, we tracked the same cohort within the same pond or raceway throughout the length of the study. Fish were randomly netted from the ponds/raceways. Since C, N, P and Ca analyses required dry tissue while RNA content can only be measured from wet tissue, we had to use different fish for elemental analyses than for RNA measurements. Fish were anesthetized and sacrificed using the ice-slurry immersion method (Blessing et al 2010). After fish were sacrificed; mass and total length were recorded, while mass and diameter was recorded for individual eggs. For egg collection of most species (except bluegill and largemouth bass) we were able to sample the eggs within 24-48 hours after fertilization (eggs water hardened and un-eyed) and again after the embryo had formed (eyed). Bluegill eggs were sampled only at the eyed stage, while we were unable to collect any eggs from the largemouth bass. Eggs were sorted underneath a light to ensure they were viable and 10-25 eggs (depending on species/egg size) were pooled together to constitute a sample to ensure sufficient biomass to assay elemental contents; samples were placed into individual vials (five replicate samples per population for each sampling event). Eggs for RNA analysis were flash frozen using dry ice and transported back to the lab and placed at -80C for future analysis, while the eggs for nutrient analyses were placed on ice and stored at -20C. For larval fish, the unit of replication for RNA was an individual fish, with three replicates placed directly in RNAlater™ for future RNA analysis. RNAlater™ is a tissue storage solution that rapidly permeates tissue to stabilize and protect the integrity of the RNA. However, when we attempted to use RNAlater™ on embryos it created a hypertonic environment, causing them to shrivel; therefore, we continued to use dry ice for the remainder of my experiment.

To analyze egg nutrients, samples were dried at 60°C until reaching a constant mass, weighed, and ground into a fine powder. For body nutrients, whole fish were gutted, dried at 60°C until reaching a constant mass, then ground into a fine powder. For early larval stages, fish were pooled in batches of 5-10 individuals per sample to obtain enough mass for analysis; for larger stages, a sample consisted of a single individual. C and N (one replicate per ground
sample) were analyzed using a FLASH 2000 CN analyzer. Body P content (two replicates per ground sample, because P tends to be more variable and less expensive to measure than body C and N) was determined by digesting samples with HCl to convert P to SRP, which was then assayed with a Lachat autoanalyzer. For Ca content analysis (two replicates per ground sample) dried and ground samples were combusted and dissolved in HCl solution and contents were determined using a Perkin Elmer atomic absorption (AA) spectrophotometer.

To quantify RNA, we used a Promega SV Total RNA Isolation System kit. Whole fish were homogenized in the manufacturer supplied lysis buffer using a Qsonica sonicator. Following sonication RNA contents were manually extracted and purified following manufacturer supplied protocol. Following extraction, the total RNA concentration of each sample was determined using a Nanodrop 2000 (Thermo Scientific). RNA concentration of fish was calculated as % wet mass. RNA samples were taken from all fish species with the exception of the largemouth bass since we were unable to collect eggs from them. Samples were taken at weeks 0, 1, 3, 6 with the exception of channel catfish and rainbow trout, because we were unable to collect a sample at those exact intervals, so the closet week was substituted. These intervals were chosen because they should be good representations of growth and RNA concentration changes in the early development of fish.

Obtaining Ca, P and RNA measurements allowed us to focus on how Ca:P and RNA:P ratios change with development. A correlation between P and Ca will inform how much P is being used in bone formation (Hendrixson et al 2007, Pilati and Vanni 2007). Ca and Ca:P should increase with age, while the RNA:P should decline with age as bones comprise relatively more P and as individual growth rates decline with age. By examining the relationships between these variables, we will develop a more complete understanding of the developmental dynamics of body stoichiometry.

Statistical Analyses:

To analyze differences across species and over time, we organized my data three ways. First, we ran my analyses on untransformed body nutrient concentrations and ratio data over time (age) in weeks. However, because species differ greatly in size but we sampled all species over the same age range, it is possible that ontogenetic changes may vary just because of inherent interspecific variation in size. For example, while we predict that body P will increase greatly with age in all species, the rate of change may be slower in long-lived, large species. Therefore,
to assess general patterns, we also standardized body size and/or elemental content before analyses. To standardize body size, we expressed size (total length (TL)) as a percentage of adult TL. Adult size (average TL for each species) was obtained from FishBase (Froese, R. and D. Pauly. Editors. 2017. FishBase). To standardize elemental content, we expressed it as a percentage of the maximum mean value for that element. For example, body P at a given time was expressed as the maximum mean body P observed for that species. Thus, we examined trends three ways: 1) variation in unstandardized elemental content as a function of age; 2) standardized elemental content (% of maximum) as a function of age; and 3) standardized elemental content as a function of standardized body size (% of adult size). In standardizing the data, we hoped to minimize the influence of species-specific growth rates.

We compared trends in body chemistry and stoichiometry using generalized additive models (GAM). GAMs allow for the fitting of non-linear smoothed (splined) predictor variables, with the smoothing procedure non-parametric nonlinear functions to the data as opposed to pre-defined parameters. We fit models with standardized body composition as the dependent variable and either week or percent of (standardized) adult size as the independent variables, and controlled for hatchery as a random factor. To determine statistically significant differences among species in trends through time or with body size, species was treated as a random factor in the model and tested for interaction with week or percent of adult size. GAMs require comparisons of non-linear patterns to be made to a “reference species”, which was specified as bluegill for these analyses. For the sake of this analysis, we chose bluegill since there is more data published in the literature about bluegill body stoichiometry than any of the other species in our study. We fit GAMs using the mgcv package in the R statistical environment (R Core Team 2015) using the gam() function, and non-linear smoothing was performed using the s() function. The form of the model across time, fit for relative maximum % C, N, P, Ca, RNA and C:N, C:P, N:P, Ca:P are as follows:

**Body composition ~ (Week) + (Week*Species) + (Hatchery±)**

While the form of the model used for body composition across % of adult size is:

**Body composition ~ (% of Adult Size) + (% of Adult Size *Species) + (Hatchery±)**

Results from the GAMs indicated that many of the trends through time and across size were not following the same patterns across species. However, we wanted to take a closer look at what species were significantly different from each other yet, since species in our data set
were at a wide range of adult sizes this presented a problem. So as an alternative approach we chose a subset of the data for which we had the same % of adult size (all individuals 5-8% of adult size) and used ANOVA on the raw data for all body nutrient concentrations to compare differences among species. By using standardized size (5-8% of adult size), it was valid to use the raw data to compare species. All ANOVA results showed significant differences in group means so we then used Tukey’s post-hoc tests to compare differences among species. P-values set at =0.05 for all statistical analyses.

Non-standardized body P was used to predict non-standardized Ca and RNA to explore trends at the species level. We used a multiple regression to assess the relationship among fish body P (relative to standardized body P), fish body calcium, and fish RNA content. Multiple regression also included time as a factor for repeated measures, and hatchery to control for potential differences in fish composition depending on where they were reared.

Results:

Body C generally declined over the course of the experiment, although the pattern of decline varied among species (Figure 2A). Body C in most species was highest during the egg stage and then declined over time and as standardized adult size increased (Figure 2 A, B, C). However, in some soft-rayed species (brown trout, fathead minnow, and channel catfish), body C increased in the early weeks and then declined (Fig. 2A). Standardized body C showed a decline in trout species with trends in channel catfish and fathead minnow being more variable (Figure 2B). Changes in standardized body C for spiny species showed a steeper decline, except for largemouth bass which showed no substantial change over time (Figure 2B). Standardized body C showed similar trends when plotted against standardized adult size, but differences among species were generally less than when plotted against age, especially within each group (soft-rayed and spiny-rayed; Fig. 2C).

Results from the GAM indicated that trends in standardized body C were significantly different across week in all species when compared to the reference species bluegill (P<0.05; Table 2). There was also a significant hatchery effect (P<0.05; Table 2). GAM of standardized body C as a function of standardized adult size indicated that all species were significantly different from bluegill (P<0.05; Table 2) and also indicated a significant hatchery effect (P<0.05; Table 2).
ANOVA of raw body C at 5-8% adult size show significant differences across some species (P<0.05; Figure 3). Body C was highest in rainbow trout (48.6% ± 0.006) followed by brown trout (46.8%±0.03SE) and channel catfish (46% ±0.02SE). Body C was lowest in St. Mary’s saugeye (40% ±0.002SE) and Hebron saugeye (42% ± 0.005SE) (P<0.05; Figure 3). Post-hoc Tukey analysis revealed that rainbow trout body C was significantly higher than body C in both St. Mary’s saugeye and Hebron saugeye while brown trout and channel catfish had a significantly higher body C than St. Mary’s saugeye (P<0.05; Figure 3). No other significant differences in body C was found among the other species (P<0.05; Figure 3).

Body N in general increased over development in all species, at least over the first 5-6 weeks (Figure 4A). Soft-rayed species tended to show increasing and then decreasing N over time, although the relative increase and decrease varied among species (Figure 4A). Bluegill followed a similar trend to the soft-rayed species, while muskellunge steadily increased and largemouth bass stayed steady with a slight decline (Figure 4A). All three species of Percidae showed the greatest increase in body N, and body N stayed high throughout sampling (Figure 4A). Standardized body N followed the same temporal trends as the raw body N but in bluegill, muskellunge, and percids some of the changes were more pronounced (Figure 4B). Standardized body N varied with standardized adult size in a manner similar to the variation in standardized body C with age. Percid species and muskellunge and largemouth bass body N became more similar to each other when plotted against standardized adult size, while (Fig. 4C), while soft rayed species and bluegill did not (Fig. 4C).

The GAM indicated that differences in standardized N across week were not significant in any species except saugeye when compared to the reference species bluegill (P>0.05; Table 2). No significant hatchery effect was found (P>0.05; Table 2). GAM of standardized N across standardized adult size indicated that no species was significantly different from bluegill (P>0.05; Table 2). No significant hatchery effect was found (P>0.05; Table 2).

ANOVA results showed significant species differences in raw N at 5-8% body size (P<0.05; Figure 3). The highest N at this size was found in yellow perch (12.8% ± 0.002SE) followed by some of the other spiny-rayed species; bluegill (12.4% ±0.002SE), muskellunge (12.4%± 0.002SE) and St. Mary’s walleye (12.4% ±0.002SE) (P<0.05; Figure 3). The lowest N was found in channel catfish (10.4% ± 0.008SE) and brown trout (10.8% ± 0.002SE) (P<0.05; Figure 3). Post-hoc Tukey analysis revealed yellow perch, bluegill, muskellunge and St. Mary’s
walleye had significantly higher N than channel catfish and brown trout, while largemouth bass and Hebron walleye had significantly higher N than channel (P<0.05; Figure 3), no other significant differences in body N was found among other species (P<0.05; Figure 3).

Body P in all species increased from egg stage to later stages. In all species, body P started at about 1% during egg stage and increased over development (Figure 5A). In the “soft-rayed” species body P tended to be more variable, increasing and decreasing on individual weeks but generally increasing over time (Figure 5A), whereas body P in “spiny” species steadily increased over time. For most taxa, body P was still increasing over time when our sampling ended (Fig. 5A). standardized body P increased sharply for all species, but soft-rayed species tended to be more variable both within and among species than spiny-rayed species (Figure 5B). Soft-rayed species were highly variable even when standardized body P was expressed as a function of standardized adult size (Fig. 5C). For all spiny species, and for fathead minnow (for which we were able to include individuals >80% of adult size, standardized body P seems to stabilize at the maximum value as fish reach 10-20% of adult size (Figure 5C).

GAM indicated that trends in standardized body P vs. week were significantly different for all species when compared to the reference species bluegill (P<0.05; Table 2). However, there was no significant hatchery effect (P>0.05; Table 2). GAM of standardized body P vs. standardized adult size indicated that all species except fathead minnow were significantly different from bluegill, and that there was a significant hatchery effect (P<0.05; Table 2).

ANOVA of raw body P at 5-8 % adult size showed significant differences in some species (P<0.05; Figure 3). With the exception of the yellow perch highest body P was found in the spinier species, Hebron saugeye and largemouth bass had the highest P (2.8%±0.002SE) while the lowest body P was found collectively in rainbow trout, brown trout, channel catfish, yellow perch and fathead minnow (2% ±0SE). Post-hoc Tukey analysis revealed largemouth bass and Hebron saugeye had significantly higher P than rainbow trout, brown trout, channel catfish, fathead minnow and yellow perch (P<0.05; Figure 3). while no other significant differences in body P was found among other species (P<0.05; Figure 3).

Body Ca increased over time as fish grew but was highly variable among species (Figure 6). Fish species in the “soft-rayed” group showed a wider range of Ca values, both within and among species, while the “spiny-rayed” largemouth bass, muskellunge and bluegill showed an early increase and then leveled off (Figure 6A). Species in the Percidae family had the sharpest
increase in Ca (Figure 6A). Standardized body Ca showed similar overall patterns over time except trends exhibited steeper increases (Figure 6B). When standardized body Ca was expressed as a function of standardized size, the soft-rayed species and percids showed very similar patterns, increasing sharply over time and then leveling off; in contrast, body Ca of non-percid spiny species varied less over time (Figure 6C).

GAM on standardized Ca vs. week indicated that all species except brown trout, channel catfish, largemouth bass and yellow perch were significantly different from bluegill (P<0.05; Table 2). There was a significant hatchery effect on max Ca vs week (P>0.05; Table 2). GAM on standardized Ca vs standardized size indicated that all species except fathead minnow and rainbow trout were significantly different from bluegill (P<0.05; Table 2). There was no significant hatchery effect on max Ca vs standardized adult size (P>0.05; Table 2).

ANOVA of raw body Ca at 5-8% adult size showed significant differences in some species (P<0.05; Figure 3). Highest body Ca was found in channel catfish (8.8% ± 0.005SE), St. Mary’s saugeye (7.2% ± 0.006SE) and brown trout (6.8 % ± 0.008SE), while the lowest was found in bluegill (2.6%± 0.006SE) and Hebron walleye (2.8%±0.002SE). Post-hoc Tukey analysis revealed channel catfish body Ca was significantly higher than all species except St. Mary’s saugeye and brown trout (P<0.05; Figure 3), while St. Mary’s saugeye and brown trout had significantly higher Ca than bluegill, Hebron saugeye and Hebron walleye and largemouth bass (P<0.05; Figure 3). No other significant differences in body Ca was found among other species (P<0.05; Figure 3).

Body C:N decreased over time and with increasing size (Figure 7A). For spiny species, there is a steep decline in body C:N followed by a leveling off, while in the soft-rayed species the decline is more gradual (Figure 7A). Of the spiny species, members of the percid family had the steepest decline in C:N (Figure 7A). Body C:P decreased over time in all species with the exception of the trout species and channel catfish, for which C:P was very variable (Figure 7B). Body N:P decreased over time in all species with the exception of brown and rainbow trout (Figure 7C). The trend in trout N:P is highly variable, similar to trout C:P, indicating that trends in P are driving the relationships (Figure 7B,C). Ca:P ratios tended increase over time and with increasing body size in most species with the exception of channel catfish and fathead minnows (Figure 7D). Channel catfish Ca:P decreased over time and increasing size, while fathead
minnows Ca:P increased from embryo to larvae then decreased for several weeks until leveling off around week 6. (Figure 7D).

Results from GAM indicated that trends in C:N ratios were significantly different across week in all species except largemouth bass when compared to the reference species bluegill (P<0.05; Table 2). There was also a significant hatchery effect on CN ratios across week (P<0.05; Table 2). GAM on C:N ratios across standardized size indicated that all species except fathead minnow and channel catfish were significantly different from bluegill (P<0.05; Table 2). There was also a significant hatchery effect on C:N vs standardized size (P<0.05; Table 2). C:P ratios across week in all species except largemouth bass were significantly different when compared to bluegill (P>0.05; Table 2), and there was no hatchery effect. GAM of C:P across standardized size indicated that all species except fathead minnow were significantly different from bluegill (P<0.05; Table 2). There was also a significant hatchery effect on C:P vs standardized size (P<0.05; Table 2). Trends in N:P ratios were significantly different across week in all species except largemouth bass when compared to the reference species bluegill (P<0.05; Table 2); however, there was no significant hatchery effect (P>0.05; Table 2). N:P ratios were significantly different across standardized size in all species except fathead minnow when compared to the reference species bluegill (P<0.05; Table 2). There was also a significant hatchery effect on N:P vs standardized size (P<0.05; Table 2). Ca:P ratios were significantly different across week in all species except walleye and saugeye when compared to the reference species bluegill and there was a significant hatchery effect (P<0.05; Table 2). Ca:P ratios were significantly different across standardized size in all species except walleye and saugeye when compared to the reference species bluegill (P<0.05; Table 2). There was also a significant hatchery effect on Ca:P vs standardized size (P<0.05; Table 2).

ANOVA on body C:N at 5-8% adult size showed significant differences for some species (P<0.05; Figure 3). Channel catfish had the highest C:N (4.43% ±0.11SE) followed by brown trout (4.35% ± 0.24SE) and rainbow trout (4.206%±0.06SE) while the other species had lower values (3.4%±3.7SE). Channel catfish and brown trout had significantly higher C:N compared to all species except rainbow trout, while rainbow trout was significantly different from all species except channel catfish, brown trout, and fathead minnow (P<0.05; Figure 3), while no other significant differences in C:N was found among other species (P<0.05; Figure 3). C:P differed significantly across some species (P<0.05;Figure 3). Highest C:P was found in rainbow
trout (73.1%±1.91SE) and brown trout (63.4% ± 2.93SE); while the lowest values were found in Hebron saugeye (39.47% ±1.59SE) and St. Mary’s saugeye (39.64% ± 0.53SE). Rainbow trout had significantly higher C:P than all species except brown trout (P<0.05; Figure 3), while brown trout had significantly higher C:P than Hebron and St. Mary’s saugeye and walleye, bluegill and largemouth bass (P<0.05; Figure 3). Fathead minnow and yellow perch had significantly higher C:P than Hebron and St. Mary’s saugeye, largemouth bass, bluegill and Hebron walleye (P<0.05; Figure 3). Channel catfish and muskellunge had significantly higher C:P than Hebron and St. Mary’s saugeye (P<0.05; Figure 3), while no other significant differences in body C:P was found among other species (P<0.05; Figure 3). Body N:P was significantly different across some species (P<0.05; Figure 3). N:P was highest in rainbow trout (14.9% ± 0.46SE) followed by yellow perch (14.13% ± 0.47SE) and fathead minnow (13.35% ± 0.91SE). N:P was lowest in Hebron and St. Mary’s saugeye (9.36%± 0.40SE; 9.81%± 0.17SE). Rainbow trout N:P was significantly higher than all other species except brown trout, fathead minnow, yellow perch and muskellunge (P<0.05; Figure 3), yellow perch was significantly higher than Hebron and St. Mary’s saugeye, largemouth bass, channel catfish and bluegill (P<0.05; Figure 3). Fathead minnow and muskellunge N:P was only significantly higher than Hebron and St. Mary’s saugeye (P<0.05; Figure 3), while brown trout was only significantly higher than Hebron saugeye (P<0.05; Figure 3). ANOVA results for body Ca:P indicated the highest Ca:P at 5-8% of adult size was found in the “softer rayed” species. Channel catfish had the highest Ca:P (6.2%± 0.46SE) followed by brown trout (5.6%± 0.68SE), rainbow trout (4.9%± 0.09SE) and fathead minnow (4.29%± 1.4SE). While lower Ca:P ratios were found in the bonier species with the lowest Ca:P found in bluegill (1.6%± 0.38SE). Channel catfish Ca:P was significantly higher in Hebron saugeye, bluegill, largemouth bass, Hebron walleye, muskellunge and yellow perch (P<0.05; Figure 3). Rainbow and brown trout were significantly different from Hebron saugeye bluegill largemouth bass and Hebron walleye (P<0.05; Figure 3). Fathead minnow was significantly different from Hebron saugeye (P<0.05; Figure 3). No other significant differences in body Ca:P was found among other species (P<0.05; Figure 3).

RNA increased from egg to freshly hatched larvae through week 3, and then declined in most species (Figure 8). Muskellunge RNA was still increasing at week 6 when sampling stopped (Figure 8). Changes in RNA body concentration were most dramatic in soft-rayed species and Percidae, while muskellunge and bluegill had much more modest changes (Figure
Standardized RNA followed similar trends with age, but the changes between weeks were more dramatic (8B). Expressed as standardized RNA as a function of standardized size, the overall trend was that RNA increased with size, and for most species growing closer to >15% of adult size during the study (fathead minnow, bluegill, and yellow perch), RNA then declined dramatically (Figure 8C).

Results from the GAM indicated that trends in RNA concentrations were significantly different across week in all species except brown trout, rainbow trout, and muskellunge when compared to the reference species bluegill (P<0.05; Table 2). RNA concentrations were only significantly different for standardized size in fathead minnow and saugeye when compared to the reference species bluegill (P<0.05; Table 2).

Non-standardized body P was used to predict non-standardized Ca and RNA to explore trends at the species level (Figure 9A). Trends in P vs. Ca and RNA varied by species however, for each species only Ca or RNA were significant. Results from multiple linear regression using standardized data indicated that Ca, RNA, week and hatchery explained 70% of the variation in P (Table 3; Figure 9 B). Ca, RNA, week, and hatchery were statistically significant (Table 3). P was positively correlated with Ca, RNA and week but negatively correlated with hatchery (Table 3).

**Discussion:**

*Elemental changes during development*

The goal of my thesis was to explore the ontogenetic changes in body stoichiometry that occurs during development. To address that goal we characterized body elemental changes over early development in ten different species of fish. Specifically, our objectives were to see if body elemental composition changed significantly with time and if interspecifically there were different patterns of change. In order to address that goal, we tested two hypotheses. For my first hypothesis we wanted to investigate what the catalyst for stoichiometric change was over early development, i.e. are changes due to growth rate and high P demands needed for building bones and/or RNA? We predicted that as all fish species grew, the development of bones and scales would result in increased whole-body P and Ca levels. In support of our prediction, body P and Ca increased across all species as they grew from embryo stage (Figure 5,6). Also, we found using multiple linear regression, standardized P was significantly and positively correlated with Ca and RNA supporting our predictions that as fish grow and develop bones and scales Ca and
RNA are an important pool of P matching findings from (Pilati and Vanni 2007 and Boros et al 2016) (Figure 9). We also predicted that the P and Ca concentrations would start out low at the egg stage and increase rapidly, and that changes in Ca and P would universal across all the species. While this was true for P, it was not the case for Ca, which changed much more modestly and was highly variable across species (Figure 5,6). Only a few studies have examined ontogenetic changes in body composition of fish, but some of these include some of the same species we studied. Showalter et al (2016) studied bluegill sunfish stoichiometry over early development in a eutrophic lake, and found trends in body Ca and P similar to ours. Showalter et al (2016) found that the timing of rapid change in body P and Ca occurred around 15-20mm total length, in our study we found that bluegill experienced rapid changes in body P and Ca around weeks 2-3 and during that time they were between 12-16mm (Figure 5,6). Similarly, Boros et al (2015) observed trends similar to those we observed for fathead minnow. Boros et al (2015) found a spike in P after post embryo to larval stage this corresponds with our finding of an increase in P at weeks 2 and 3, our Ca findings also mirrored Boros et al(2015). Ca from embryo to larvae and then highly variable concentrations after that (Figure 6).

Our second prediction of our first hypothesis was that as body P and Ca increase, body C and N should decrease. Body C did decrease during development; in most species, it was highest during egg stage, then declined, after which it was relatively consistent in the “spiny” fish while being more variable in the “soft” species (Figure 2). Over all C trends matched predictions and the overall decline could likely be due to fish metabolizing C stored as lipids while not consuming enough excess C to store more. Comparing our results to Boros et al (2015) we found that our % C in FHM followed the same general trends, however, the over all C content was lower in our experiment. Bluegill C trends post embryo followed that of Showalter et al (2016) (Figure 2). Body N trends did not follow our predictions; rather, it generally increased during development in most species and then leveled off, eventually declining in some species (Figure 4). Body N in bluegill followed different trends from the other spiny-rayed fish in our study but had a similar trend in body N (an increase then a decline) that was observed for bluegill in a eutrophic lake (Showalter et al. 2016). Trends we observed in body N and P also do not follow stoichiometric predictions that these two elements should trend in opposing directions, as P increases with body size (Sterner and Elser 2002, Vanni 1996 and Davis and Boyd 1978); although body P clearly increased with body size, we did not observe the predicted
corresponding decrease in body N (Figure 4,5). Our findings could be due to the short time period that we examined; negative correlations between body N and P derive from studies that include adults, so perhaps a wider range of ages would have revealed the predicted negative correlation. Indeed, in most species, body elemental composition seems to be stabilizing with increasing size.

Our third set of predictions, involving body nutrient ratios, was partially supported. We predicted that we would observe decreasing C:N, N:P, and C:P and increasing Ca:P. C:N decreased with age matching our initial prediction (Figure 7). The decline in C:N was driven by decreasing body C and increasing N. These declining trends were supported by literature in similar studies, Showalter et al (2016) found that bluegill C:N decreased until bluegill were around 25mm, bluegill in our study declined until week 4 when they were at an average total length of 20mm (Showalter et al 2016) Pilati and Vanni (2007) and Boros et al (2015) also found declining C:N in early stages, and similar to Boros et al (2015) our C:N patterns in fathead minnow were quite variable (Figure 7). C:P and N:P generally decreased with development in all species, in agreement with previous studies (Showalter et al 2016, Boros et al 2015, Pilati and Vanni 2007). Declining trends in N:P seem to be driven mostly by increases in P, most likely due to bone/scale formation. Ca:P generally increased with size, and over development, but was much more variable. This increase is most likely due to bone/scale formation and development (Boros et al 2015, Pilati and Vanni 2007). The leveling off of the Ca:P ratios, especially in the spiny species, could indicate that the biggest pool of P is bone (Boros et al 2015, Pilati and Vanni 2007) (Figure 7). The trends we observed in Ca:P, C:P and N:P in fathead minnow and bluegill agreed with those in Showalter et al (2016) and Boros et al (2015).

Since our study was based in a hatchery setting it is important to note that fish were not food limited, while this allowed us to explicitly look at changes during ontogeny and across species without external environmental factors it could have implications for understanding development in systems where food ration size and quality/composition vary. Since fish in this study were fed high quality food at optimal levels this could have resulted in higher lipid storage and a “dilution effect” on the other elements (N, P, Ca). In a more natural setting the varying quantity and quality of food could play a role and account for some of the variation seen in changing elemental body composition.
Our last prediction for our first hypothesis was that RNA content would increase dramatically from embryo stage to freshly hatched larvae and then decline over time as fish growth slowed. This prediction was partially supported as RNA did increase from embryo to freshly hatched larvae but changes were highly variable across species and at the end of sampling for RNA; in some species, RNA concentrations were still increasing, or at least had not declined greatly, when sampling ended (Figure 8). This could likely be due to that fact that all fish (with the exception of bluegill) were still growing (adding mass) throughout the entire course of our study (Supplemental Figure 1).

**Elemental changes across species**

Our second hypothesis focused on the interspecific variation in body composition over time. We predicted that within the general developmental patterns predicted for changing body composition, there would be significant interspecific variation based on different species of fish within different families having different morphological features (i.e. ray and scale type, body shape). This hypothesis was partially supported. Results from the GAMs across week indicated that trends over time in most standardized body elements and ratios all differed significantly among species. This was an interesting finding and implies that even as changes in elemental content and changes based on size differences were accounted for; there were differences between species and our reference species (bluegill). Exceptions were N content and Ca:P ratio. While Ca:P content of all species except brown trout, walleye, and saugeye differed significantly from bluegill, Ca:P did not vary significantly over time. Saugeye was the only species that had a significantly different N content than bluegill but did not vary with time for any species. N GAM that included standardized adult size showed no significance across time, hatchery or species.

Using standardized RNA data by standardized size we found that only saugeye and fathead minnow were significantly different than our reference species bluegill. This was not surprising as RNA production should be a strictly regulated process and high RNA levels are needed during periods of high growth (Elser et al. 1996, 2003). We did find that standardized RNA varied significantly by week and brown trout, fathead minnow, muskellunge, saugeye, walleye and yellow perch were significantly different than our reference species.

This section of our discussion focuses on our ANOVA findings when species were 5-8% adult size. In support of our second hypothesis, body C content did not vary significantly among
species within the same families, however, it did vary significantly across species from different families. The highest C content was found in the soft-rayed species of brown trout, rainbow trout, and channel catfish, while the lowest was found in the spiny-rayed saugeye (Figure 3). Species from the ictalurid and salmonid families differed significantly from esocids and percids. These findings are also supported by the literature. Hendrixson et al (2007) found that the ictalurid black bullhead (Ameiurus melas) tended to become more C rich as they grew while bonier species became C poor. Body N data also supported my hypothesis, as salmonid and ictalurid species differed from the spinier families while interestingly fathead minnow species was not significantly different from spiny species (Figure 3). Body P results matched our predictions, with the exception of yellow perch. Body P was higher in all of the spinier species except yellow perch (Figure 3). Yellow perch at 5-8% of adult size is still very small 10mm so bones and scales have not yet ossified, while the other species of percids would have. Body Ca varied the most across species and families and there were no clear trends in the ANOVA nor in the GAMs that indicated significant differences between spiny vs soft-rayed species. Finally, soft-rayed families had higher C:nutrient, N:P and Ca:P ratios than their spinier counterparts this is likely being driven by trends in P and supports findings in other studies on adult species (Sterner and George 2000, Vanni et al. 2002, Hendrixson et al. 2007, McIntyre and Flecker 2010) (Figure 3). Although these findings only represent a snapshot in time (5-8%) of size they still show interspecific differences in development and are supported by findings from the GAMs indicating that there are significant elemental differences across species.

**Conclusion:**

In summary, we found evidence that fish elemental stoichiometry varies over development. Our results suggest that while there are some interspecific differences in development, variation through time is a more important predictor of changing elemental composition. While it is hard to compare our findings to other studies based on the limited amount of data out there, it is important to note that we did find some similarities to other studies (Pilati and Vanni 2007, Boros et al 2015, Showalter et al 2016, Leal et al 2017) indicating that fish do not follow strict species-specific elemental homeostasis during ontogeny. This may suggest that changing elemental trends could be driven by interspecific differences in lipid storage, muscle and bone development. In a broader sense, our findings highlight the dynamic changes that occur as fish develop from embryos during their early life stages. While our study
was based in a hatchery setting it can have implications for natural aquatic environments as developing fish experience rapid growth, changing nutrient dynamics and can experience high mortality (Boros et al 2015) thus playing roles as important nutrient sources and sinks.
References


Figures

**Figure 1**

- A: % Body C vs. Age
- B: % Body N vs. Age
- C: % Body P vs. Age
- D: % Body Ca vs. Age
- E: C:N vs. Age
- F: C:P vs. Age
- G: N:P vs. Age
- H: Ca:P vs. Age
- I: % RNA vs. Age
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Figure 3
Figure 4
Figure 5
Figure 6
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Figure 8
Figure 9
Supplemental Figure 1
### Tables

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**Table 1**
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| Model | Cmax ~ (% of adult size) + (% of adult size*Species) + (Hatchery±) | Model Parameters | <2e-16 | <2e-16 | 4.65e-16 | 2.34e-13 | <2e-16 | <2e-16 | <2e-16 | <2e-16 | <2e-16 |
|-------|------------------------------------------------------|-------|-------|-------|----|-----|-----|-----|-----|-----|
| % of Adult Size | | | | | | | | | | |
| Brown Trout | <2e-16 | 2.29e-09 | 1.65e-06 | 3.35e-06 | 3.63e-12 | <2e-16 | <2e-16 | <2e-16 | <2e-16 | <2e-16 |
| Channel Catfish | 2.26e-05 | 0.0016 | 1.18e-06 | | | 4.34e-11 | 1.13e-09 | 4.52e-12 | 0.0397 |
| Fathead Minnow | 7.68e-09 | 0.0306 | | | | | | | |
| Largemouth Bass | <2e-16 | <2e-16 | 0.000534 | | | 4.22e-05 | 1.77e-08 | 5.14e-07 | 6.00e-05 | <2e-16 |
| Muskellunge | <2e-16 | 2.92e-09 | 1.14e-12 | | | 4.30e-06 | 0.000031 | 0.002712 | 0.0371 | <2e-16 |
| Rainbow Trout | 2.27e-06 | 9.83e-13 | | | | | | | |
| Saugeye | <2e-16 | 1.76e-07 | 0.001628 | 6.24e-06 | 2.56e-09 | 0.000016 | 0.018527 | 1.98e-07 | 6.00e-05 |
| Walleye | <2e-16 | 2.16e-15 | 1.73e-09 | | | 2.01e-11 | 2.28e-11 | 3.00e-06 | 3.00e-06 | <2e-16 |
| Yellow Perch | 6.64e-07 | 3.52e-07 | 9.00e-05 | | | 0.00053 | 0.04300 | 0.000511 | 0.0219 | <2e-16 |
| Hatchery | <2e-16 | 0.0167 | | | | | | | |

Table 2
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<th>P-Value</th>
<th>R²</th>
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<td>-0.0033</td>
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Table 3
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**Supplemental Table 1**