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Ward, D, Schroeder, L, Pomeroy, E, Roy, J, Buck, LT, Stock, J, Martin-Gronert, M, Ozanne, S, Silcox, M and Viola, B (2021) Early life malnutrition and fluctuating asymmetry in the rat bony labyrinth. The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology, 304

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Early life malnutrition and fluctuating asymmetry in the rat bony labyrinth

Short title: Malnutrition and bony labyrinth shape

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Keywords: Developmental instability, inner ear, bony labyrinth, phenotypic plasticity, isocaloric protein-restricted diet, bilateral asymmetry

Grant Support Information:
University of Toronto PhD Pilot Research Grant to DLW
NSERC Discovery Grant to MTS
SSHRC Insight and Connaught New Researcher Grants to BV
Henry Sidgwick Research Fellowship from Newnham College, Cambridge to EP
European Research Council Grant 617627 to JTS

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request to DW and EP.

Ethics Approval: Approval from the University of Toronto Ethics Review Board was not required for this study, as the samples were deceased prior to data collection.

Abstract:

Maternal malnutrition during gestation and lactation is known to have adverse effects on offspring. We evaluate the impact of maternal diet on offspring bony labyrinth morphology. The bony labyrinth develops early and is thought to be stable to protect vital sensory organs within. For these reasons, bony labyrinth morphology has been used extensively to assess locomotion, hearing function, and phylogeny in primates and numerous other taxa. While variation related to these parameters has been documented, there is still a component of intraspecific variation that is unexplained. Although the labyrinthine developmental window is small, it may provide the opportunity for developmental instability to produce corresponding shape differences, as measured by fluctuating asymmetry (FA). We hypothesized that (1) offspring with poor maternal diet would exhibit increased FA, but (2) no unilateral shape difference. To test these hypotheses, we used two groups of rats (*Rattus norvegicus*; Crl:WI[Han] strain), one control group and one group exposed to a isocaloric, protein-restricted maternal diet during gestation and suckling. Individuals were sampled at weaning, sexual maturity, and old age. A Procrustes ANOVA identified statistically significant FA in all diet-age subgroups. No differences in level of FA were identified among the subgroups, rejecting our first hypothesis. A principal components analysis identified no unilateral shape differences, supporting our second hypothesis. These results indicate that bony labyrinth morphology is remarkably stable and likely protected from a poor maternal diet during development. In light of this result, other factors must be explored to explain intraspecific variation in labyrinthine shape.

Introduction

The bony labyrinth is the ossified structure surrounding the organs of the inner ear (Gray, 1918). These organs sense sound, motion, and angular acceleration and correspond with the cochlea, vestibule, and semicircular canals in the bony labyrinth (Milton, 1808) (Figure 1). The function of these organs is thought to be related to the shape of the surrounding bone (Ifediba et al., 2007).

Interspecific variation in bony labyrinth shape related to locomotion, agility, and phylogeny has been documented across many species (Kirk and Gosselin-Ildari, 2009; Malinzak et al., 2012; Perier et al., 2016; Pfaff et al., 2017, 2015). Some of these findings have been used to infer aspects of the behavior of extinct species (e.g., Bernardi and Couette, 2017; Ryan et al., 2012; Silcox et al., 2009; Spoor et al., 1994; Walker et al., 2008). For example, a greater semicircular canal radius relative to body mass has been found to correspond with greater agility in primates and primate relatives (Spoor et al., 2007; Spoor and Zonneveld, 1995, but see Rae et al., 2016), and some inner ear shape changes in human ancestors are hypothesized to correspond with adaptations for bipedalism (Le Maître et al., 2017; Spoor et al., 1996, 1994). This work has often been based on very small sample sizes within particular species, with patterns of intraspecific variability in the bony labyrinth only starting to be appreciated (e.g., Billet et al., 2012; Gonzales et al., 2019; Schellhorn, 2018). With respect to hominins, it is possible that differences identified among and within closely related groups, such as Neanderthals and recent *Homo sapiens* are non-functional (Beals et al., 2016; Li et al., 2017; Spoor et al., 2003; Uhl et al., 2016; Wu et al., 2014). Sexually dimorphic bony labyrinth variation is one example of non-functional

shape variation within recent humans that has been long debated (Osipov et al., 2013; Spoor and Zonneveld, 1998; Uhl et al., 2020; Ward et al., 2020). More recent research indicates that other non-functional morphological variation is linked to population movement and dispersal out of Africa (Ponce de León et al., 2018). These studies have highlighted the existence of diverse factors influencing both inter- and intra-specific bony labyrinth variation.

The combination of developmental instability and plasticity has been under-explored with respect to its potential to produce bony labyrinth shape diversity through ontogeny. In humans, the bony labyrinth has been found to reach adult shape and size at around 19 weeks gestation (Jeffery and Spoor, 2004), the earliest of any region in the skeleton. Once adult morphology is achieved, the bone surrounding the labyrinth is known to remodel at rates significantly lower on average than those in the rest of the skeleton (Bloch and Sørensen, 2010; Frisch et al., 2000; Sørensen, 1994; Sørensen et al., 1992). The mechanism slowing remodeling is thought to protect the sensory functions of the ear by maintaining a static morphology throughout the lifetime of the individual (Jáuregui et al., 2016; Zehnder et al., 2006). Therefore, the bony labyrinth's short window of development, followed by relative stability, should mean that its shape is most susceptible to developmental and environmental perturbations during gestation, within the maternally regulated environment.

Stability early in development is thought to correspond with later life health outcomes (Barker et al., 1993, 1989; Barker and Osmond, 1988), suggesting that stability early in development would correspond with later life health outcomes. The Thrifty Phenotype Hypothesis proposes

a connection between poor fetal and infant nutrition and later development of type 2 diabetes and cardiovascular disease (Barker et al., 1993; Hales and Barker, 2001), but other related side effects and non-metabolic organ systems are less explored. Hales and Barker (2001) suggested that poor fetal nutrition, such as protein restriction, alters how energy is allocated to various systems during development, largely to protect the brain, at the expense of other organs. This results in changes in kidney, pancreas, and liver function, as well as in hormonal and sympathetic nervous system regulation, and in adipose tissue (Antonow-Schlorke et al., 2011; Barker, 1998). These changes may serve as adaptations that are beneficial to survival in continued potential poor nutrition after weaning and into adulthood, but become maladaptive in good nutritional circumstances (Hales and Barker, 2001). Some of these adaptations can be reversed if nutrition is restored at specific points of early development, or individuals are only exposed to nutritional deficits for a short period of time. However, exposure until weaning leads to permanent functional and physiological changes and the underlying mechanisms are still being explored (e.g., Berardino et al., 2020; Crowder et al., 2019; Fernandez-Twinn et al., 2005; McKerracher et al., 2019). The effects of suboptimal maternal nutrition on the human bony labyrinth, in particular, are currently unknown.

In lieu of human data, which cannot be collected ethically, model organisms are an excellent alternative where developmental environments can be controlled experimentally. Rodents are already used both for models of cranial growth (Hallgrímsson and Lieberman, 2008) and for models of fetal malnutrition (Boersma and Tamashiro, 2015). Developing rodent basicrania have been shown to exhibit similar patterns of integration and reactions to environmental

perturbations as the human basicranium, and therefore have been proposed as good models of human cranial development in human evolutionary biology (Hallgrímsson and Lieberman, 2008). In particular, rats have also been shown to replicate "brain-protecting" mechanisms associated with the Thrifty Phenotype Hypothesis (Barbiro-Michaely et al., 2007). With analogous function, anatomy, and reduced bone remodeling, the rat bony labyrinth is also good model for humans (Blanks and Torigoe, 1989; Gray, 1907; Hunt, 1924; Li et al., 2015; Sørensen et al., 1990). The vestibular system is the earliest functioning sensory system in rats, reaching full function prenatally (Gottlieb, 1971). The membranous labyrinth reaches maturity at 17 days gestation (Morsli et al., 1998), but the inner ear signals biochemically like that of an adult at 17-19 days old (Meza et al., 1996). Vestibular function and full locomotor skill set is completely developed by 22 days old (Meza et al., 1996; Morsli et al., 1998).

As with humans, poor gestational conditions in rats have been found to be detrimental to offspring in a diversity of systems, resulting in various coping pathologies (Boersma and Tamashiro, 2015). Litters of mothers exposed to some external stress while gestating, such as the presence of a cat or a restraint, are known to be negatively affected (Barlow et al., 1978; Braastad, 1998). These effects range from smaller litters and sex ratio changes, to a reduction of offspring body size and various developmental delays. Although neurological delays vary depending on the timing and predictability of the changing variable (Friede and Weinstock, 1984), deficits in vestibular function, including impairment of balance and movement, are common. Offspring exposed to stress during gestational development miss neurological development milestones (Mesquita et al., 2007), specifically those related to motor function

development. Offspring then will also exhibit stunted motor function at adulthood (Patin et al., 2004). Specifically, protein deficiency (Woodward, 1998) during gestation results in smaller body size, gene expression changes in the liver and muscle tissue, hyperinsulinemia, hypertension, severe brain development delay, and endocrine changes (Almeida and Mandarim-de-Lacerda, 2005; Boersma and Tamashiro, 2015; Dahri et al., 1991; Fernandez-Twinn et al., 2005; Gonzalez et al., 2014; Gressens et al., 1997; Langley-Evans et al., 1999, 1996; Mortensen et al., 2010). Individuals can recover from side effects, such as neurological delays, if exposure to protein restriction is limited in time (Dahri et al., 1991; Gressens et al., 1997).

Protein deficiency has been shown to produce different effects on skeletal development in rats than caloric deficiency (Miller and German, 1999). Such deficiencies do not change the mineral content of bone, but do change the shape of endochondral bone, largely as a reduction in cortical thickness in long bones (Glick and Rowe, 1981) and as a delay in bone and tooth growth overall (Bonjour et al., 2001; Diorio et al., 1973; Nakamoto and Miller, 1977). These effects are largely the same as seen in protein deficient developing humans, a global challenge identified by the World Health Organization (WHO “e-Library of Evidence for Nutrition Actions”). More specifically, protein restriction has been shown to cause small but significant changes in overall cranial size in rats, while altering the growth trajectory of cranial measurements much more strongly after birth, causing more changes in the face than in the neurocranium (Miller and German, 1999). The rat skull and long bones may be affected differently by protein restriction on the cellular level and depending on when protein is restricted (Nakamoto and Miller, 1977).

The effect of prenatal maternal protein deficiency on the bony labyrinth has not been studied before.

Fluctuating asymmetry (FA) is a good indicator of an organism's ability to handle genetic and environmental stress (Palmer and Strobeck, 1992, 1986; Tomkins and Kotiaho, 2002; Valen, 1962). Fluctuating asymmetry is one of several types of asymmetry that can be quantified as random deviations from perfect symmetry. These deviations away from the midline in bilaterally symmetric species increase with less-than-ideal environments, when individuals are exposed to nutritional, disease, or emotional stress (Palmer and Strobeck, 1992). Quantified as the difference between the left and right from the midline, or between left and right structures, FA can be represented as a normal or even leptokurtic (with a greater concentration around the mean) distribution (Tomkins and Kotiaho, 2002). Unlike other types of asymmetry, such as directional symmetry, FA is not thought to have an adaptive advantage (Palmer and Strobeck, 1992; Thornhill et al., 1999; Valen, 1962), but instead can act as a marker of growth constraint that may actually be disadvantageous if affecting function or attractiveness to potential mates (Hallgrímsson, 1998; Thornhill et al., 1999). As with growth stunting, FA has been used to assess poor environmental conditions during development in humans as well as model organisms (DeLeon, 2007; Hallgrímsson et al., 2003; Mooney et al., 1985; Ozener, 2010; Willmore et al., 2005).

In summary, the effects of maternal malnutrition during gestation warrant further exploration because of the detrimental long-term consequences on offspring, human or otherwise. The

effects of protein restriction, a widespread form of malnutrition in humans today (WHO "e-Library of Evidence for Nutrition Actions"), have not been explored in the bony labyrinth. However, with its short and early development, the bony labyrinth has the potential to be a marker of early life stress, in a structure with critical sensory function. Conversely, a better understanding of the impact of developmental perturbations on the bony labyrinth may help to explain the intraspecific variation seen in this part of the skeleton.

In the following study, we examine the impact of gestational and lactational malnutrition on offspring bony labyrinth morphology through unilateral shape differences and FA, using a rat model. Due to previously identified preference for brain and skull protection from poor nutrition, in combination with the important function of the vestibular system, we propose that the morphology of the labyrinth will be buffered from adverse developmental conditions. Specifically, we hypothesize that the labyrinthine morphology of offspring with mothers fed a control diet will not differ in shape or size from those whose mothers were provided a protein-restricted diet throughout gestation and during early development. However, we hypothesize that protein-restricted offspring will have more FA than control offspring. Fluctuating asymmetry levels quantify environmental stress, but also should not impact labyrinthine function (Hallgrímsson et al., 2003; Palmer and Strobeck, 1986).

Materials and Methods

Study Sample

Data in this study include 67 male Stock Wistar Han rats (*Rattus norvegicus*; Crl:WI[Han] strain) as a model organism. All rats studied here are offspring of two groups of mothers bred for a study on insulin production and diabetes (Martin-Gronert et al., 2016). This initial study was conducted in accordance with Home Office Animals (Scientific Procedures, UK) Act 1986 and approved by the University of Cambridge Animal Welfare and Ethical Review Board. Male rats were purchased from Charles River (Tranent, UK) and dams were bred in-house. All individuals were housed in specific pathogen free, ventilated cages with environmental enrichment, at 22°C with a 12-hour light to dark cycle. Day 1 of gestation was marked at the expulsion of the virginal plug, after mating.

The control group of dams was fed a standard diet (20% protein, wt/vol.) throughout gestation and up until the offspring reached 12 days old (weaning). The experimental group was fed a protein-restricted diet (8% protein, wt/vol.) throughout gestation and during suckling. At birth, lactating dams in the experimental group were continued on the protein-restricted diet. At weaning, the offspring were separated from the mothers and all individuals were placed on the standard diet (LAD1 diet, Special Diets Services, Witham, UK) regardless of group. All diets were fed *ad libitum*. Individuals from each litter were sacrificed by CO_2 asphyxiation and decapitation at 22 days (immediately after weaning), at approximately three months old (at adulthood or sexual maturity), and at 15 months (at "old age") (Figure 2). Following analyses of body composition, weight, length and insulin signalling proteins (Martin-Gronert et al., 2016), the rats were dissected and frozen for long-term storage.

All rat heads were separated and μCT scanned at the Cambridge Biotomography Centre in May and June of 2016 by DLW, with assistance from LTB, JTS, and EP. Scanner parameters were 116-140 kv and 78-125μA. The resulting scans have isotropic voxels with sizes ranging between 17μm^3 and 29μm^3, depending on the size of the specimen. Because the samples were not living when scanned, scanning and shape data collection did not need to be approved for study by the University of Toronto Research Ethics Board. In some cases, individuals included in the original experiments could not be included in the final sample because the temporal was damaged during dissection. Only individuals with bilaterally complete bony labyrinths are included here. A total of 67 individuals were processed, 37 from the protein-restricted group and 30 from the control group (Table 1).

Data Collection

All CT data were processed in Avizo® 8.0 (FEI, Hilsboro, USA). The volume of both right and left bony labyrinths were segmented from the cranium of each individual by JR using the half-maximum-height (HMH) principle, which has been shown to be reliable for visually distinguishing bone density material from air density material in μCT data (Gunz et al., 2012; Spoor, 1993). Surface files were generated from the segmented volume and transferred to Meshlab (Cignoni et al., 2008). In Meshlab, each labyrinth surface file was cropped, cleaned, and repaired as needed. Then the surfaces were smoothed using the Laplacian Smooth filter (smoothing steps: 3) to remove surface roughness while minimizing the effect on morphological features and overall volume. The smoothed surfaces were returned to Avizo and translated back into a volume. At this point, the Skeletonization module was applied, reducing the

labyrinthine volume to its midline structure (Figure 1), following the protocol published by Gunz and colleagues (2012).

Semilandmarks were placed on the labyrinthine skeleton based on the protocol published by Gunz and colleagues (2012). Sets of sliding semilandmarks were placed along the anterior semicircular canal (ASC), the common crus (CC), the posterior semicircular canal (PSC), and the lateral semicircular canal (LSC), moving counterclockwise from the ampulla of the ASC to the posterior end of the LSC (Figure 1). JR collected all semilandmark data. To assess intra-observer error, JR repeated landmark collection three times for one individual per diet and age group, with each data collection bout occurring more than a week apart. Each semilandmark set was exported as an ASCII lineset, resampling each set of semilandmarks to 100 per structure, equidistant. As the bony labyrinths are two separate bilateral structures, they exhibit matching and not object symmetry (Mardia et al., 2000). Therefore, to compare the right and left sides, the Z dimension of each left-side configuration of semilandmarks was multiplied by negative 1. This mirrors the configuration and facilitates morphological comparison between the left and right sides (Klingenberg and Leamy, 2001). All semilandmark data were saved and formatted as Morphologika files (Adams and Otárola-Castillo, 2013) for processing in R Studio (R version 3.5.2 “Eggshell Igloo”, RStudio Team, 2020) and in MorphoJ (Klingenberg, 2011).

Data Processing & Assessment

Before statistical testing, intra-observer error was assessed in unilateral semilandmark data. To do so, the mean landmark standard deviation (Table 2) was calculated as described in von

Cramon-Taubadel and colleagues (2007). Deviation ranged between 0.042 mm and 0.042 mm between all replicated individuals.

To process and analyze landmark data, we used the Geomorph (Version 3.3.1) and RRPP (Version 0.6.0) packages in R (Adams et al., 2020; Collyer and Adams, 2020; Collyer and Adams, 2018) with the gpagen, plotTangentSpace, and bilat.symmetry functions (Adams and Otárola-Castillo, 2013; Klingenberg et al., 2002), as well as MorphoJ (Klingenberg, 2011). To compare configurations of semilandmarks for unilateral shape differences, we used the gpagen function to slide semilandmarks along a tangent to the curve of each semicircular canal and the common crus and Procrustes register semilandmarks (accounting for orientation, location, and size of the coordinate cloud) only from the right side. To compare individuals and groups for unilateral differences, we used the plotTangentSpace function to perform a Principal Components Analysis (PCA), and visually represented shape variation.

Before assessing FA with bilateral shape data, we first slid and registered bilateral landmarks using the gpagen function for each diet-age group. Then, we inspected the resulting registered landmark configurations for outliers by plotting the average Procrustes distance from every configuration to the mean configuration for every age and diet group. To assess FA, we used the bilat.symmetry function in Geomorph, which is specifically geared towards assessing bilateral asymmetry in sets of three-dimensional landmark-based data. This function applies a multivariate analysis of variance (Procrustes MANOVA) test for morphological matching

symmetry (Mardia et al., 2000). This calculation is built on a matrix of tangent Procrustes distances between the left and right configurations.

The bilat.symmetry function's calculation produces a summary of the deviation in landmark configurations accounted for by variation among individuals, by directional asymmetry (DA), by FA, and by measurement error. Variation due to DA calculates differences across a group related to side (left-right); variation due to FA examines differences within individuals due to random left-right variation, and measurement error is calculated by examining individuals for whom replicated landmark configurations have been produced (Klingenberg et al., 2002; Klingenberg and McIntyre, 1998; Leamy, 1984; Palmer and Strobeck, 1986; Woodard and Neustupa, 2016). The bilat.symmetry package provides p values for each component of the Procrustes ANOVA by comparing means between sources of variation with the F-statistic, and produces R squared values indicating the contribution of each source to shape variation (Zelditch et al., 2012). Statistical significance of the influence of individual variation (random factor) is tested against DA (fixed factor), significance for DA is tested against FA (interaction term), and significance for FA is tested against measurement error (Klingenberg et al., 2002; Klingenberg and McIntyre, 1998; Mardia et al., 2000; Zelditch et al., 2012). The Procrustes ANOVA was performed on each of the diet-age subgroups to compare how proportions of FA varied among groups, and test whether FA is greater than observer error.

To test the difference in levels of FA, we calculated FA scores for each individual in MorphoJ (Klingenberg, 2011). Semilandmarks were slid and Procrustes registered in the Geomorph

package, and then exported. These data were imported into MophoJ, and FA scores were calculated for each individual according to Klingenberg and Monteiro (2005). Each diet-age subgroup was assessed for normality and skew in FA scores with Shapiro-Wilks and equivalency of variance testing, and then a Kruskal-Wallis test was performed to identify differences in medians between groups.

Results

Unilateral Shape Assessment

First a PCA was completed to assess the impact of diet and age on unilateral shape. The PCA plot shows shape variation among all individuals based on unilateral labyrinthine landmarks. This plot displays complete overlap of diet and age subgroups (Figure 3). The groups defined by the control diet and low protein diets for 22 day, three month, or 15 month age groups occupied approximately the same shape space. The sum of principal components 1 through 4 encompassed over 56% of all variation in the sample (Figure 4). PC1 appears to represent difference in overall height versus width of the semicircular canals, but this variation is slight and not associated with a particular subgroup (Figure 6). No identifiable shape trends were noted in examination of wireframes found of the extremes of principal components two through four (Figure 5).

Bilateral Shape Assessment

Calculations assessing bilateral data began with Procrustes distance plots for each diet-age subgroup. These figures, which plot the Procrustes distance of each specimen's right and

mirrored left landmark configurations from the subgroup mean, identified no outliers (Figure 6). The subsequent Procrustes ANOVA assessing asymmetry found the greatest proportion of variation to be related to individual differences, followed by FA, DA, and then replication error (Table 2, Figure 7). All factors consistently contributed to shape variation across all diet and age groups. Individual variation accounted for 67 to 73% of variation. Fluctuating asymmetry was a statistically significant ($p < 0.01$) proportion of overall variation in each group and proportions were also consistent between groups, ranging between 20 and 26% of overall variation. Proportions of DA contributed between 2 and 7% of variation, and replication error contributed between 1 and 3% of variation. Fluctuating asymmetry was the only contributor to shape variation that reached statistical significance.

Fluctuating asymmetry scores, calculated according to Klingenberg and Montiero (2005) are consistent between all groups (Figure 8). The control 22 day and control three month groups each have one upper outlier, but the level of FA in these two individuals is not dissimilar to the upper extremes of the low protein three month group. The median FA score decreases slightly through increasing age groups in the control sample, while the median low protein FA score increases then decreases. Although this trend fails to reach statistical significance, it would be worth exploring how and when reduced remodeling is expressed in the bone surrounding the labyrinth as a potential factor influencing developmental stability. As the data were non-normally distributed according to Shapiro-Wilk testing and did not exhibit equivalence of variance, a Kruskal-Wallis test was applied to examine for difference in group medians. This

test did not reach statistical significance and no post-hoc testing was conducted. The results indicate no difference in levels of FA between any of the diet-age subgroups.

Discussion

A previous study conducted on this sample concluded that maternal diet had a significant impact on offspring body composition (Martin-Gronert et al., 2016). Body weight, body length, and size of overall fat deposits of offspring of protein-restricted mothers were statistically significantly reduced compared to the control group (Martin-Gronert et al., 2016). This study identified changes to insulin signaling proteins in the protein-restricted offspring (Martin-Gronert et al., 2016).

In the current study we used a combination of unilateral and bilateral analyses to show a lack of shape change or shape variation in the bony labyrinth as a result of maternal protein restriction during early development. Unilateral shape variation was consistent among all dietary and age groups, supporting our first hypothesis, predicting no change. The absence of shape differences associated with developmental stage or with dietary protein content indicates that, despite body size differences both between age groups and diet groups (Martin-Gronert et al., 2016), the overall shape of the bony labyrinth is very stable. Stability in labyrinthine morphology is important, as the structure is so closely related to vital balance and hearing functions (Ifediba et al., 2007). Therefore, shape consistency, even in the context of otherwise detrimental nutritional stress, suggests that labyrinthine morphology is constrained and guarded to protect function.

Our second hypothesis posited that maternal protein restriction would produce higher levels of FA in the bony labyrinth, but that these levels would be consistent across age groups. Statistically significant levels of FA were identified in all diet-age subgroups, in greater proportion than intra-observer error. Despite the presence of FA in all groups, the amounts of individual FA were not statistically significantly different between any pairing of groups, causing us to reject our second hypothesis. Neither diet nor age had an impact on the amount of FA.

Consistency in the amount of FA regardless of dietary protein content may indicate several possible scenarios. Most likely, labyrinthine morphology is prioritized during early development. Canalization of labyrinthine growth has been hypothesized in other research as a mechanism to preserve functionally-important morphology of the bony labyrinth (Jáuregui et al., 2016; Jeffery and Spoor, 2004; Zehnder et al., 2006). Our results, both from unilateral and bilateral analyses, support these hypotheses. The protection of function also supports the use of labyrinthine morphology to infer function and phylogeny, connections which have been sought in fossil and living primates (Beaudet et al., 2019; Kirk and Gosselin-Ildari, 2009; Malinzak et al., 2012; Perier et al., 2016; Pfaff et al., 2017, 2015; Rook et al., 2004). This study does not help to explain patterns of intraspecific variation, however, as identified individual variation did not covary with age or dietary protein content.

Consistent FA may indicate that the mother is successfully buffering the effects of malnutrition during gestation. Reduction in overall body size and other differences between the control and

experimental group identified by Martin-Gronert and colleagues (2016) suggest that the mother is not able to protect offspring from all the effects of a low-protein diet. Indeed, numerous other side-effects known to result from protein deficiency in early development (Almeida and Mandarim-de-Lacerda, 2005; Boersma and Tamashiro, 2015; Fernandez-Twinn et al., 2005; Gressens et al., 1997; Langley-Evans et al., 1999, 1996; Mortensen et al., 2010), point to the existence of mechanisms prioritizing certain structures during development. A broader exploration of other physiology might illuminate both canalized structures as well as those protected by the maternal environment.

Statistically significant levels of FA, greater than variation introduced by intra-observer error, could indicate that the environment is contributing to labyrinthine morphological variation in these groups. As environmental factors are universal between both the experimental and control groups, they are the most likely contributors to FA identified. These factors might be stress related to the captive environment, handling of rats, or additional unknown variables (Borysenko and Borysenko, 1982; Holmes et al., 2005; Meerburg et al., 2008). The proportion of FA might also be representative of a baseline level seen across the rat skeleton, or the integration of different regions of the basicranium may be influencing labyrinthine morphological variation (Hallgrímsson and Lieberman, 2008; Lieberman et al., 2000; Martínez-Abadías et al., 2012). This result points to interesting patterns of developmental instability in the cranium (Martínez-Abadías et al., 2012) that warrant further exploration through examination of FA across additional regions of the cranium and skeleton in the future.

Conclusion

Maternal malnutrition is known to produce a number of significant short and long-term side effects on offspring in humans and model organisms (Hales and Barker, 2001). These effects are still of great concern among living people, and warrant exploration to elucidate evolutionary trends and intergenerational health. Some of these side effects influence the developmental trajectory of the skull (Miller and German, 1999). In this study we investigated the impact of a protein restricted diet on a functionally constrained, but early-developing region of the rat basicranium.

Using unilateral and bilateral shape analyses, we identified the extent to which labyrinthine morphology is protected from malnutrition throughout gestation and before weaning. We found that, despite a low protein maternal diet from gestation and until weaning, bony labyrinth morphology is not altered, either in terms of absolute inter-individual variation or in levels of FA. These results support several previous hypotheses and research findings (e.g. Gonzalez et al., 2014). First, as no shape differences are seen between age groups, regardless of diet, our findings show stability through development and into adulthood (Bloch and Sørensen, 2010; Frisch et al., 2000; Jeffery and Spoor, 2004; Sørensen, 1994; Sørensen et al., 1992). Second, as we investigated a specific source of variation, malnutrition, our results support the case for canalization in the rat bony labyrinth and suggest that maternal diet protein content is not an important variable in its development (Ward et al., 2016). As we know the basicranium to be similar in reaction to external stress in rodents and humans (e.g., Hallgrímsson and Lieberman, 2008), these results could point to a potentially similar response

to protein restriction in both species. Finally, with the stability we identify, bony labyrinth morphology can be more confidently used for phylogenetic and locomotor reconstructions in extinct and extant species (among others, see: Le Maître et al., 2017; Perier et al., 2016; Ryan et al., 2012; Silcox et al., 2009; Spoor et al., 2007, 1994; Walker et al., 2008).

Future research should seek to compare levels of FA within the skeleton overall in relation to malnutrition, as well as explore other environmental variables with the potential to contribute toward shape variation in the bony labyrinth. This study emphasizes the importance of the bony labyrinth as a tool for understanding development, evolution, and intergenerational health in humans, our relatives, and model organisms alike. Model organisms are an excellent tool for bridging the gap between evolutionary anthropology and human biology studies, and this is especially true for the bony labyrinth.

Acknowledgments
This research was supported by a University of Toronto PhD Pilot Research Grant to DLW, a NSERC Discovery Grant to MTS, SSHRC Insight and Connaught New Researcher Grants to BV; the Henry Sidgwick Research Fellowship from Newnham College, Cambridge to EP, and a European Research Council grant 617627 to JTS. DLW is grateful to Michelle Cameron and David Katz for feedback on study design and preliminary results, and to Matthew McNeil for coding support.

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Tables

Table 1: Sample number and sums for every diet and age group.

	22 Days	**3 Months**	**15 Months**	**Sum**
Low Protein	10	11	16	37
Control	8	12	10	30
Sum	18	23	26	67

Table 2: Mean landmark deviation across replicates from each subgroup, calculated to assess intra-observer error (von Cramon-Taubadel et al., 2007). C_22d is the control group at 22 days old, C_3m is the control group at three months old, C_15m is the control group at 15 months old, LP_22d is the protein restricted group at 22 days old, LP_3m is the protein restricted group at three months old, and LP_15m is the protein restricted group at 15 months old.

Replicate	**C_22d**	**C_3m**	**C_15m**	**LP_22d**	**LP_3m**	**LP_15m**
Mean landmark deviation (mm)	0.0421	0.0427	0.0423	0.0422	0.0423	0.0423
Percentage of mean group centroid size	0.132%	0.137%	0.133%	0.136%	0.137%	0.136%

Table 3: R2 values resulting from the Procrustes ANOVA separated into diet and age groups, as a proportion of 100% of the variation in each group. Significance indicated: $p < 0.01$ **. C_22d is the control group at 22 days old, C_3m is the control group at three months old, C_15m is the control group at 15 months old, LP_22d is the protein restricted group at 22 days old, LP_3m is the protein restricted group at three months old, and LP_15m is the protein restricted group at 15 months old.

	Replication Error	**Fluctuating Asymmetry**	**Directional Asymmetry**	**Individual**
C_22d	0.02639	0.20101**	0.07535	0.69726
C_3m	0.02336	0.26133**	0.04453	0.67077
C_15m	0.01919	0.23935**	0.05464	0.68681
LP_22d	0.02348	0.21901**	0.04163	0.71588
LP_3m	0.01552	0.24101**	0.05345	0.69002
LP_15m	0.01239	0.22982**	0.02255	0.73525

Figure Legends

Figure 1: (Left) A transparent volume rendering of a rat skull, with the bony labyrinth seen within. (Right) The segmented bony labyrinth separated from the skull, with primary anatomical components labeled. An example of landmark data collected for this study seen within the segmented volume.

Figure 2: Visual representation of the diets of the experimental and control groups through development and adulthood.

Figure 3: Principal Components Analysis of unilateral variation across all six diet-age subgroups, with all subgroups overlapping in shape space, regardless of age or diet. Green is control 22 days, dark blue is control three months, light blue is control 15 months, purple is low protein 22 days, black is low protein three months, and red is low protein 15 months.

Figure 4: Histogram of the proportion of variation across unilateral shape variation accounted for by the first 75 principal components.

Figure 5: Wireframes of semicircular canals from the negative and positive extremes of the first four principal components, accounting for the 56% of variation across all diet and age subgroups. (Top) PC1 show a slight difference between taller semicircular canals at the negative extreme and wider canals antero-posteriorly.

Figure 6: Procrustes distances from the mean of the Control 22 day group, Control 3 month group, Control 15 month group, Low Protein 22 day group, Low Protein 3 month group, and the Low Protein 15 month group to demonstrate the absence of outliers bilaterally.

Figure 7: Stacked bar chart illustrating the R^2 values for each component of bilaterally asymmetric variation in the six diet-age subgroups, as a percentage. Green is individual variation, orange is directional asymmetry, blue is fluctuating asymmetry, and grey is intra-observer error. C_22d is control 22 days, C_3m is control three months, C_15m is control 15 months, LP_22d is low protein 22 days, LP_3m is low protein three months, and LP_15m is low protein 15 months.

Figure 8: Box and whisker plots of fluctuating asymmetry scores across all diet-age subgroups, calculated according to Klingenberg and Monteiro (2005). C_22d is control 22 days, C_3m is control three months, C_15m is control 15 months, LP_22d is low protein 22 days, LP_3m is low protein three months, and LP_15m is low protein 15 months.

Figure 1

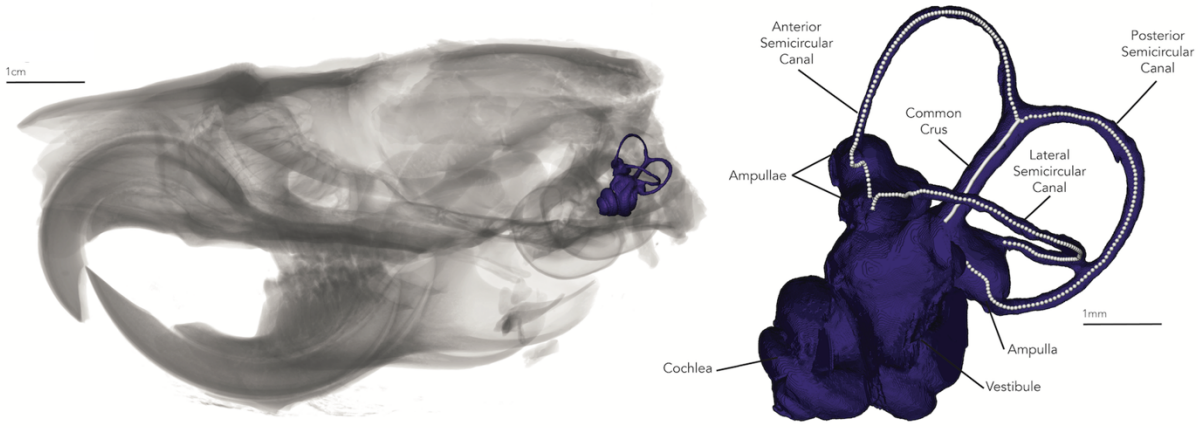


Figure 2

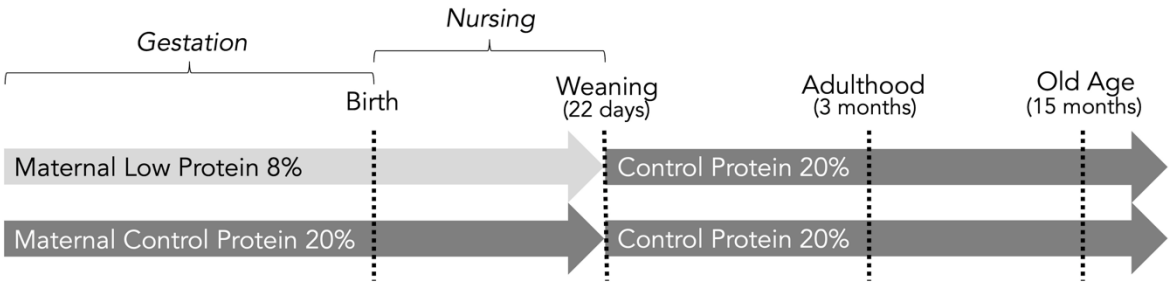
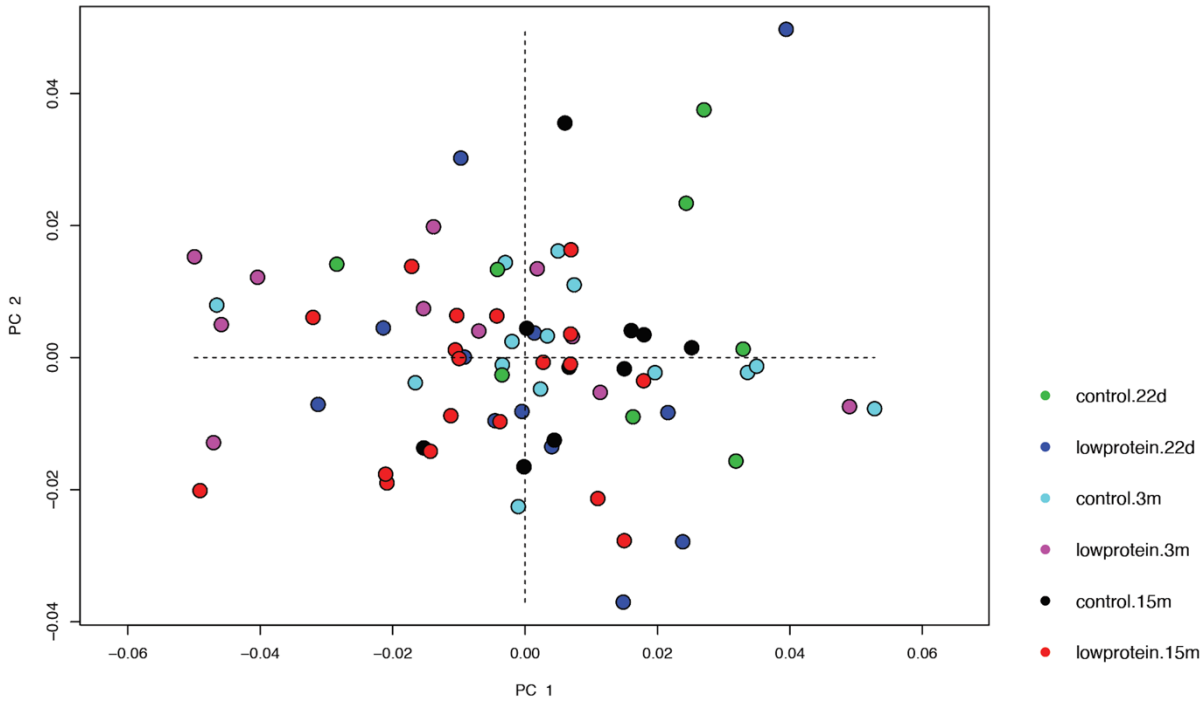


Figure 3



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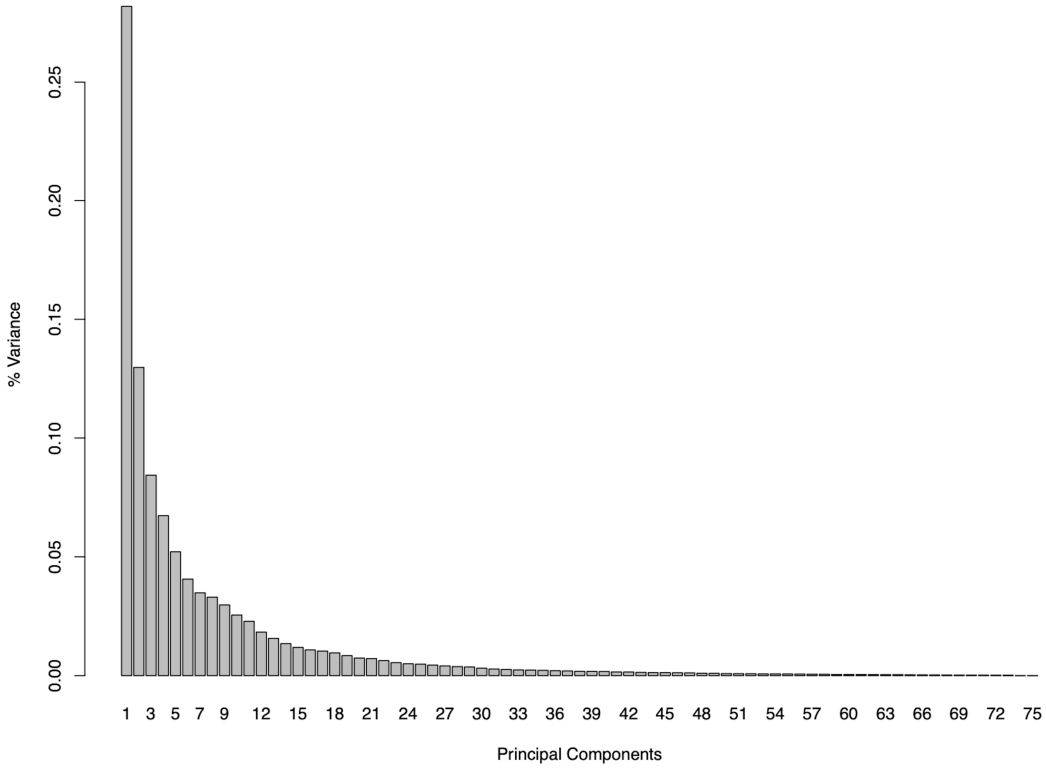

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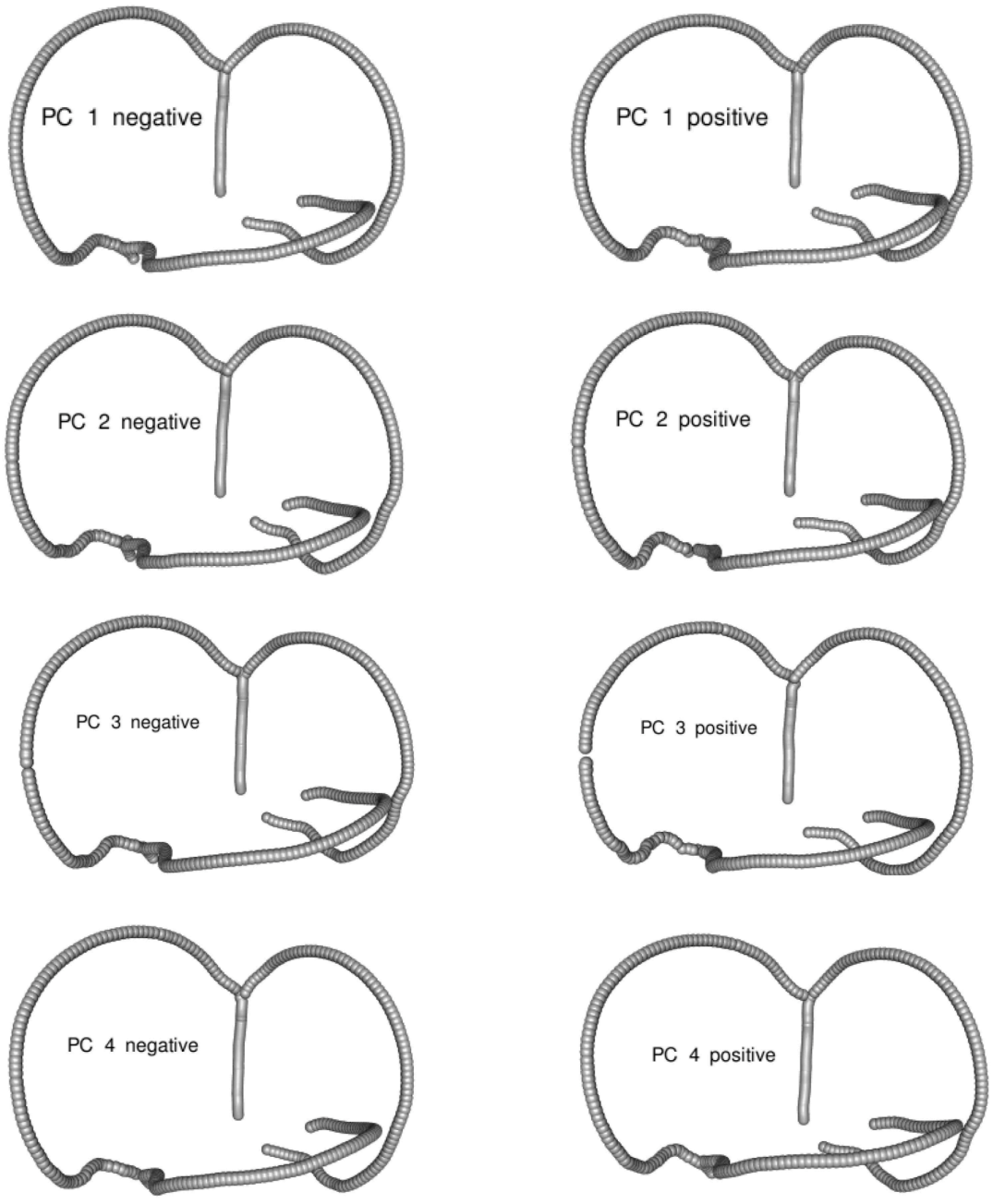

PC 1 negative
PC 1 positive
PC 2 negative
PC 2 positive
PC 3 negative
PC 3 positive
PC 4 negative
PC 4 positive

Figure 6

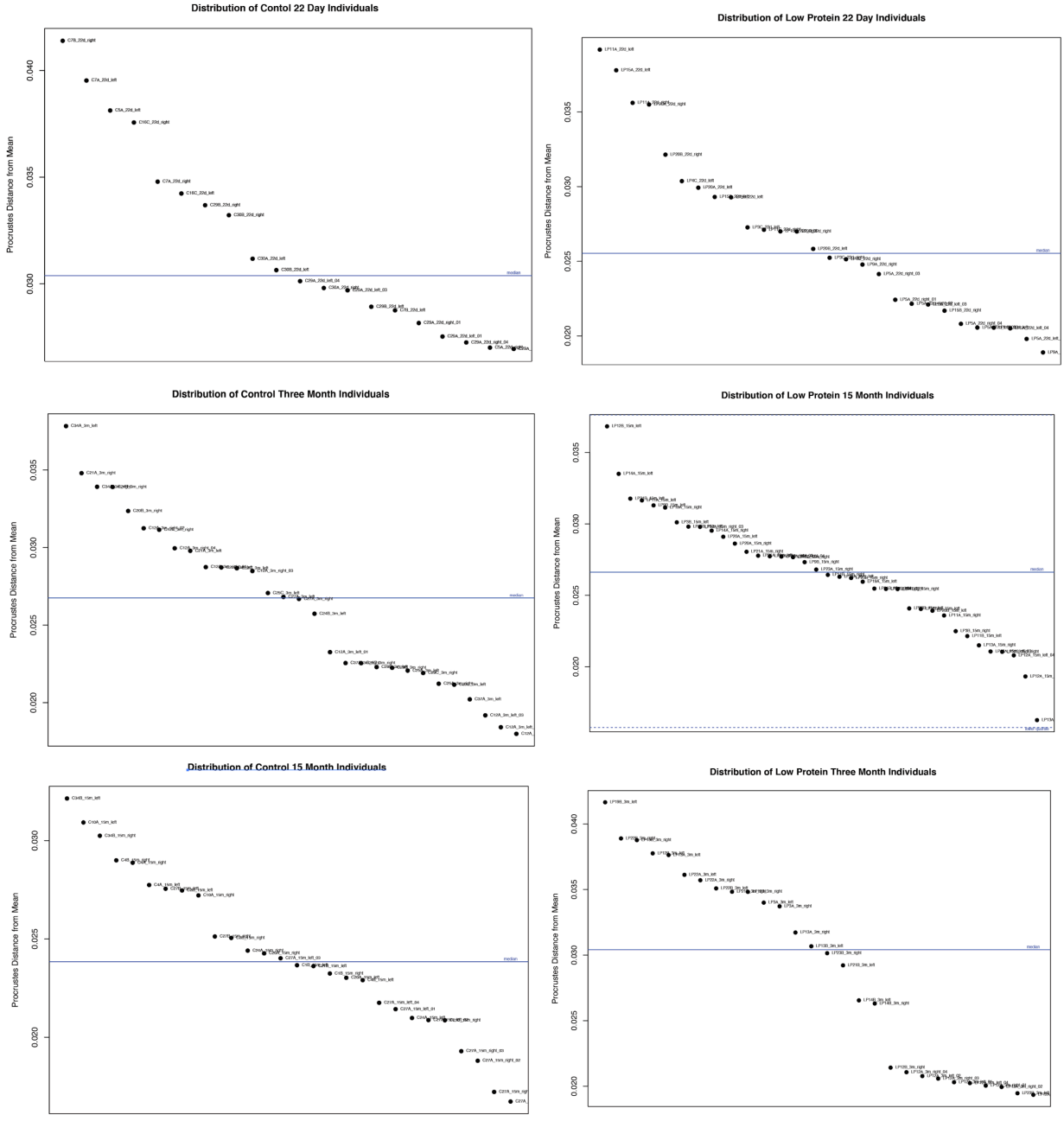

Distribution of Contol 22 Day Individuals
Procrustes Distance from Mean
0.040
0.035
0.030
median
Distribution of Low Protein 22 Day Individuals
Procrustes Distance from Mean
0.035
0.030
0.025
0.020
median
Distribution of Control Three Month Individuals
Procrustes Distance from Mean
0.035
0.030
0.025
0.020
median
Distribution of Low Protein 15 Month Individuals
Procrustes Distance from Mean
0.035
0.030
0.025
0.020
median
Distribution of Control 15 Month Individuals
Procrustes Distance from Mean
0.030
0.025
0.020
median
Distribution of Low Protein Three Month Individuals
Procrustes Distance from Mean
0.040
0.035
0.030
0.025
0.020
median

Figure 7

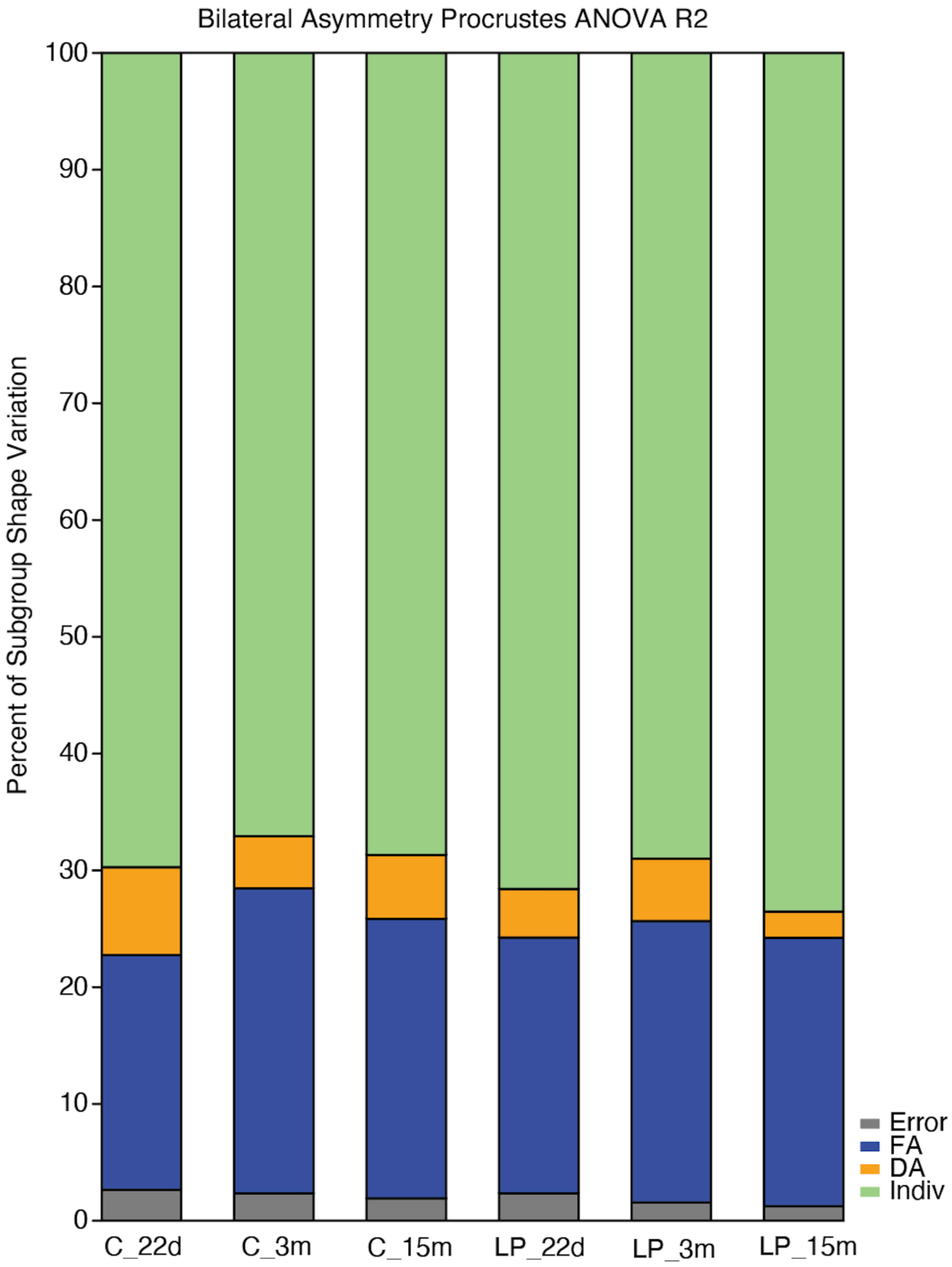
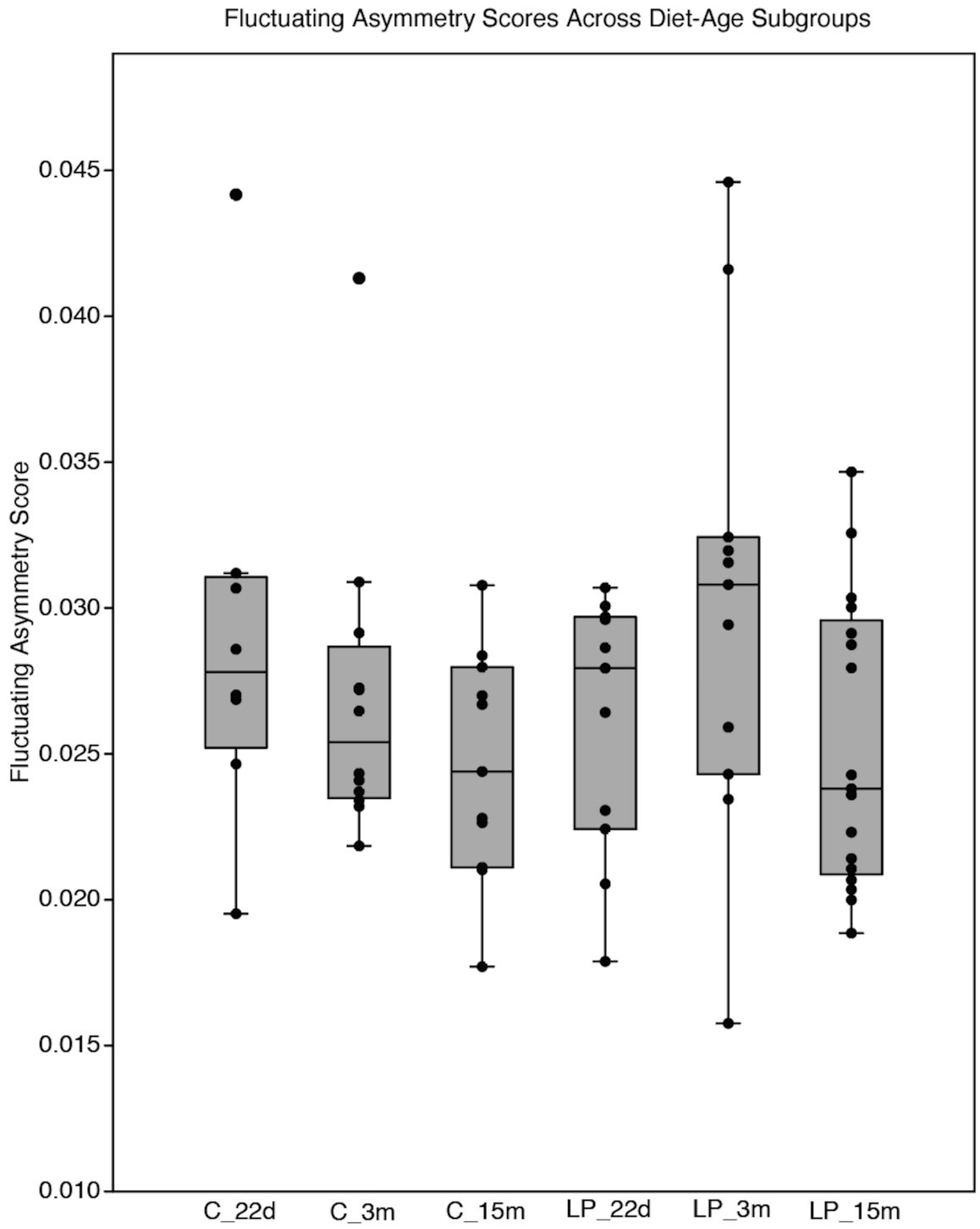

Bilateral Asymmetry Procrustes ANOVA R2
Percent of Subgroup Shape Variation
100
90
80
70
60
50
40
30
20
10
0
C_22d
C_3m
C_15m
LP_22d
LP_3m
LP_15m
Error
FA
DA
Indiv

Figure 8


Fluctuating Asymmetry Scores Across Diet-Age Subgroups
Fluctuating Asymmetry Score
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
C_22d
C_3m
C_15m
LP_22d
LP_3m
LP_15m