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The Heritability of Mandible Length in the Zebrafish (Danio rerio)

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The Heritability of Mandible Length

in the Zebrafish (*Danio rerio*)

By

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of the Requirements for the Degree
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Master's Thesis

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Danielle K. Ringo

November 6th, 2022

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A Thesis
Presented to
The Faculty of
Western Washington University

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Of the Requirements for the Degree
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ABSTRACT

Jaw shape often plays a significant role in determining feeding niche. Changes to jaw development can alter feeding mechanics and affect an organism's ability to acquire and/or process food. Derived patterns of jaw morphogenesis are therefore frequently associated with evolutionary shifts in trophic ecology. Changes in thyroid hormone signaling can have strong developmental effects on many aspects of vertebrate anatomy including the shape of the lower jaws. Zebrafish mutants (*opallus*^{b1071}) are hyperthyroid and typically undergo excessive mandible elongation. However, hyperthyroid *opallus* do not always develop elongated lower jaws. This mutant line was maintained at separate universities for many years, resulting in the establishment of a short-jawed phenotypic variant. It was originally thought that hyperthyroidism induced elongation of the mandibles in *opallus* during development, however, the short-jawed phenotypic variant suggests that the developmental determinants of mandible length are much more complicated. These two phenotypes in *opallus* provided an excellent opportunity to begin work towards investigating the developmental controls of mandible length in the zebrafish and its danionine relatives. To achieve our aims, we have 1) estimated the heritability of mandible length in *opallus* zebrafish; 2) predicted the additive genetic contributions of each parent fish to facilitate future genetic mapping studies; and 3) used an enzyme-linked immunosorbent assay (ELISA) to compare thyroid hormone levels in *opallus* and wildtype zebrafish tissues. We determined that corrected mandible length was heritable in *opallus* zebrafish and have identified the sire and dam to use for future genetic mapping and quantitative trait locus analysis. We also found that the thyroid hormone levels within the tissues of wildtype and *opallus* fish were not in agreement with results of previous studies using ELISA techniques, though this was likely due to kit selection errors.

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INTRODUCTION

The jaw morphology of fishes plays a significant role in determining their feeding niche (Cooper et al. 2017). Subtle changes to jaw morphology can impact which feeding mechanisms fishes can employ, what types of food they eat, and which ecological niches they occupy (Cooper et al. 2017). While the form, function, and evolution of the upper jaws have been extensively studied in fishes (Bellwood et al. 2015; Wainwright & Longo, 2017; Cooper et al. 2017; Roberts et al. 2021) the lower jaws, or mandibles have not. The mandibles play an equally important role in food capture and processing, making them a valuable target for evolutionary studies of fish feeding mechanics and ecological niche determination. Mandible morphology, especially mandible length, is highly variable within fishes and can provide an important advantage while feeding on certain prey types. This study focuses specifically on the elongation of the mandibles.

To understand how increasing mandible length may confer feeding advantage in fishes, we must consider the biomechanics of the jaws. The opening and closing of the jaws in fishes works as a simple lever system. The mechanical advantage (MA) of a lever system is the ratio of force input to force output (Westneat 1994). The inverse of MA is displacement advantage (DA), which measures the speed of the opening or closing of the jaws (Westneat 1994). A high MA of the jaws contributes to a stronger bite, while a low MA produces a weaker bite. The opposite is true for DA; a high DA facilitates a faster bite, while a low DA produces a slower bite (Westneat 1994). Increasing the length of a fish's mandible increases the out-lever length, which decreases MA and increases DA. This gives a fish a weaker, but faster bite. Having a fast bite is

advantageous for many species, particularly those who feed on evasive, hard-to-catch prey within the water column (Westneat 1994).

Along with facilitating a faster bite speed, elongated mandibles can support diverse diets, feeding strategies, and lifestyles in fishes. Another example being that elongated mandibles can contribute to the upturned (superior) mouth positioning observed in fishes that capture prey from below such as those who feed off the water's surface (Helfman et al. 2009). Superior mouth positioning is also present in bottom-dwelling fishes which feed on prey that swim above them (Helfman et al. 2009). The length of the mandibles can have a direct effect on the strategies a fish uses to capture its prey items.

Mandible length plays an important role in fish feeding dynamics, therefore further investigations of mandible length determination will provide insight into the diversification of fish feeding mechanics. This has not yet been done using a zebrafish (*Danio rerio*) model, nor has this been done with any of the zebrafish's close relatives in the subfamily Danioninae (*sensu* Stout et al. 2016). There is significant mandible length variation in the zebrafish and amongst its danionine relatives (Conith et al. *in press*). These differences in mandible length are a product of differences in skull development. Differences in mandible elongation during development could promote adaptive diversification if this phenotypic variation has a heritable genetic basis. This project will determine if there are additive genetic effects on mandible length in the zebrafish. Additive genetic effects are the allelic contribution to an individual by each of their parents for a specific trait. In this case, the presence of additive genetic effects on mandible length in the zebrafish would indicate that parental genetic contributions have some responsibility for mandible length determination within offspring rather than purely environmental, dominance, or

epistatic effects. Determining the additive genetic effects on mandible length is a critical first step in understanding how this trait manifests in the zebrafish and its close relatives.

Determination of additive genetic effects and variation can be accomplished by estimating the heritability of the trait. Narrow-sense heritability estimates how much phenotypic variation amongst offspring is due to additive genetic effects acquired from their parents (Lynch & Walsh 1998). Broad-sense heritability, on the other hand, considers both additive and non-additive genetic effects (Lynch & Walsh 1998).

The degree to which a trait can evolve in either a population or a diversifying lineage should be proportionate to its narrow-sense heritability. The response of a trait with a specific narrow-sense heritability to a given level of selection can be calculated using the breeder's equation: ($R=h^2S$). In this equation R represents the response to selection, h^2 represents narrow-sense heritability, and S represents the selection differential (Falconer & MacKay 1996; van Tienderen & de Jong 1994). Estimating the narrow-sense heritability of mandible length in the zebrafish will allow us to determine if the trait can respond to selective pressures, which ultimately is what drives ecological diversification. Danionine fishes exhibit species-specific differences in mandible length that are associated with disparate feeding strategies and the occupation of different tropic niches (Conith et al. *in press*). This suggests that lower jaw length variation has evolved in response to natural selection within the Danioninae. Estimating the narrow-sense heritability of mandible length in zebrafish therefore has implications for understanding the ecological diversification of this lineage.

Subtle changes to jaw morphogenesis can significantly impact fish feeding mechanics. For example, slight changes to the elongation of a single bony process in the upper jaw (the ascending arm of the premaxilla) can move fishes into new feeding niches (Cooper et al. 2017;

Cooper et al. 2020). One factor that can shift the development of the jaw bones is the production and secretion of thyroid hormone (TH). Thyroid hormone plays a major role in the stimulation of metamorphosis in zebrafish and other vertebrates (McMenamin & Parichy 2013). Metamorphosis in teleost fishes, such as the zebrafish, has been characterized by a suite of morphological changes that occur during the larva-to-juvenile transition and often involves extensive remodeling of the body (McMenamin & Parichy 2013). As zebrafish undergo metamorphosis much of their cartilaginous skeleton becomes reshaped and ossified, and substantial remodeling of the mandible is initiated (McMenamin et al. 2017; Galindo et al. 2019).

Silencing or increasing TH production impacts zebrafish jaw metamorphosis (Galindo et al. 2019; Keer et al. 2019). Assessments of zebrafish skeletal development showed that post-metamorphic hyperthyroid zebrafish mutants (*opallus*^{b1071}) developed significantly elongated lower jaws in comparison to wildtype specimens (Galindo et al. 2019; Keer et al. 2019). In such specimens, the anterior tip of the mandible extends past the upper jaw tip so that the two do not meet with the mouth is fully closed (Galindo et al. 2019). Excess TH also causes over-ossification of the lower jaw and other bony elements and can contribute to the malformation of multiple skeletal components (Keer et al. 2019). This pattern of over-ossification is consistent in other hyperthyroid vertebrates, including mice and humans (Kim & Mohan 2013; Williams & Bassett 2018).

Not all hyperthyroid zebrafish exhibit elongated lower jaws. Some *opallus* mutants obtained after the completion of the Galindo et al. (2019) study exhibit a lower jaw phenotype similar to that of wildtype zebrafish in which the upper and lower jaw tips align when the mouth is closed (Fig 1). Hereafter the long-jawed *opallus* phenotype will be referred to as "*longjaw*"

and the *opallus* phenotype with lower jaws comparable to wildtype zebrafish will be referred to as "*shortjaw*".

Hyperthyroidism alone cannot be responsible for the variation in mandible length that we observe among *opallus* specimens. The *opallus* line (both *long-* and *shortjaw*) contains a point mutation that upregulates expression of the TH precursor gene, thyroglobulin, which consequently affects thyroid stimulating hormone receptors (McMenamin et al. 2014; Tuncel 2017). No other significant genomic differences had been identified within the original mutant line when compared to AB wildtype zebrafish (McMenamin et al. 2014). The *opallus* line was maintained separately at different universities for many years, and it is unknown if inbreeding, backcrossing, or genetic drift occurred during this time. There may now be significant differences within the genome of our *opallus* population in comparison to AB wildtype specimens. Excess TH simultaneously inhibits melanophore development and promotes iridophore development in zebrafish skin (McMenamin et al. 2014; Gulliot et al. 2016). This explains why *opallus* specimens have shiny, opalescent flanks and lack the characteristic horizontal black stripes of wildtype zebrafish (McMenamin et al. 2014). We can confirm that both *opallus* phenotypes are still hyperthyroid because of their altered skin pigmentation (McMenamin et al. 2014; Gulliot et al. 2016). However, since these lines had been maintained separately for so many years, there is a strong likelihood that random genetic changes have occurred, which may be contributing to the phenotypic discrepancies in mandible length that we observe today.

The existence of these two *opallus* phenotypes provides a unique opportunity to investigate mandible length determination in zebrafish, as it is presumed that some genetic change has occurred which allowed for the development of this phenotypic variation. *Longjaw*

and *shortjaw opallus* exhibit a range of mandible lengths, which facilitates using this line to estimate additive genetic variance and narrow-sense heritability for this trait in the zebrafish. These phenotypes also raise an interesting question about how TH signaling affects the development of zebrafish mandibles. With the potential implications for evolutionary-developmental studies of fishes in mind, this project will lay the foundation for identifying which aspects of development contribute to mandible elongation in zebrafish.

Observations suggest that mandible length variation is heritable in *opallus*, but this hypothesis has not been previously tested. Along with estimating trait heritability, we can also predict the specific effects each parent may have on their offspring phenotype, also known as their *breeding value*. If we find evidence that this trait is heritable, this will allow us to continue exploring which specific genetic loci, signaling pathways, and other aspects of development contribute to mandible length determination and variation in the zebrafish. To do this, we will 1) estimate the narrow-sense heritability of mandible length in *opallus* zebrafish; 2) predict the breeding values of parents to facilitate future genetic mapping studies; and 3) use an enzyme-linked immunosorbent assay (ELISA) to compare thyroid hormone levels in *opallus* and wildtype zebrafish tissues.

METHODS AND MATERIALS

Animal Husbandry

This research was conducted using the zebrafish facility in the Cooper Lab at Western Washington University (WWU). Animal husbandry complied with WWU's Animal Care and Use Committee guidelines, and all procedures were approved prior to beginning any experiments (protocol 21-004). For this study we utilized two phenotypic variants of the *opallus*^{b1071} mutant line of zebrafish: *shortjaw* and *longjaw*. The *opallus* line was an appropriate study system for this research because: 1) *opallus* jaw development has been studied previously (Galindo et al. 2019; Keer et al. 2019); 2) our *opallus* population exhibits a wide variety of mandible lengths; 3) zebrafish husbandry is simple and inexpensive; and 4) zebrafish have short generation times that promote the feasibility of a heritability study and associated future work.

Zebrafish were kept under controlled, standard laboratory conditions. Fish were housed on a multi-tank rack system or in free-standing 10-gallon tanks with constant water circulation. Water temperature was maintained at 26-28.5°C, while pH was maintained between 6.8-7.5 (Avdesh et al. 2012; Aleström et al. 2020; Westerfield 2000). Fish were also kept at a 14:10 hour (light: dark) lighting schedule to simulate a natural lighting environment (Avdesh et al. 2012; Aleström et al. 2020, Westerfield 2000). Fish were fed twice daily with standard commercial fish flakes and live, algae-enriched brine shrimp hatchlings (*Artemia sp.)*. Because of space limitations within the laboratory, we could not effectively prevent resource competition amongst fish within the same tank. The number of fish housed within each tank varied from clutch to clutch.

Embryos harvested from natural matings or *in vitro* fertilization were incubated in embryo water-filled Petri dishes at 28°C for five days post fertilization (dpf). Methylene blue, an anti-fungal, was added to the dishes for the first 24 hours and then removed. After 5 dpf larvae were transferred into tanks and fed 50 mL of live *Paramecia sp.* culture per tank each day. At 10 dpf, larvae were fed small amounts of live brine shrimp that were gradually increased. At 20 dpf larvae were placed on the rack system with slow water flow that was gradually increased over several weeks. All lab members involved with zebrafish husbandry were thoroughly trained in the appropriate animal care protocols.

Heritability Study

For this study, we wanted to determine how much of a genetic effect parents have on the mandible length phenotypes of their offspring. To estimate narrow-sense heritability, five sires with varying mandible lengths (corrected mandible lengths ranging between 0.076-0.111mm) were each bred to three different dams (corrected mandible lengths ranging from 0.066-0.091mm). Fifteen clutches of offspring were produced in total, with clutch size ranging from 9 to 64. This nested, full-sibling, half-sibling design followed Walsh (2007) and Conner and Hartl (2004; Fig 2.) We chose a nested breeding design because it allowed us to consider the effects of both dam and sire into the additive genetic variance estimation (Falconer & MacKay 1989; Conner & Hartl 2004). The full-sibling, half-sibling breeding design also allows us to limit maternal effects, as each sire is bred to multiple dams. Similarly, this breeding design better isolates the effects of dominance because the background noise of maternal effects are reduced. We can therefore obtain a more robust estimate of narrow-sense heritability by using this breeding design.

Mutant zebrafish lines like *opallus* often take longer to mature than wildtype zebrafish. Because of this, all fish were raised for at least 3 months. All clutches were maintained until multiple females became noticeably gravid, which we used as an indicator of adulthood. Clutches were euthanized by submersion in ice water. Euthanized specimens were first fixed in 10% formalin, then gradually stepped into a 25% tap water, 75% ethanol solution for storage.

Measurements of mandible length (mm) and standard body length (mm) were taken from each preserved specimen using digital calipers. Mandible length was measured from the retroarticular joint to the anterior tip of the dentary bone. Standard length was measured from the tip of the rostrum (anterior tip of the head) to the anterior edge of the caudal fin (tail fin). Measurements of mandible length were standardized for each fish's standard length before analysis because mandible length increases with length in adult *opallus*, which like all fishes, continue to grow throughout life (Helfman et al. 2009). We then natural log transformed these data to achieve a normal distribution. Hereafter, when referencing mandible length in this study, we are referring to the corrected mandible length (MLc) data that have been standardized for body size and undergone natural log transformation (Table S1).

In order to assess narrow-sense heritability, we employed two different statistical methods. The first method is described as a traditional analysis of variance (ANOVA) model because it utilizes the mean squares and expected mean squares used in an ANOVA to calculate variance estimates. Dam, sire, and error variance estimates are used to calculate total phenotypic variance (σ^2_P), additive genetic variance (σ^2_A), and eventually the narrow-sense heritability (h^2) using the following equations (Walsh 2007; Conner & Hartl 2004; Lynch and Walsh 1998).

Equation 1: $\sigma^2_P = \sigma^2_s + \sigma^2_d + \sigma^2_w$

Equation 1 was used to calculate the total phenotypic variance (σ^2_P) which is equal to the sum of variance within sires (σ^2_s), dams (σ^2_d), and full siblings (σ^2_w).

Equation 2: $\sigma^2_A = 4\sigma^2_s$

Equation 2 was used to calculate the additive genetic variance (σ^2_A) using variance within sires (σ^2_s).

Equation 3: $h^2 = \sigma^2_A / \sigma^2_P$

Equation 3 is the narrow-sense heritability (h^2) equation (σ^2_A = additive genetic variance; σ^2_P = total phenotypic variation).

One of the major difficulties of the traditional ANOVA approach to estimating heritability is that it relies heavily on the assumption that all families are balanced (i.e., equal clutch sizes; Conner & Hartl 2004). The traditional approach makes its estimations utilizing ANOVA, where calculating means is critical for producing variance values. When families are extremely unbalanced, means from each group cannot be compared to each other reliably or proportionally. For our study, clutch size ranged from 9 to 64 individuals, deviating from the assumption of equally sized families in the traditional method. Although we could have randomly sub-sampled clutches to select 9 individuals from each, this would have sacrificed a great deal of statistical power. To maintain statistical power, we used a second heritability estimation model that was less sensitive to unbalanced family sizes.

The second approach we used to estimate heritability was a Markov Chain Monte Carlo generalized linear mixed model (MCMCglmm), as described by de Villermeruil (2018; Hadfield 2022). The MCMCglmm method is an excellent alternative to the traditional ANOVA approach to heritability estimations because it has the flexibility to include unbalanced families and

produces a 95% confidence interval that can be used to assess significance of the heritability estimate.

We first constructed a simple pedigree matrix containing family relationships. To do this, we used the `MakeD` function from the nadiv package (version 2.7.1; Wolak 2012) to create an additive genetic matrix, or pedigree, by calculating the kinship between each individual. Then, using the `Dinv` function from the same package, we were able to produce the inverse of this matrix, giving us the non-additive genetic effects that could be incorporated into the model as "dominance effects" (Wolak 2012). Once our dominance and pedigree matrices were prepared, we could set the rest of the parameters for our model using the MCMCglmm package (version 2.33). After testing different priors, we chose a Fisher prior (R list defined as V=1, nu=1; G lists [G1 and G2] defined as V=1, nu=1, alpha.mu=0, alpha.V=1000). Our model was set to have one default fixed effect with an intercept of 1, two random effects, and a gaussian distribution. Our Markov chain ran 500,000 times, dropped the first 1,000 to account for convergence of the chain, and stored every 50th run. This produced posterior distributions for three variance components: additive, dominance, and error (Fig S1). We defined heritability using the following equation (de Villermeruil 2018):

$$Heritability = additive / (additive + dominance + error)$$

This equation calculated a final posterior distribution for the heritability of corrected mandible length in *opallus* zebrafish (Fig 3). To obtain a single h^2 estimate, we took the mean of this final posterior distribution. The `HPDinterval` function from the MCMCglmm package was used to identify the lower and upper limits of a 95% confidence interval based on the 500,000 Markov chain runs (Hadfield 2022). All analyses were conducted using R version 1.4.1717 (R Core Team 2022).

Prediction of Parent Breeding Values

We also wanted to predict the additive genetic effect that each parent could contribute to their offspring, also known as their breeding values. Breeding values can be used to identify sires and dams that are likely to produce offspring with a specific phenotype because of their allelic contributions for that specific trait (Robinson 1991). Best linear unbiased predictions (BLUPs) were used to achieve this goal, as they utilize linear models to predict random effects (Robinson 1991). Using the same MCMCglmm script from our heritability study, we added the argument `pr=TRUE` to store the random effect posterior distributions from all parents and offspring in the pedigree (Hadfield 2022; de Villermeruil 2018). From this, we extracted the BLUPs from the 10,000 Markov chain iterations for every individual and adjusted them based on the intercept for each run. Then, we took the means of each individual's posterior distribution to produce a final breeding value prediction for each parent fish as well as a corrected mandible length prediction for each offspring. We then used the predicted breeding values to evaluate which parents would contribute combinations of alleles that produce offspring with either long or short mandibles. These predicted breeding values for each parent were then plotted over the predicted MLc ranges of their full-sibling families. We also wanted to gauge the validity of these breeding values by comparing the actual phenotype and predicted breeding values for each parent fish. We therefore plotted each parent's predicted breeding value against their corrected mandible length and used a Pearson correlation to calculate the correlation coefficients and p values for dams and sires. All analyses were conducted using R version 1.4.1717 (R Core Team 2022).

Thyroid Hormone Assay

To quantify the levels of TH in the tissues of *opallus* and wildtype fish (AB strain) we used a thyroxine (T4) competition enzyme-linked immunosorbent assay kit (ELISA; Cusabio, Houston, TX). Thyroxine was the target for this assay because it provides a more representative measure of the total TH present in body tissues in comparison to triiodothyronine (T3). While T4 is the inactive form of TH, the large majority of TH that is present in blood and other tissues is T4, which is converted into T3 intracellularly (Waung et al. 2012). Quantifying T4 concentrations therefore provides a better estimate of total TH production (Hu et al. 2019).

ELISAs carry out colorimetric reactions and employ spectrophotometry to obtain their measurements (Gan & Patel 2013). The amount of color that is developed in each test well determines the optical density that will be measured by a spectrophotometer (Gan & Patel 2013). Darker coloration in a well results in greater optical density. Bound antibody concentration can be inferred by comparing optical density reads of samples extracted from fish tissues to those from manufacturer-supplied concentration standards. The ELISA kit used here measures optical density inversely to T4 concentrations within the samples (i.e., higher T4 concentrations should result in lower optical density values).

Opallus and wildtype AB specimens were bred for this assay. A previous study from Chang et al. (2012) demonstrated that T4 levels in zebrafish peak at 21 dpf so our specimens were raised under standard conditions for this length of time. At 21 dpf specimens were euthanized by submersion in ice water, rinsed with 1x phosphate buffered saline (PBS), then homogenized using a TissueLyserII (QIAGEN, Germantown, MD) tissue disruptor. Samples were frozen at -20°C overnight, subjected to two freeze-thaw cycles to break the cellular membranes, and then centrifuged for 5 minutes at 5000 x g. Supernatants from the centrifuged

tissue homogenates were then pipetted off and assayed according to the kit manufacturer's instructions.

Optical densities of all samples (at least 3 replicates per treatment) were read at 450 nm using a Victor^3 V Multilabel Plate Reader (PerkinElmer, Waltham, MA) with a correction wavelength of 595 nm. The optical density values of replicates were averaged and corrected using the optical density values determined for blank (empty) wells (Table 1). A sigmoidal standard curve was constructed using the corrected optical density reads for each of six concentration standards (0 ng/mL, 20 ng/mL, 40 ng/mL, 80 ng/mL, 160 ng/mL, and 320 ng/mL; Fig S2). Thyroxine concentration estimates for our wildtype and *opallus* samples were then interpolated from the standard curve using the GraphPad Prism 9 (version 9.3.1) software. A one-tailed T-test was used to determine if TH tissue levels in *opallus* were significantly different from those in AB specimens.

RESULTS AND DISCUSSION

Mandible Length is Heritable in the Zebrafish

We estimated an h^2 value of 0.584 using the traditional ANOVA method for calculating narrow-sense heritability. Narrow-sense heritability is measured on a scale of 0 to 1. A value that is closer to 1 indicates that the proportion of the phenotypic variance that is determined by additive genetic effects is high. Alternatively, a value that is closer to 0 indicates that the proportion of the phenotypic variance that is determined by non-additive or environmental effects is high. This initial estimate of heritability was quite high. However, our data violated the model assumption of equally balanced families.

Using the MCMCglmm approach, we estimated a h^2 value of 0.416 with a 95% confidence interval of 0.287 - 0.547 (Fig 3). These findings indicate that the proportion of the phenotypic variance that can be attributed to additive genetic variation is significant and suggests that variation in corrected mandible length is heritable in *opallus* zebrafish. Because the confidence interval did not include zero and only included values greater to or equal than those associated with adaptive traits in wild populations (0.1-0.2; Visscher et al. 2006), we interpret these results as evidence for a substantial level of heritability for corrected mandible length in *opallus*.

Our narrow-sense heritability estimate of 0.416 is consistent with what is considered highly heritable when compared to other h^2 estimates for quantitative traits in fishes. The threespine stickleback (*Gasterosteus aculeatus*) is a popular model for estimations of quantitative trait heritability because it exhibits significant phenotypic variability (Cresko et al. 2008). Hundreds of studies have been published about quantitative traits in the stickleback,

providing us with a wide spread of h^2 estimations to compare our estimate to (Cresko et al. 2008). Previous stickleback h^2 estimations that indicate significant trait heritability include 0.04 - 0.54 for the number of anal fin rays, 0.58 for the total number of gill rakers, and 0.34- 0.68 for number of dorsal fin rays (Cresko et al. 2008). Another study from Hagen and Gilbertson reported a h^2 of 0.84 ± 0.034 for number of lateral armor plates in the stickleback (1973). McGuigan et al. found that under high salinity conditions, there was no statistically detectable heritability of stickleback body size (h^2 = 0.007), while under low salinity conditions, there was significant heritability of body size (h^2 = 0.313; 2012). Based off these estimates of narrow-sense heritability in fishes as discussed above, there is sufficient evidence that a trait with a h^2 of 0.416 can be considered significantly heritable (Cresko et al. 2008; Hagen & Gilbertson 1973; McGuigan et al. 2012). This supports that mandible length variation in *opallus* zebrafish is not predominantly determined by environmental, epistatic, or dominance effects.

Breeding Values of Parents

BLUPs were used to calculate breeding values to select ideal parents for future genetic mapping as well as to estimate how much offspring may resemble their parents' phenotype (Robinson 1991; Table 2). Here, we present the breeding values as absolute units, where the scale and magnitude of said predicted breeding values are unique to each study (Falconer & MacKay 1996). An individual with a high breeding value relative to other specimens will likely produce offspring with the intended phenotype.

Sire breeding values were as follows: Sire 1 (-2.54), Sire 2 (-2.67), Sire 3 (-2.66), Sire 4 (-2.70), and Sire 5 (-2.81). This indicates that Sire 1 would likely produce offspring with longer mandibles because he had the greatest breeding value overall. Similarly, Sire 5 would likely

produce offspring with shorter mandibles because he had the lowest breeding value. We also found that there is a very strong relationship between corrected mandible length and predicted breeding values of these sires. Our Pearson correlation of these two variables produced a r value of 0.935 ($p= 0.019$) indicating that sire phenotype is a good predictor of breeding value as well as offspring phenotype for our sampled males for our population (Fig 4). Therefore, if we wanted to choose sires that were likely to produce offspring with the desired phenotype of long mandibles, we could base our sire selection off their breeding values alone.

We calculated the breeding values for each dam as well. We found that there was no consistent pattern of dam or sire contributing more additive genetic effects, as dam breeding value was sometimes greater than that of their mated sire, and sometimes lower (Fig 5). Dam 1,2 had the greatest breeding value of -2.42 while Dam 5,2 had the smallest breeding value of -2.90 of the fifteen females examined. Our Pearson correlation comparing corrected mandible lengths of dams to their breeding values produced a correlation coefficient of -0.07 ($p= 0.98$). This indicates that the breeding values of our sampled dams were poor predictors of actual jaw phenotype and offspring phenotype. Following a similar scenario to that discussed above, if we intended to produce offspring with long mandibles, we would need to select dams with long mandibles, rather than relying on their breeding values alone.

The corrected mandible lengths of offspring fell between the breeding values of both parents for all fifteen clutches (Fig 5). This was expected as offspring phenotypes are an intermediate of the additive genetic contributions of both parents, therefore their mean phenotypes should fall somewhere in the middle.

Parent breeding values were calculated with the intention of using these values to select ideal parents for future genetic mapping and QTL analyses. We choose the male with the longest

mandibles and highest breeding value (Sire 1) because of the strong correlation between the two variables (r= 0.935). However, since dam breeding value was poorly correlated with actual phenotype (r= -0.07), we did not rely on the predicted breeding value to make this selection. Instead, we chose the female with the shortest mandibles (Dam 1,2). Choosing parents with extreme phenotypes (extreme *longjaw* male and extreme *shortjaw* female) will allow us to produce a F1 generation with significant phenotypic variation that will be necessary for genetic mapping. The full-sibling offspring produced by these two individuals, or Clutch 2, will serve as our F1 generation (MacKay 2001). Following the QTL breeding design as described by MacKay (2001), two individuals from the F1 generation will then be self-crossed to produce a F2 generation that can be used for genetic mapping.

Thyroxine Concentrations in Zebrafish Tissues

Based off previous studies, we hypothesized that *opallus* tissues would contain significantly greater levels of T4 when compared to wildtype. However, we found that *opallus* does not produce significantly different concentrations of T4 during the onset of metamorphosis in comparison to AB zebrafish (Fig 6). We found that AB tissues contained approximately 15.11 ng/mL of thyroxine, while *opallus* tissues contained approximately 15.86 ng/mL of thyroxine. Although we found higher levels of T4 in *opallus*, a one-tailed t-test yielded a p-value of 0.236. We must therefore fail to reject the null hypothesis.

These concentration estimates were not what we expected to find, as these sample concentrations were smaller than the detection range of our ELISA kit (20-320 ng/mL). From the standard curve, we can also see that between 0 and 20 ng/mL the optical density plateaus (Fig S2). This suggests that sample concentrations within this range cannot be accurately measured

because there is not enough of a difference to differentiate between the optical densities. Similarly, a previous study that compared T4 concentrations between *opallus* and wildtype zebrafish reported a much greater difference. McMenamin et al. (2014) showed that *opallus* fish produced approximately three times as much T4 as their wildtype counterparts, with a significant p-value of <0.01. The authors did not report the detection range of their ELISA kit, the age at which their samples were taken, what type/s of samples were assayed (tissue homogenates, serum, plasma, etc.), nor the actual concentration of T4 measured (only relative abundance estimates were provided). With so many unknowns, it is difficult to compare our results to those of McMenamin and colleagues.

Due to timing and resource constraints, we were not able to purchase and test an alternate ELISA kit with a different detection range. Without previous concentration ranges from which to make estimates we could not determine which detection range would be appropriate for our samples. We suggest that in the future a kit with a narrower detection range should be used (0-20 ng/mL may be ideal). While the results of this thyroid hormone assay are inconclusive, we now have the necessary training with ELISA techniques for future investigations.

CONCLUSIONS

Mandible length is an important target for evolutionary and developmental studies of fish feeding mechanics due to its role in determining trophic niche (Conith et al. *in press*; Helfman et al. 2009; Westneat 2004; Cooper et al. 2017). Changes in mandible length can affect biomechanical traits which heavily impact fishes' abilities to successfully exploit different food resources (Westneat 2004; Westneat 1994; Conith et al. *in press*). Amongst the danionine fishes, species with elongated mandibles have significantly faster feeding strikes, larger mouth gapes, and faster gape speeds (Conith et al. *in press*). Mandible length in this lineage has also undergone positively correlated evolution with both feeding strike speed and gape size (Conith et al. *in press*). Modulation of TH signaling has also been identified as an important factor in the evolutionary diversification of fish feeding mechanics (Cooper et al. 2020; Galindo et al. 2019; Conith et al. *in press*; Shkil et al. 2012). This project provides a justification for preceding with a genetic mapping study directed at identifying the genomic differences in *longjaw* and *shortjaw* fish which contribute to the determination of mandible length during zebrafish development. This work has the potential to provide greater insight into the changes in danionine skull morphogenesis which have promoted ecological diversification of this lineage and in other fishes.

A trait with narrow-sense heritability that is significantly different from zero can respond to selection and is therefore capable of adaptation (Falconer & MacKay 1996; van Tienderen & de Jong 1994). Our h^2 estimate of 0.416 indicates that zebrafish mandible length should be capable of responding to selection. Because this estimate is greater than the range which is typical of adaptive traits in the wild (0.1-0.2; Visscher et al. 2006), it is plausible that zebrafish

mandible length may be capable of adaptation. It is also plausible that in other danionine fishes, mandible length is heritable and therefore able to respond to selection as well. Recent work indicates that not only has this trait evolved in the Danioninae, but that these changes are linked to important differences in feeding mechanics that have arisen during the last 50 million years (Conith et al. *in press*).

Differences in mandible length have been observed among danionine species that utilize different feeding strategies (Conith et al. *in press*). Longer or shorter jaws confer different MAs when the mandible is both abducted (rotated away from the upper jaw during mouth opening) and adducted (rotated toward the upper jaw during mouth closing; Westneat 1994; Westneat 2004). Lower MAs can facilitate faster jaw movement (e.g., faster feeding strikes), while higher MAs can promote greater force transmission (e.g., harder bites), either of which can enhance feeding performance depending on the food source (Westneat 1994; Westneat 2004). Mandible elongation can also shift the location of the mouth opening to a more superior positioning which can improve fishes' ability to feed from the surface (Helfman et al. 2009). One example of this is the giant danio (*Devario aequipinnatus*), a species which has significantly longer mandibles than many other danionines and has also specialized on surface feeding (McClelland 1839). Furthermore, if the mandible is lengthened the maximum distance between the upper and lower jaws (i.e., maximum gape distance) is increased without altering the gape angle, this can allow species to acquire larger prey (Westneat 1994; Westneat 2004). Because mandible length is variable among the Danioninae (which likely share genetic mechanisms of mandible length determination) and affects fish feeding niche, this study is relevant to understanding the trophic diversification of the danionine fishes.

Danionine fishes like the zebrafish begin exogenous feeding at very small sizes where they experience water as a more viscous fluid than they will encounter as adults (Galindo et al. 2019; Hernandez 2000; McMenamin et al. 2017). These fishes must therefore undergo developmental transitions between functional jaw morphologies which allow for successful feeding in different physical environments and ecological niches. These developmental transitions are facilitated by the production and secretion of TH. Thyroid hormone is an important regulator of skeletal development as increased TH levels triggers metamorphosis in fishes (McMenamin & Parichy 2013; Williams & Bassett 2018). Thyroid hormone signaling has recently been recognized as an important target for evolutionary and developmental studies of fish feeding (Cooper et al. 2020; Galindo et al. 2019; Conith et al. *in press*). Changes in TH signaling have also been identified to play a role in the adaptive diversification of the Cypriniformes (Shkil et al. 2012), the order of ray-finned fishes to which the Danioninae belong (Stout et al. 2016).

While TH is necessary for normal metamorphosis-induced remodeling of the jaws, the mechanisms by which TH elicits different responses in different tissues or organs (e.g., different bones) are not yet fully understood (Galindo et al. 2019; Bassett & Williams 2016; Berry et al. 1998; Shkil et al. 2012). Shkil et al. (2012) found that altering TH profiles during early development influenced the timing and formation of some cranial elements in cyprinid fishes, while leaving others unaffected. Under hyperthyroid conditions, changes in the ossification time of certain skull bones resulted in abnormal head morphologies (Shkil et al. 2012). Other cranial elements developed normally in regard to both timing and shape formation (Shkil et al. 2012). Although hyperthyroidism is a condition of the entire organism, different tissues exhibit different

responses to elevated TH production. The findings of this study suggest that different skull bones can possess different mechanisms of responding to TH.

Localized changes in the developmental responsiveness of specific skull bones could allow for small-scale changes to fish feeding biomechanics. This could promote "evolutionary tinkering" or "fine tuning" of fish feeding ability that might be more likely to confer adaptive advantages than large-scale changes in overall skull morphology (Laudet 2011; Stewart 2015; Carroll 2006). The findings of this study indicate that there is heritable variation for the degree to which zebrafish mandibles respond to TH during development. This could potentially facilitate localized changes to lower jaw morphology that allow fishes to shift between different food sources while preserving the basic integration of their cranial mechanics.

The development of upper jaw elements in the giant danio are similar to those of hypothyroid zebrafish (Galindo et al. 2019), while their lower jaws resemble those of hyperthyroid *longjaw* zebrafish (Fig 7*;* Conith et al. *in press*). During their development, both cranial regions should experience similar, if not identical, TH blood levels. It is therefore plausible that changes in the response of different skull elements to TH could have promoted the evolution of this species' particular feeding mechanisms. In comparison to the zebrafish, their developing upper jaws may be less sensitive to TH, while their developing mandibles may be more sensitive. The giant danio employs a feeding strategy that is very different from that of the zebrafish in terms of the functional abilities of both the upper and lower jaws (Conith et al. *in press*). It also occupies a different trophic niche (Conith et al. *in press*; Talwar & Jhingran 1991; McClelland 1839; Parichy 2015; McClure et al. 2006). It is unlikely that changes in TH production, which would expose all tissues to new TH levels during metamorphosis, could have produced these regional differences in zebrafish and giant danio skulls. Evolutionary tinkering

via the changes in tissue-specific responses to TH signaling therefore represents a more plausible adaptive scenario.

The findings of our study are highly relevant to understanding how the developmental responses of individual bones to TH are determined. They confirm that there is heritable variation in the response of zebrafish mandible elongation to TH. This justifies moving forward with a genetic mapping study with intentions of identifying the genomic regions that determine this response. Such work has the potential to inform our understanding of how specific developmental changes have promoted the adaptive diversification of the Danioninae and other fishes.

FUTURE DIRECTIONS

Quantitative Trait Locus Analysis

Evolutionary and developmental investigations seek to understand how changes in development shape the evolution of species. The calculation of genotype-to-phenotype maps is a critical component of these efforts. QTL mapping represents a valuable way to determine which regions of an organism's genome influence specific traits, but QTL mapping in fishes requires a significant investment of funding, time, and other resources. These resources cannot reasonably be invested in attempting to map traits that are predominantly determined by environmental factors (i.e., traits with low narrow-sense heritability). Because we have evidence of a high level of narrow-sense heritability for this trait in *opallus* zebrafish, we have the justification for investing resources in an attempt to identify genomic regions that affect mandible length; a project that the Cooper Lab has been funded by the National Science Foundation to complete.

Two F1 individuals were chosen from Clutch 2 of the heritability study because their parents (Sire 1 and Dam 1,2) represented phenotypic extremes on either end of the mandible length spectrum (extreme *longjaw* sire, extreme *shortjaw* dam). This suggested that their F2 offspring would exhibit the variation necessary for a successful mapping study if mandible length was found to be heritable. We have since bred and raised F2 offspring from this pair to facilitate a mapping cross and QTL analysis. At this time, an estimated 1,300 F2 specimens have been reared to adulthood. These should be sufficient sample sizes for the QTL analyses that will move forward.

Caudal fin samples will be taken from each fish and stored at -80°C in 100% pure ethanol before fixation in 10% formalin and storage in 75% ethanol. Standard length (mm) and mandible

length (mm) measurements will be taken from each specimen following the same procedure outlined in our heritability study. DNA from the caudal fin will be extracted and sequenced once sampling has concluded.

This work may also have applications for human craniofacial medicine. Mandibular prognathism is a relatively common craniofacial disorder in humans (Chang et al. 2006). Many patients with severe mandibular prognathism experience difficulty with chewing, speaking, and breathing, and often require corrective orthodontic surgery (Jacobson et al. 1974; Li et al. 2020). These individuals tend to undergo the greatest lower jaw growth during pubescence (Gomes & Lima 2006). The parallel between zebrafish developing their elongated mandibles during metamorphosis and humans experiencing the greatest mandible elongation during puberty suggests there may be underlying hormonal causes in both species.

Zebrafish are powerful models for understanding human health (Carnovali et al. 2019; Bradford et al. 2017; Howe et al. 2013). This is especially relevant within the realm of craniofacial malformation, as many previous studies of human craniofacial diseases have utilized the zebrafish model (Cooper et al. 2013; Raterman et al. 2020; Machado & Eames 2017; Van Otterloo et al. 2016). A recent study has identified a single-nucleotide polymorphism within the human gene *MYO1H* that is associated with mandibular prognathism (Atteeri et al. 2021). Our QTL study will test the hypothesis that the zebrafish ortholog, *myo1ha*, is associated with mandible elongation in *opallus*.

RNA-Sequencing and Transcriptomics

We also plan to use RNA-sequencing and transcriptomic analyses to examine the differences in gene expression in the developing mandibles of different zebrafish lines. We will

dissect off the mandibles of mid-metamorphic *shortjaw* and *longjaw opallus* zebrafish, wildtype (AB) zebrafish, a hypothyroid transgenic line of zebrafish whose thyroid follicles have been chemically ablated *Tg(tg:nVenus-2a-nfnB)*$^{wp.rt8}$, and giant danio.

We will use the RNeasy Plus Micro kit (QIAGEN, Germantown, MD) to extract RNA samples from the homogenized mandible tissues from each sampling group. Standard RNA-seq procedure will be followed to construct libraries, sequence samples, and process sequence reads. We hope to be able to identify differential expression among these lines and species to gain insight into how gene transcription during this critical time of development affects mandible length.

FIGURES

Figure 1. Comparison of mandible morphology between *longjaw* and *shortjaw opallus* phenotypic variants. A) *Longjaw opallus* with significantly elongated mandibles. B) *Shortjaw opallus* with lower jaw morphology which closely resembles that of a wildtype zebrafish.

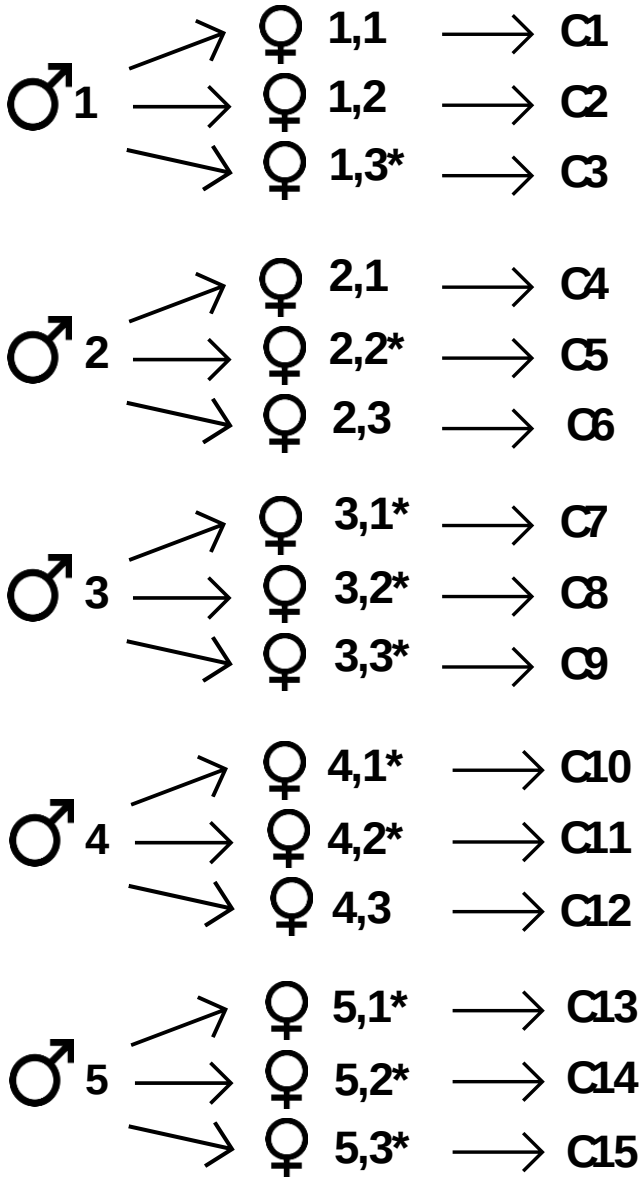

Figure 2. Diagram of the nested, full-sibling, half-sibling breeding design used for the heritability study. Pairings marked with an asterisk required *in vitro* fertilization. Pairings without asterisks were mated naturally.

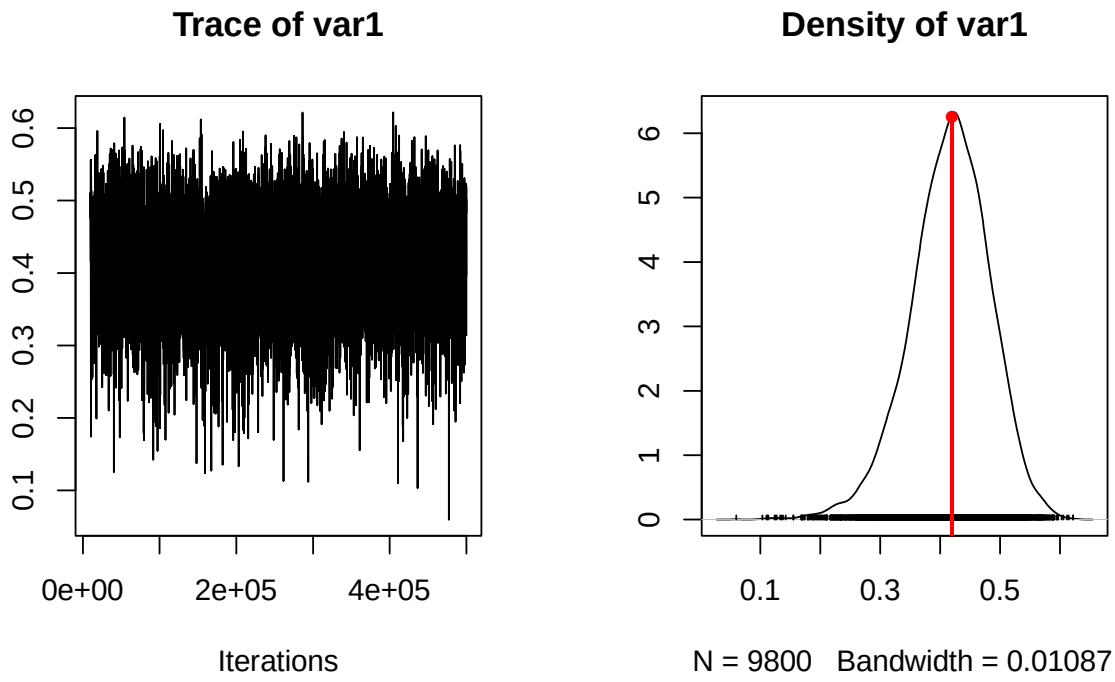

Figure 3. Trace (left) and posterior density (right) of the heritability of the mandible length in the zebrafish. Trace and density plots are used to evaluate model sensitivity and identify the upper and lower limits of a 95% confidence interval. The trace (left) plot allows us to check convergence and autocorrelation. Our heritability trace plot follows no specific pattern, indicating that there is a good spread and that the autocorrelation values between Markov chain iterations are weak. The posterior density (right) plot shows the likelihood of heritability falling within the values on the curve. The curve corresponds with our 95% confidence interval (0.287 - 0.547), with the final h^2 estimate resting at the peak of the curve (0.416), highlighted by the red line.

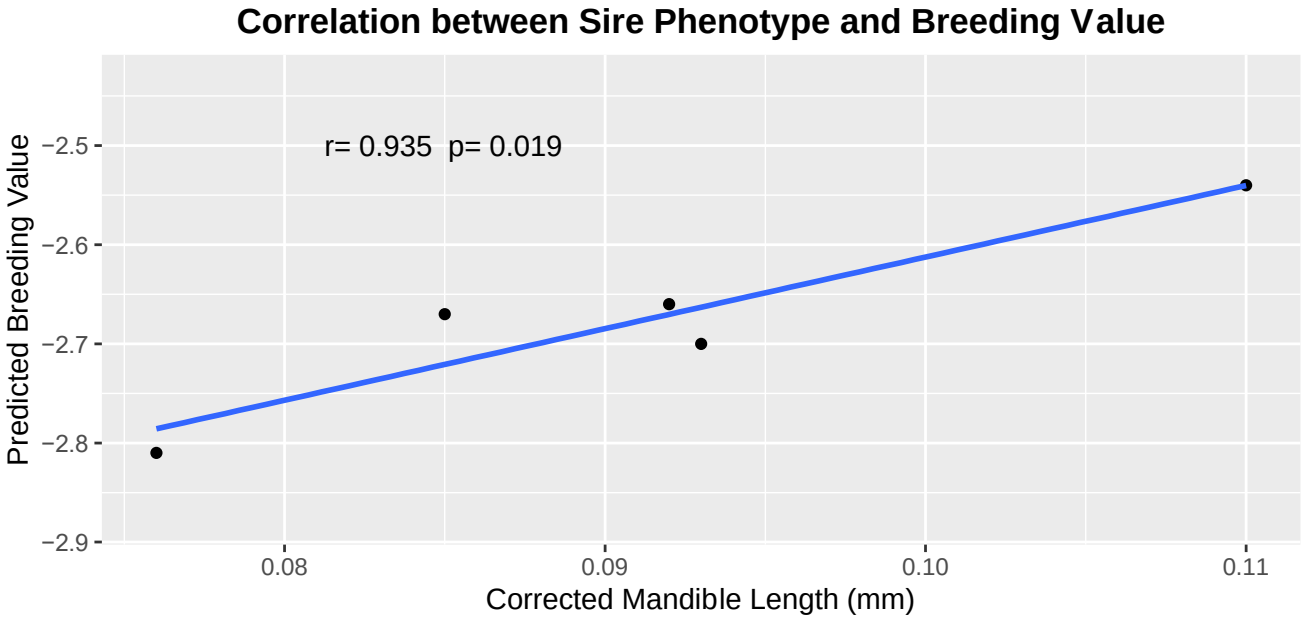

Figure 4. Correlation between actual phenotype (corrected mandible length measurements) and predicted breeding value for our sampled sires. The correlation coefficient of 0.935 (p= 0.019) indicates that there is a strong relationship between physical mandible length and breeding value within our sampled sires. Because of this strong relationship, we can use sire phenotype or breeding value as a reliable predictor of offspring phenotype.

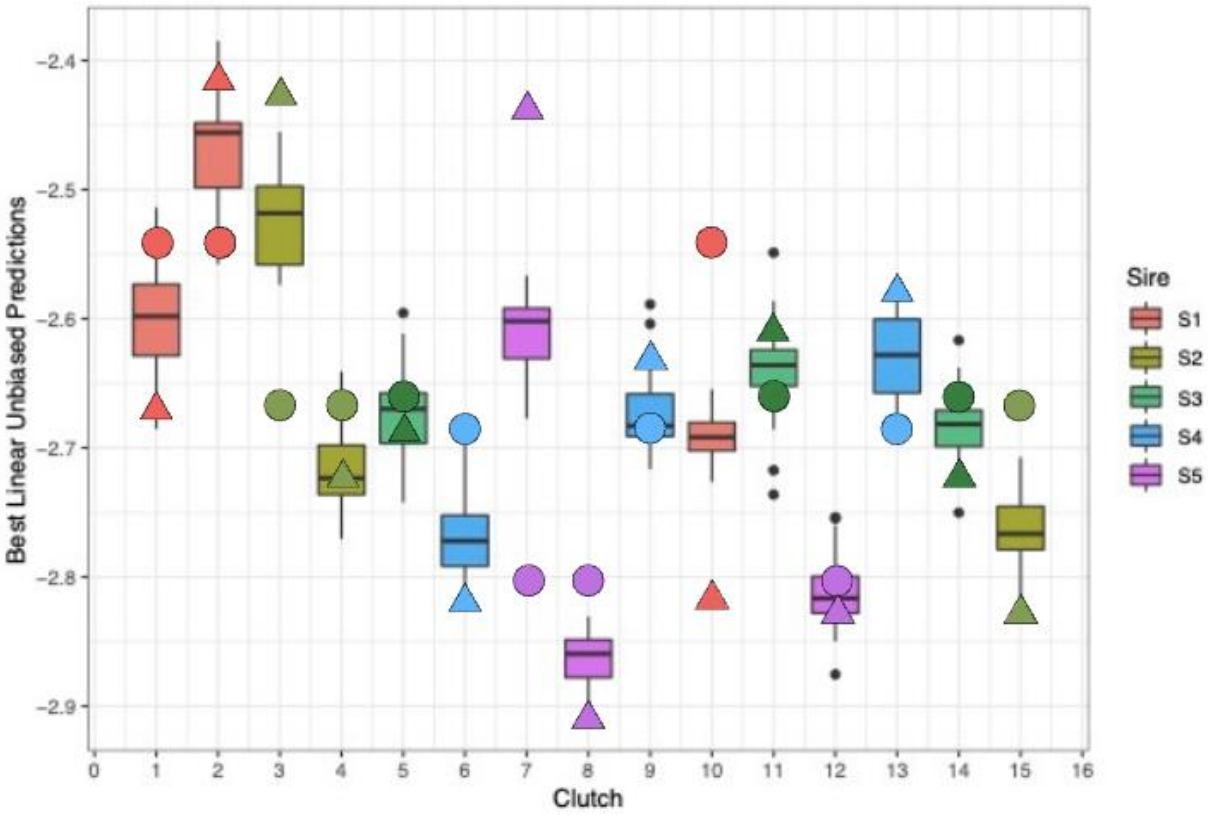

Figure 5. Predicted corrected mandible length of full-sibling families (boxplots) with predicted parent breeding values overlayed. Circles represent breeding values for sires, triangles represent breeding values for dams for each family. Breeding values and corrected mandible length values are based off 500,000 Markov chain iterations. The corrected mandible lengths of all offspring families fell between the breeding values of both parents for all fifteen clutches. This was expected as offspring phenotypes are an intermediate of the additive genetic contributions of both parents, therefore their mean phenotypes should fall within the middle.

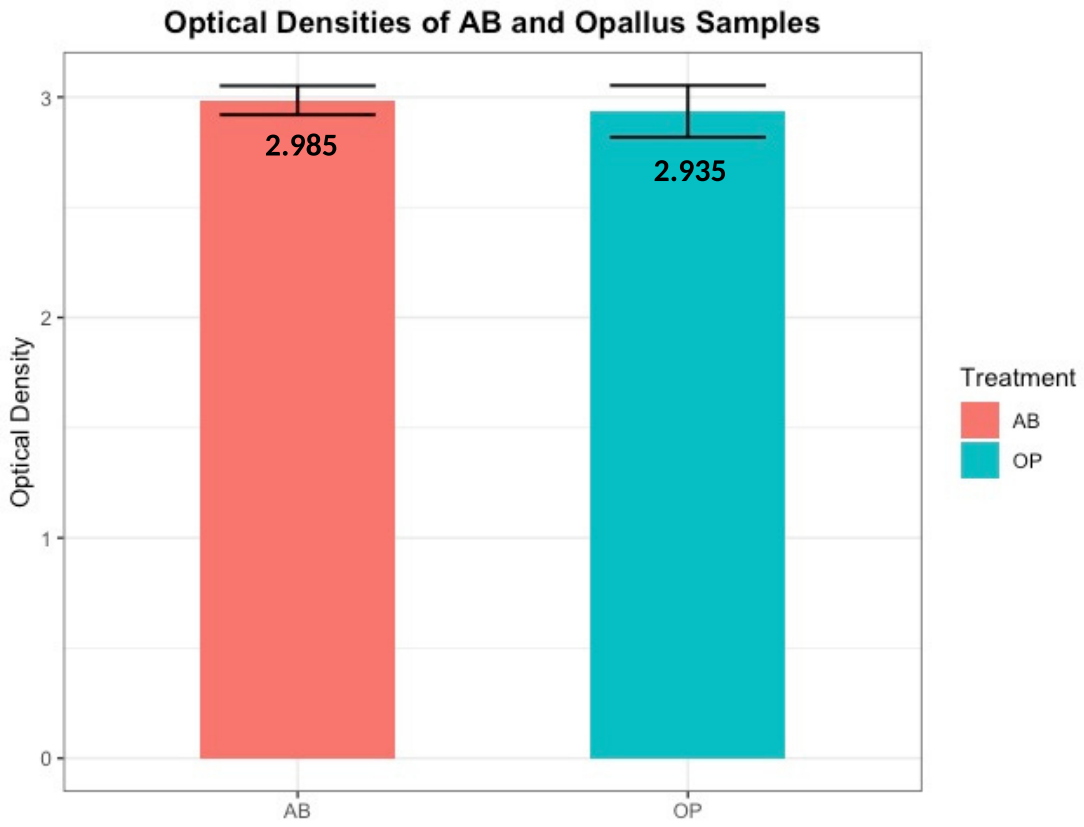

Figure 6. Optical densities of AB and *opallus* (OP) samples from our thyroid hormone (T4) assay. A one-tailed t-test found that there was not a significant difference in optical densities of AB samples versus *opallus* samples (p=0.236). Optical density is inversely proportional to thyroxine concentration (i.e., higher optical density indicates lower sample thyroxine concentration). While *opallus* samples were technically hyperthyroid when compared to AB, we do not see the significant difference as reported by previous authors such as McMenamin et al. (2014).

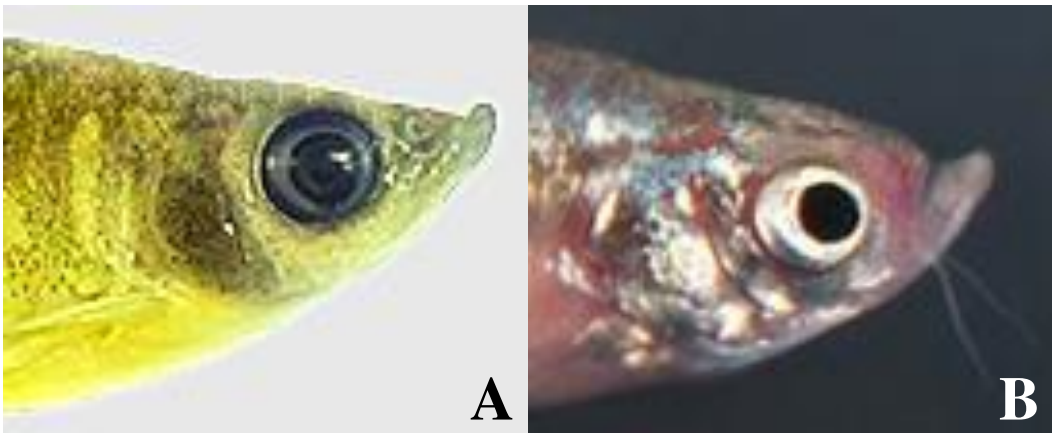

Figure 7. Comparison of A) giant danio (*Devario aequipinnatus*) with mandibular prognathism to a B) *longjaw opallus* zebrafish (*Danio rerio*).

TABLES

Table 1. Optical density reads from ELISA testing. Blanks and standards were assayed in triplicate while AB samples were assayed in 4 replicates, and *opallus* samples were assayed in 5 replicates. Optical densities for each treatment were averaged then corrected for the blank well optical density. Optical density is inversely proportional to thyroxine concentration in each sample.

	Optical Densities (read at 450nm)					
Treatment	1	2	3	4	5	Average
Blank	0.0436653	0.0436849	0.0432144	-	-	0.043531*
S0 (0 ng/mL)	3.04398	2.93545	3.09399	-	-	2.9809
S1 (20 ng/mL)	3.0178	3.02891	3.00809	-	-	2.97468
S2 (40 ng/mL)	1.47605	1.38144	1.53786	-	-	1.4216
S3 (80 ng/mL)	0.672284	0.723632	0.636415	-	-	0.6339
S4 (160 ng/mL)	0.373311	0.299975	0.345131	-	-	0.2959
S5 (320 ng/mL)	0.170689	0.165481	0.160492	-	-	0.122
AB	3.00472	3.02128	2.88841	3.02832	-	2.9421
Opallus	3.06343	3.02832	2.77115	2.93593	2.87866	2.8919

Note: Average for the blank group was not blank corrected for redundancy.

Table 2. Breeding value predictions for parents used in heritability study. BLUPs were adjusted for each stored Markov chain iteration's unique intercept then averaged to produce a single breeding value, as shown below. Scale and magnitude of each breeding value is relative. Individuals with greater breeding values compared to other individuals sampled will contribute more alleles for mandible length to their offspring.

Individual	Mated Sire	Clutch	BLUP
S1	-	-	-2.545715
S2	-	-	-2.670249
S3	-	-	-2.660009
S4	-	-	-2.698923
S5	-	-	-2.808823
D1,1	S1	1	-2.6531316
D1,2	S1	2	-2.4240059
D1,3	S1	10	-2.8188557
D2,1	S2	3	-2.4281098
D2,2	S2	4	-2.7583452
D2,3	S2	15	-2.835774
D3,1	S3	5	-2.6869453
D3,2	S3	14	-2.7056934
D3,3	S3	11	-2.6182272
D4,1	S4	6	-2.8303147
D4,2	S4	9	-2.6460266
D4,3	S4	13	-2.5743684
D5,1	S5	7	-2.4481195
D5,2	S5	8	-2.9000775
D5,3	S5	12	-2.8107068

S= Sire
D= Dam

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SUPPLEMENTAL MATERIALS

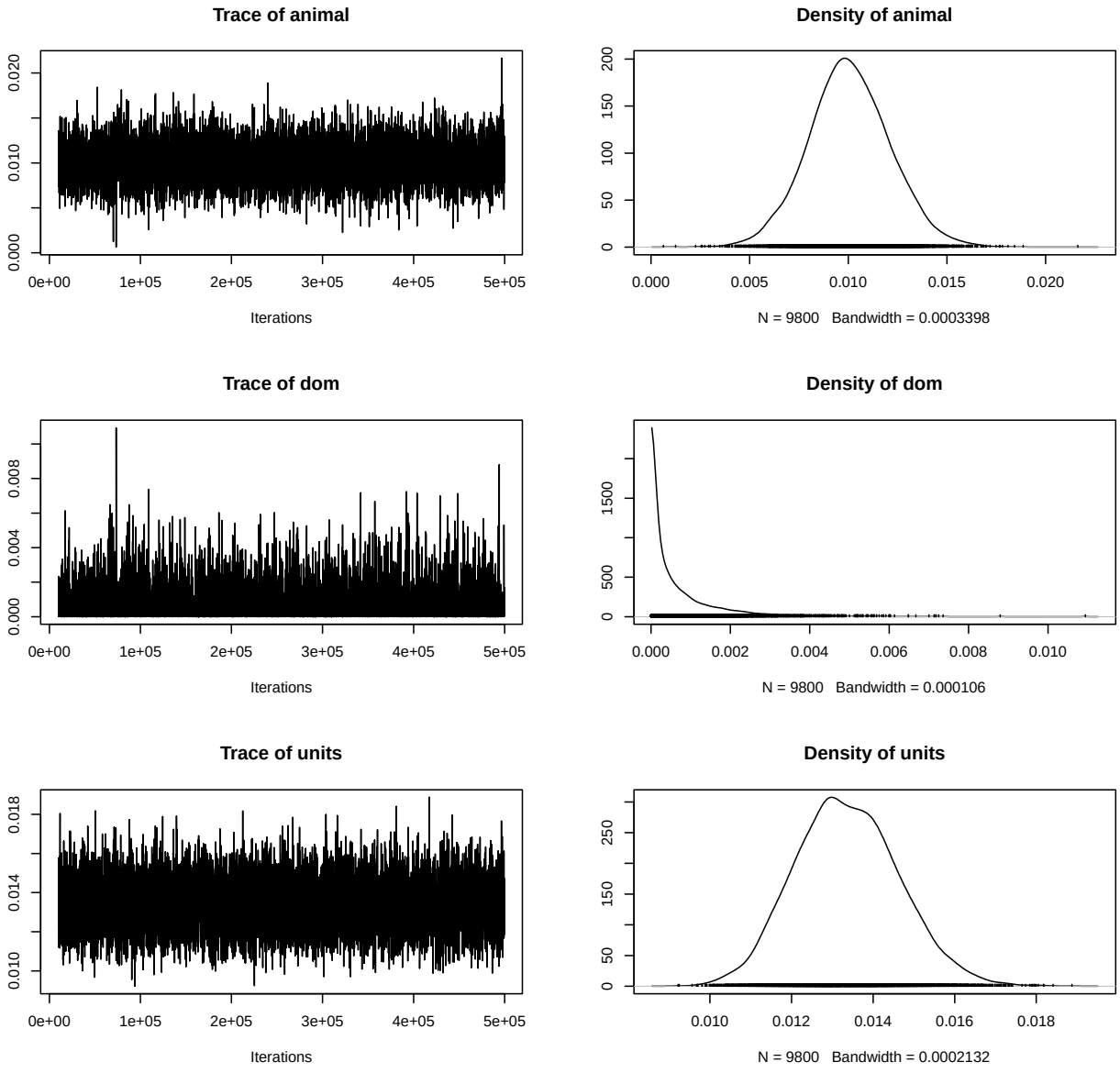

Figure S1. Trace and density plots of variance components from model sensitivity (VCV) analysis. Trace (left) plots allow us to check convergence and autocorrelation for the three variance components incorporated in our model. Our trace plots follow no specific pattern, indicating that there is a good spread and that the autocorrelation values between iterations are weak. Density plots (right) show the estimated posterior distributions of the three variance components. Posterior distributions appear as expected. These plots were used to confirm the model was calibrated correctly before moving onto the estimation of heritability.

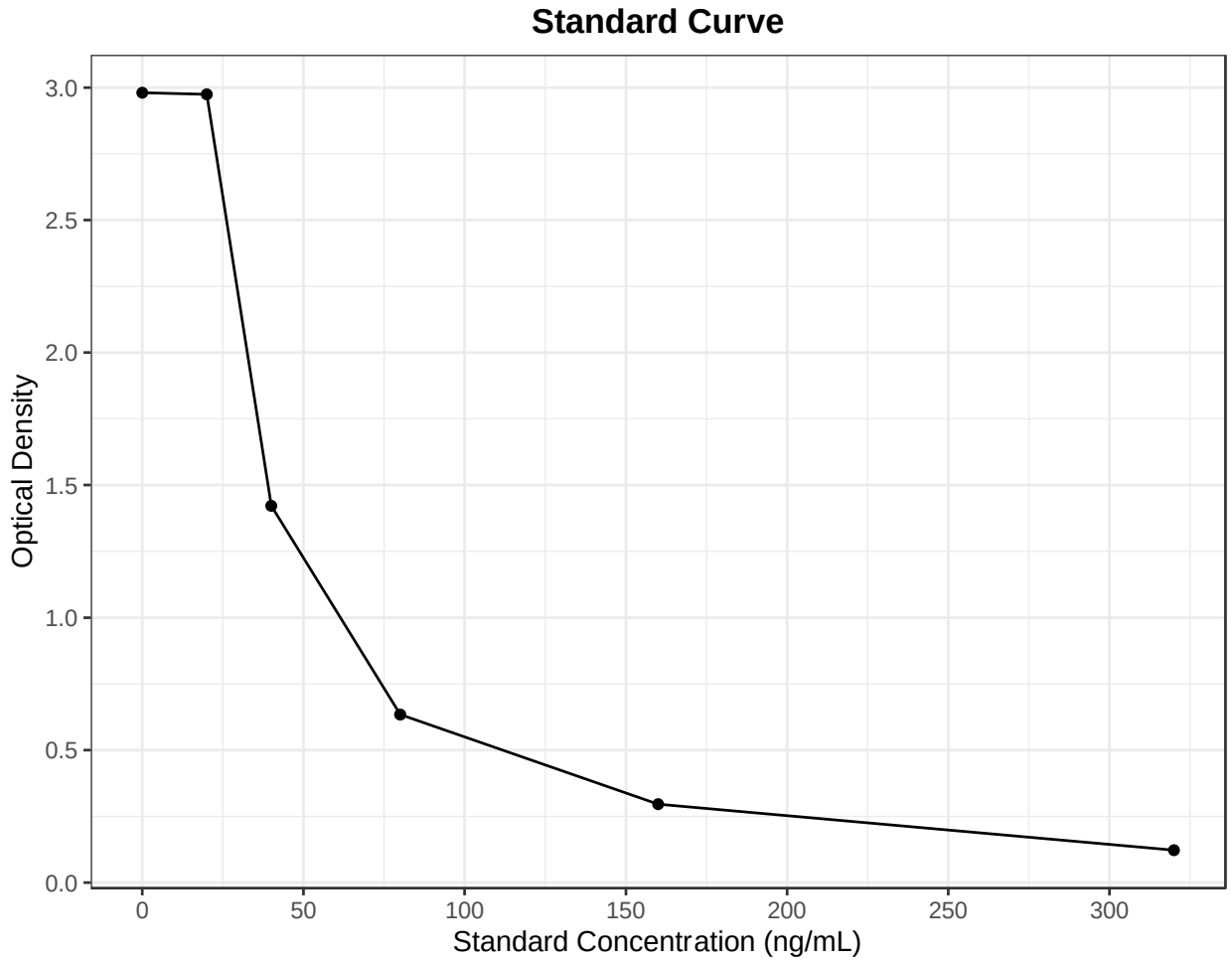

Figure S2. Standard curve generated from our ELISA test. Averages of triplicates were blank corrected then plotted to produce a standard curve. Concentrations of AB and *opallus* samples were interpolated from this standard curve using GraphPad Prism 9 Software (version 9.3.1) .

Table S1. Heritability Study Data

Individual	Sire	Dam	Clutch	Sex	SL (mm)	(ML mm)	ML norm (mm)
1	1	1,1	1	f	21.54	1.65	0.07660167
2	1	1,1	1	f	25.71	2.08	0.08090237
3	1	1,1	1	f	22.83	1.8	0.07884363
4	1	1,1	1	m	18.43	1.89	0.10255019
5	1	1,1	1	m	20.45	1.65	0.0806846
6	1	1,1	1	f	22.06	2.09	0.09474161
7	1	1,1	1	f	23.72	2.04	0.08600337
8	1	1,1	1	f	21.79	1.82	0.08352455
9	1	1,1	1	f	21.08	2.06	0.09772296
10	1	1,1	1	f	20.42	1.67	0.08178257
11	1	1,1	1	m	20.64	1.81	0.0876938
12	1	1,1	1	m	22.83	1.89	0.08278581
13	1	1,1	1	m	21.2	1.48	0.06981132
14	1	1,1	1	f	20.46	1.67	0.08162268
15	1	1,1	1	f	26.2	2.64	0.10076336
16	1	1,1	1	m	20.25	1.56	0.07703704
17	1	1,1	1	f	21.77	1.88	0.08635737
18	1	1,1	1	f	24.72	1.83	0.07402913
19	1	1,1	1	f	20.17	1.55	0.0768468
20	1	1,1	1	f	19.81	1.61	0.08127208
21	1	1,1	1	m	20.73	1.47	0.07091172
22	1	1,1	1	m	20.24	1.45	0.07164032
23	1	1,1	1	m	16.51	1.1	0.06662629
24	1	1,1	1	f	22.05	1.98	0.08979592
25	1	1,1	1	f	20.9	1.49	0.07129187
26	1	1,1	1	f	24.33	2.01	0.08261406
27	1	1,1	1	m	23.63	1.78	0.07532797
28	1	1,1	1	f	19.67	1.37	0.06964921
29	1	1,1	1	f	22.62	2.11	0.09328028
30	1	1,1	1	f	21.34	1.86	0.08716026
31	1	1,1	1	f	20.76	1.55	0.07466281
32	1	1,1	1	f	20.59	1.67	0.08110733
33	1	1,1	1	f	22.24	1.48	0.06654676
34	1	1,1	1	f	19.16	1.58	0.08246347
35	1	1,1	1	f	19.38	1.15	0.05933953
36	1	1,1	1	f	19.99	1.21	0.06053027
37	1	1,1	1	f	19	1.61	0.08473684

38	1	1,1	1	f	17.9	1.15	0.06424581
39	1	1,1	1	f	19.8	1.32	0.06666667
40	1	1,1	1	f	21.25	1.55	0.07294118
41	1	1,1	1	f	23.66	1.62	0.06846999
42	1	1,1	1	f	22.13	1.86	0.0840488
43	1	1,1	1	f	22.78	1.81	0.07945566
44	1	1,1	1	f	20.69	1.65	0.07974867
45	1	1,1	1	f	22.18	1.64	0.07394049
46	1	1,1	1	m	22.56	1.46	0.06471631
47	1	1,1	1	m	19.9	1.45	0.07286432
48	1	1,1	1	f	21.2	1.71	0.08066038
49	1	1,1	1	f	22.14	1.54	0.06955736
50	1	1,1	1	f	21.55	1.58	0.07331787
51	1	1,1	1	m	20.88	1.24	0.05938697
52	1	1,1	1	f	18.7	1.13	0.06042781
53	1	1,1	1	f	21.7	1.68	0.07741935
54	1	1,1	1	f	19.11	1.62	0.08477237
55	1	1,1	1	f	22.81	1.37	0.06006138
56	1	1,1	1	f	19.38	1.51	0.07791538
57	1	1,1	1	f	21.19	1.14	0.05379896
58	1	1,1	1	m	20.55	1.53	0.07445255
59	1	1,1	1	m	21.7	1.37	0.06313364
60	1	1,1	1	m	23.7	1.8	0.07594937
61	1	1,1	1	f	21.33	1.42	0.0665729
62	1	1,1	1	f	19.53	1.28	0.06554019
63	1	1,1	1	f	22.12	1.46	0.06600362
64	1	1,1	1	f	22.05	1.94	0.08798186
65	1	1,1	1	m	19.02	1.35	0.07097792
66	1	1,1	1	f	20.11	1.32	0.06563899
67	1	1,1	1	f	20.77	1.28	0.06162735
68	1	1,1	1	f	23.18	1.72	0.0742019
69	1	1,1	1	f	21.44	1.57	0.07322761
70	1	1,1	1	f	23.28	2.01	0.08634021
71	1	1,1	1	f	22.09	1.29	0.05839746
72	1	1,1	1	m	17.08	1.35	0.07903981
73	1	1,1	1	f	23.31	1.62	0.06949807
74	1	1,1	1	f	18.57	1.02	0.0549273
75	1	1,1	1	f	22.58	1.7	0.07528787
76	1	1,1	1	f	23.16	1.84	0.07944732
77	1	1,1	1	f	21.78	1.43	0.06565657

78	1	1,1	1	f	24.8	1.45	0.05846774
79	1	1,2	2	f	23.4	2.2	0.09401709
80	1	1,2	2	f	22.03	2.12	0.09623241
81	1	1,2	2	m	24.11	2.09	0.08668602
82	1	1,2	2	m	23.76	2.27	0.09553872
83	1	1,2	2	f	23.48	2.25	0.09582624
84	1	1,2	2	f	22	2.7	0.12272727
85	1	1,2	2	f	23.14	1.89	0.08167675
86	1	1,2	2	f	23.61	2.33	0.098687
87	1	1,2	2	f	23.64	2.42	0.10236887
88	1	1,2	2	f	22.59	2.11	0.09340416
89	1	1,2	2	f	21.79	1.75	0.08031207
90	1	1,2	2	m	20.84	1.98	0.0950096
91	1	1,2	2	f	22.05	1.84	0.08344671
92	1	1,2	2	f	20.24	1.95	0.09634387
93	1	1,2	2	m	24.2	2.56	0.10578512
94	1	1,2	2	m	21.31	2.04	0.0957297
95	1	1,2	2	m	23.53	1.99	0.08457289
96	1	1,2	2	m	22.42	1.79	0.07983943
97	1	1,2	2	f	22.11	2.14	0.09678878
98	1	1,2	2	f	21.42	1.59	0.07422969
99	1	1,2	2	f	19.61	1.49	0.07598164
100	1	1,2	2	m	23.71	2.44	0.10291016
101	1	1,2	2	m	23.46	1.55	0.06606991
102	1	1,2	2	m	22.53	1.5	0.0665779
103	1	1,2	2	m	23.17	1.48	0.0638757
104	1	1,2	2	m	21.54	1.39	0.0645311
105	3	3,1	3	f	22.89	2.43	0.1061599
106	3	3,1	3	f	26.73	2.01	0.07519641
107	3	3,1	3	f	21	2.37	0.11285714
108	3	3,1	3	f	25.87	1.97	0.07614998
109	3	3,1	3	f	21.66	1.92	0.08864266
110	3	3,1	3	f	23.59	1.7	0.07206443
111	3	3,1	3	f	25.22	2.26	0.08961142
112	3	3,1	3	f	23.82	2.28	0.09571788
113	3	3,1	3	f	24.59	1.98	0.08052054
114	13	13,1	7	f	24.36	1.5	0.06157635
115	13	13,1	7	f	23.16	1.56	0.06735751
116	13	13,1	7	f	21.12	1.53	0.07244318
117	13	13,1	7	f	22.88	1.68	0.07342657

118	13	13,1	7	f	19.96	1.21	0.06062124
119	13	13,1	7	m	20.25	1.68	0.08296296
120	13	13,1	7	f	19.44	1.54	0.07921811
121	13	13,1	7	f	19.87	1.82	0.09159537
122	13	13,1	7	f	22.43	1.83	0.08158716
123	13	13,1	7	m	20.27	1.73	0.0853478
124	13	13,1	7	m	19.3	1.39	0.07202073
125	13	13,1	7	m	19.88	1.6	0.0804829
126	13	13,1	7	f	20.9	1.92	0.09186603
127	13	13,1	7	f	20.04	1.63	0.08133733
128	13	13,1	7	f	23.67	1.99	0.08407267
129	6	6,1	5	f	21.21	1.62	0.07637907
130	6	6,1	5	m	19.94	1.34	0.0672016
131	6	6,1	5	m	21.78	1.28	0.05876951
132	6	6,1	5	m	18.93	1.3	0.06867406
133	6	6,1	5	m	20.32	1.08	0.05314961
134	6	6,1	5	f	19.23	1.35	0.07020281
135	6	6,1	5	f	23.25	1.65	0.07096774
136	6	6,1	5	f	19.59	1.57	0.08014293
137	6	6,1	5	m	20.75	1.48	0.0713253
138	6	6,1	5	m	19.09	1.21	0.06338397
139	6	6,1	5	m	17.8	1.32	0.0741573
140	6	6,1	5	m	20.27	1.59	0.07844105
141	6	6,1	5	f	22.1	1.49	0.06742081
142	6	6,1	5	f	21.86	1.42	0.06495883
143	6	6,1	5	f	20.89	1.47	0.0703686
144	6	6,1	5	m	18.15	1.11	0.06115702
145	6	6,1	5	f	19.22	1.41	0.07336108
146	6	6,1	5	f	20.5	1.22	0.0595122
147	6	6,1	5	f	21.81	1.88	0.08619899
148	6	6,1	5	f	19.61	1.81	0.09229985
149	6	6,1	5	f	20.95	1.5	0.07159905
150	6	6,1	5	m	16.09	0.99	0.0615289
151	6	6,1	5	m	17.88	1.14	0.06375839
152	6	6,1	5	m	17.35	1.24	0.07146974
153	6	6,1	5	m	19.34	1.38	0.07135471
154	6	6,1	5	f	19.65	1.47	0.07480916
155	6	6,1	5	f	20.92	1.27	0.06070746
156	6	6,1	5	f	24.57	1.63	0.06634107
157	6	6,1	5	f	20.76	1.3	0.06262042

158	6	6,1	5	m	19.22	1.28	0.06659729
159	6	6,1	5	m	17.14	1.49	0.08693116
160	6	6,1	5	m	19.06	1.37	0.07187828
161	6	6,1	5	m	17	1.27	0.07470588
162	6	6,1	5	m	21.1	1.47	0.06966825
163	6	6,1	5	f	19.41	1.13	0.05821741
164	6	6,1	5	f	19.9	1.16	0.05829146
165	11	11,2	6	f	18.29	1.03	0.05631493
166	11	11,2	6	f	19.54	1.2	0.06141249
167	11	11,2	6	f	19.27	1.07	0.05552673
168	11	11,2	6	f	22.01	1.54	0.0699682
169	11	11,2	6	f	19.28	1.53	0.07935685
170	11	11,2	6	f	21.11	1.19	0.05637139
171	11	11,2	6	f	21.65	1.12	0.0517321
172	11	11,2	6	m	22.79	1.23	0.05397104
173	11	11,2	6	f	26.83	1.71	0.06373463
174	11	11,2	6	f	23.19	1.31	0.05648987
175	11	11,2	6	m	20.81	1.26	0.06054781
176	11	11,2	6	m	21.08	1.32	0.0626186
177	11	11,2	6	m	20.87	1.24	0.05941543
178	11	11,2	6	m	21.3	1.26	0.05915493
179	11	11,2	6	f	26.68	1.78	0.06671664
180	11	11,2	6	f	25.43	1.83	0.07196225
181	11	11,2	6	f	21.87	1.25	0.05715592
182	11	11,2	6	f	20.5	1.25	0.06097561
183	11	11,2	6	f	20.49	1.66	0.08101513
184	11	11,2	6	f	18.57	1.18	0.06354335
185	11	11,2	6	f	20.96	1.27	0.0605916
186	11	11,2	6	m	20.24	1.33	0.06571146
187	11	11,2	6	m	19.81	1.04	0.05249874
188	11	11,2	6	m	21.78	1.32	0.06060606
189	11	11,2	6	f	18.16	1.31	0.07213656
190	11	11,2	6	f	17.44	1.23	0.07052752
191	11	11,2	6	f	19.03	1.15	0.0604309
192	11	11,2	6	f	20.05	1.29	0.06433915
193	11	11,2	6	m	20.62	1.21	0.05868089
194	11	11,2	6	m	24.09	1.27	0.05271897
195	11	11,2	6	f	18.54	1.23	0.06634304
196	11	11,2	6	f	19.51	1.3	0.0666325
197	11	11,2	6	f	19.74	1.22	0.06180344

198	11	11,2	6	f	21.27	1.34	0.06299953
199	11	11,2	6	f	20.2	1.11	0.0549505
200	11	11,2	6	f	22.49	1.43	0.06358382
201	11	11,2	6	f	17.14	0.94	0.05484247
202	1	1,3	10	f	26.18	1.75	0.06684492
203	1	1,3	10	f	21.7	1.45	0.06682028
204	1	1,3	10	f	22.5	1.3	0.05777778
205	1	1,3	10	f	22.91	1.51	0.06591008
206	1	1,3	10	f	21.53	1.48	0.06874129
207	1	1,3	10	m	22.22	1.43	0.06435644
208	1	1,3	10	f	22.4	1.51	0.06741071
209	1	1,3	10	f	21.26	1.47	0.06914393
210	1	1,3	10	f	21.58	1.32	0.06116775
211	1	1,3	10	f	24.81	1.58	0.063684
212	1	1,3	10	f	25.42	1.57	0.06176239
213	1	1,3	10	f	21.69	1.37	0.06316275
214	1	1,3	10	f	20.54	1.19	0.05793574
215	1	1,3	10	f	22.91	1.7	0.0742034
216	1	1,3	10	f	19.33	1.27	0.06570098
217	1	1,3	10	f	21.43	1.58	0.07372842
218	1	1,3	10	f	21	1.59	0.07571429
219	3	3,4	4	f	25.33	1.6	0.06316621
220	3	3,4	4	f	25.15	1.52	0.06043738
221	3	3,4	4	f	25.44	1.57	0.06171384
222	3	3,4	4	f	17.77	1.28	0.07203151
223	3	3,4	4	f	17.63	1.24	0.07033466
224	3	3,4	4	f	19.78	1.73	0.08746208
225	3	3,4	4	f	21.7	1.53	0.07050691
226	3	3,4	4	m	19.96	1.25	0.06262525
227	3	3,4	4	m	18.09	1.11	0.06135987
228	3	3,4	4	f	24.82	1.49	0.06003223
229	3	3,4	4	f	19.79	1.26	0.06366852
230	3	3,4	4	f	18.86	1.22	0.06468717
231	3	3,4	4	f	18.95	1.43	0.07546174
232	3	3,4	4	f	20.34	1.54	0.07571288
233	3	3,4	4	f	19.37	1.44	0.07434177
234	3	3,4	4	f	18.86	1.31	0.06945917
235	3	3,4	4	f	26.16	1.8	0.06880734
236	3	3,4	4	f	27.07	1.74	0.0642778
237	3	3,4	4	f	26.39	1.41	0.05342933

238	3	3,4	4	f	27.27	1.59	0.05830583
239	3	3,4	4	f	26.34	1.58	0.05998481
240	3	3,4	4	f	19.85	1.22	0.06146096
241	3	3,4	4	f	20.67	1.16	0.05611998
242	3	3,4	4	m	19.19	1.19	0.06201146
243	3	3,4	4	f	20.23	1.14	0.05635195
244	3	3,4	4	f	20.81	1.35	0.06487266
245	3	3,4	4	f	18.55	1.4	0.0754717
246	13	13,2	8	f	24.2	1.42	0.05867769
247	13	13,2	8	f	24.13	1.49	0.06174886
248	13	13,2	8	f	21.42	1.05	0.04901961
249	13	13,2	8	f	21.55	1.18	0.05475638
250	13	13,2	8	f	19.22	1.06	0.05515088
251	13	13,2	8	f	19.92	1.16	0.05823293
252	13	13,2	8	f	17.94	1.02	0.05685619
253	13	13,2	8	f	23.17	1.17	0.05049633
254	13	13,2	8	f	21.75	1.27	0.0583908
255	13	13,2	8	f	20.78	1.12	0.05389798
256	13	13,2	8	f	21.91	1.13	0.05157462
257	13	13,2	8	f	20.05	1.17	0.05835411
258	13	13,2	8	f	24.38	1.13	0.04634947
259	13	13,2	8	f	23.5	1.2	0.05106383
260	13	13,2	8	f	21.31	1.15	0.05396527
261	13	13,2	8	f	23.72	1.16	0.04890388
262	13	13,2	8	f	19.08	1.19	0.06236897
263	13	13,2	8	m	18.04	1.07	0.05931264
264	13	13,2	8	m	17.62	0.92	0.05221339
265	13	13,2	8	m	18.86	1.04	0.05514316
266	13	13,2	8	m	16.74	0.97	0.05794504
267	13	13,2	8	m	19.15	1.12	0.05848564
268	13	13,2	8	m	19.23	1.1	0.05720229
269	13	13,2	8	f	24.29	1.29	0.05310828
270	13	13,2	8	f	25.62	1.56	0.06088993
271	13	13,2	8	f	23.89	1.36	0.05692758
272	11	11,3	9	f	19.59	1.53	0.07810107
273	11	11,3	9	f	19.97	1.32	0.06609915
274	11	11,3	9	f	17.92	1.49	0.08314732
275	11	11,3	9	f	22.12	1.47	0.0664557
276	11	11,3	9	f	19.91	1.78	0.08940231
277	11	11,3	9	f	21.93	1.48	0.06748746

278	11	11,3	9	f	18.75	1.38	0.0736
279	11	11,3	9	f	23.67	1.39	0.05872412
280	11	11,3	9	f	19.22	1.38	0.07180021
281	11	11,3	9	f	17.73	1.41	0.07952623
282	11	11,3	9	m	16.77	1.08	0.06440072
283	11	11,3	9	f	21.62	1.41	0.06521739
284	11	11,3	9	f	18.69	1.19	0.06367041
285	11	11,3	9	f	18.88	1.35	0.07150424
286	11	11,3	9	f	18.75	1.22	0.06506667
287	11	11,3	9	f	14.24	0.87	0.06109551
288	11	11,3	9	m	18.89	1.79	0.09475913
289	11	11,3	9	m	20.39	1.29	0.06326631
290	11	11,3	9	m	19.09	1.3	0.06809848
291	11	11,3	9	m	19.02	1.26	0.06624606
292	11	11,3	9	m	16.68	1.2	0.07194245
293	11	11,3	9	m	21.32	1.34	0.06285178
294	11	11,3	9	m	18.68	1.12	0.05995717
295	11	11,3	9	f	18.99	1.49	0.07846235
296	11	11,3	9	f	19.21	1.23	0.06402915
297	11	11,3	9	f	20.77	1.39	0.06692345
298	11	11,3	9	f	20.82	1.38	0.06628242
299	13	13,3	12	f	23.76	1.31	0.05513468
300	13	13,3	12	f	24.41	1.14	0.04670217
301	13	13,3	12	f	21.47	1.25	0.05822077
302	13	13,3	12	f	18.17	1.08	0.05943864
303	13	13,3	12	f	19.17	0.99	0.05164319
304	13	13,3	12	f	21.34	1.23	0.05763824
305	13	13,3	12	m	21.12	1.31	0.06202652
306	13	13,3	12	m	16.01	1.1	0.06870706
307	13	13,3	12	f	24.78	1.44	0.05811138
308	13	13,3	12	f	23.81	1.32	0.05543889
309	13	13,3	12	f	21.53	1.21	0.05620065
310	13	13,3	12	f	22.86	1.26	0.05511811
311	13	13,3	12	f	19.53	1.09	0.05581157
312	13	13,3	12	f	19.7	1.14	0.05786802
313	13	13,3	12	m	24.27	1.3	0.05356407
314	13	13,3	12	f	22.02	1.34	0.06085377
315	13	13,3	12	f	22.78	1.31	0.05750658
316	13	13,3	12	f	22.95	1.37	0.05969499
317	13	13,3	12	f	17.85	1.29	0.07226891

318	13	13,3	12	f	20.1	1.48	0.07363184
319	13	13,3	12	m	21.25	1.41	0.06635294
320	13	13,3	12	m	17.34	1.04	0.05997693
321	13	13,3	12	f	22.44	1.27	0.05659537
322	13	13,3	12	f	18.21	1.11	0.06095552
323	13	13,3	12	f	20.97	1.37	0.06533143
324	13	13,3	12	m	17.46	1.29	0.07388316
325	13	13,3	12	m	24.21	1.41	0.0582404
326	13	13,3	12	m	19.01	1.36	0.07154129
327	13	13,3	12	m	19.29	1.12	0.05806117
328	13	13,3	12	m	20.24	1.16	0.05731225
329	13	13,3	12	m	21.46	1.34	0.06244175
330	13	13,3	12	m	19.8	1.23	0.06212121
331	13	13,3	12	m	16.52	0.9	0.05447942
332	13	13,3	12	m	20.23	1.19	0.05882353
333	13	13,3	12	f	24.11	1.37	0.0568229
334	13	13,3	12	f	24.91	1.42	0.05700522
335	13	13,3	12	f	20.15	1.3	0.06451613
336	13	13,3	12	f	23.43	1.23	0.0524968
337	13	13,3	12	f	25.21	1.47	0.05831019
338	13	13,3	12	f	23.3	1.21	0.05193133
339	13	13,3	12	f	23.56	1.38	0.05857385
340	13	13,3	12	m	22.49	1.16	0.05157848
341	13	13,3	12	m	21.55	1.42	0.06589327
342	13	13,3	12	m	22.98	1.45	0.06309835
343	13	13,3	12	f	21.13	1.32	0.06247042
344	13	13,3	12	f	25.02	1.39	0.05555556
345	13	13,3	12	f	19.31	1.18	0.06110823
346	13	13,3	12	f	21.2	1.24	0.05849057
347	13	13,3	12	f	17.81	1.13	0.0634475
348	13	13,3	12	m	19.78	1.29	0.06521739
349	13	13,3	12	m	19.12	1.18	0.06171548
350	13	13,3	12	m	20.39	1.23	0.06032369
351	13	13,3	12	m	19.23	1.05	0.05460218
352	13	13,3	12	m	18.82	1.18	0.06269926
353	13	13,3	12	f	27.17	1.56	0.05741627
354	13	13,3	12	m	15.17	0.85	0.05603164
355	13	13,3	12	m	19.42	1.28	0.06591143
356	13	13,3	12	m	20.2	1.11	0.0549505
357	13	13,3	12	m	23.23	1.29	0.05553164

358	13	13,3	12	m	20.24	1.25	0.06175889
359	6	6,3	14	f	21.77	1.15	0.05282499
360	6	6,3	14	f	22.83	1.36	0.05957074
361	6	6,3	14	f	20.65	1.37	0.06634383
362	6	6,3	14	f	19.64	1.29	0.06568228
363	6	6,3	14	f	20.57	1.41	0.06854643
364	6	6,3	14	f	24.91	1.65	0.06623846
365	6	6,3	14	f	21.57	1.52	0.07046824
366	6	6,3	14	f	22	1.38	0.06272727
367	6	6,3	14	m	18.75	1.13	0.06026667
368	6	6,3	14	f	22.18	1.33	0.05996393
369	6	6,3	14	f	20.48	1.29	0.06298828
370	6	6,3	14	f	19.33	1.18	0.06104501
371	6	6,3	14	f	20.25	1.42	0.07012346
372	6	6,3	14	f	20.16	1.36	0.06746032
373	6	6,3	14	f	19.47	1.58	0.08115049
374	6	6,3	14	m	20.12	1.54	0.07654076
375	6	6,3	14	m	19.45	1.35	0.06940874
376	6	6,3	14	f	20.54	1.31	0.06377799
377	6	6,3	14	f	19.78	1.37	0.06926188
378	6	6,3	14	f	18.91	1.47	0.07773665
379	6	6,3	14	f	19.47	1.29	0.06625578
380	6	6,3	14	f	20.76	1.56	0.07514451
381	6	6,3	14	m	20.34	1.41	0.06932153
382	6	6,3	14	m	17.96	1.27	0.07071269
383	6	6,3	14	m	18.8	1.38	0.07340426
384	6	6,3	14	f	22.39	1.64	0.07324699
385	6	6,3	14	f	23.05	1.37	0.05943601
386	6	6,3	14	f	19.15	1.3	0.06788512
387	6	6,3	14	f	17.69	1.41	0.07970605
388	6	6,3	14	f	20.13	1.38	0.0685544
389	6	6,3	14	f	20.96	1.47	0.07013359
390	6	6,3	14	f	19.34	1.53	0.07911065
391	6	6,3	14	f	19.49	1.35	0.06926629
392	6	6,3	14	f	21.23	1.25	0.05887894
393	6	6,3	14	m	21.59	1.29	0.05974988
394	6	6,3	14	f	19.01	1.66	0.08732246
395	6	6,3	14	f	22.33	1.61	0.07210031
396	6	6,3	14	f	22.39	1.47	0.06565431
397	6	6,3	14	f	23.12	1.49	0.06444637

398	6	6,3	14	f	22.98	1.58	0.06875544
399	6	6,3	14	f	20.25	1.48	0.07308642
400	6	6,3	14	f	19.87	1.41	0.07096125
401	6	6,3	14	f	20.98	1.48	0.07054337
402	6	6,3	14	f	19.9	1.3	0.06532663
403	6	6,2	11	f	21.66	1.54	0.0710988
404	6	6,2	11	f	23.26	1.95	0.08383491
405	6	6,2	11	f	22.2	1.56	0.07027027
406	6	6,2	11	f	21.89	1.38	0.06304249
407	6	6,2	11	f	21.45	1.39	0.06480186
408	6	6,2	11	m	20.81	1.27	0.06102835
409	6	6,2	11	m	20.43	1.51	0.07391092
410	6	6,2	11	f	23.93	1.7	0.07104053
411	6	6,2	11	f	21.63	1.89	0.08737864
412	6	6,2	11	f	19.5	1.67	0.08564103
413	6	6,2	11	m	18.61	1.26	0.06770553
414	6	6,2	11	m	21.62	1.58	0.07308048
415	6	6,2	11	m	18.47	1.34	0.07255008
416	6	6,2	11	m	19.47	1.34	0.06882383
417	6	6,2	11	f	21.69	1.3	0.05993545
418	6	6,2	11	f	22.18	1.8	0.08115419
419	6	6,2	11	f	18.32	1.33	0.07259825
420	6	6,2	11	f	19.27	1.43	0.07420861
421	6	6,2	11	f	19.3	1.46	0.07564767
422	6	6,2	11	m	18.67	1.88	0.1006963
423	6	6,2	11	m	20.96	1.63	0.07776718
424	6	6,2	11	m	19.29	1.36	0.07050285
425	6	6,2	11	m	22.15	1.38	0.06230248
426	6	6,2	11	m	21.08	1.55	0.07352941
427	6	6,2	11	m	21.99	1.79	0.08140064
428	6	6,2	11	f	21.46	1.51	0.07036347
429	6	6,2	11	f	17.96	1.24	0.06904232
430	6	6,2	11	f	21.66	1.72	0.07940905
431	6	6,2	11	f	20.02	1.51	0.07542458
432	6	6,2	11	m	19.07	1.22	0.06397483
433	6	6,2	11	m	20.54	1.34	0.06523856
434	6	6,2	11	m	23.9	1.75	0.07322176
435	6	6,2	11	m	20.04	1.57	0.07834331
436	6	6,2	11	m	23.2	1.76	0.07586207
437	6	6,2	11	f	20.21	1.59	0.07867392

438	6	6,2	11	f	19.86	1.44	0.07250755
439	6	6,2	11	f	19.09	1.25	0.06547931
440	6	6,2	11	f	20.89	1.75	0.08377214
441	6	6,2	11	m	19.6	1.63	0.08316327
442	6	6,2	11	m	18.87	1.6	0.08479067
443	6	6,2	11	m	19.35	1.46	0.0754522
444	6	6,2	11	m	20.09	1.49	0.07416625
445	6	6,2	11	m	20.41	1.54	0.07545321
446	6	6,2	11	f	22.54	1.63	0.07231588
447	6	6,2	11	f	17.79	1.18	0.0663294
448	6	6,2	11	f	20.67	1.45	0.07014998
449	6	6,2	11	f	20.05	1.46	0.07281796
450	6	6,2	11	f	20.26	1.39	0.06860809
451	6	6,2	11	m	20.91	1.45	0.06934481
452	6	6,2	11	m	19.89	1.52	0.07642031
453	6	6,2	11	m	19.98	1.42	0.07107107
454	6	6,2	11	m	20.19	1.51	0.0747895
455	6	6,2	11	m	17.95	1.29	0.0718663
456	6	6,2	11	m	18.04	1.19	0.06596452
457	6	6,2	11	f	20.76	1.03	0.04961464
458	6	6,2	11	m	18.92	1.33	0.07029598
459	6	6,2	11	m	15.43	0.82	0.05314323
460	6	6,2	11	m	21.31	1.37	0.06428907
461	6	6,2	11	m	20.98	1.29	0.06148713
462	11	11,5	13	f	21.16	1.39	0.06568998
463	11	11,5	13	f	22.45	1.36	0.06057906
464	11	11,5	13	f	24.88	1.63	0.06551447
465	11	11,5	13	m	21.68	1.55	0.07149446
466	11	11,5	13	m	20.27	1.36	0.06709423
467	11	11,5	13	m	23.12	1.76	0.07612457
468	11	11,5	13	f	22.47	1.92	0.08544726
469	11	11,5	13	f	19.9	1.79	0.08994975
470	11	11,5	13	f	20	1.59	0.0795
471	11	11,5	13	f	22.11	1.64	0.07417458
472	11	11,5	13	m	18.48	1.68	0.09090909
473	3	3,3	15	f	23.61	1.38	0.05844981
474	3	3,3	15	f	25.62	1.34	0.05230289
475	3	3,3	15	f	25.83	1.47	0.05691057
476	3	3,3	15	f	24.86	1.28	0.05148833
477	3	3,3	15	f	23.99	1.52	0.06335973

478	3	3,3	15	f	24.42	1.47	0.06019656
479	3	3,3	15	f	23.17	1.4	0.06042296
480	3	3,3	15	f	23.22	1.35	0.05813953
481	3	3,3	15	f	20.47	1.19	0.05813385
482	3	3,3	15	f	26.3	1.28	0.0486692
483	3	3,3	15	f	16.07	1.21	0.07529558
484	3	3,3	15	f	21.31	1.56	0.07320507
485	3	3,3	15	f	22.6	1.46	0.06460177
486	3	3,3	15	f	20.96	1.38	0.06583969
487	3	3,3	15	f	20.08	1.46	0.07270916

ML= mandible length (mm)
SL= standard length (mm)
Norm ML= mandible length standardized by standard length

SCRIPTS

R-Script 1: Narrow-Sense Heritability Script (Traditional ANOVA Method)

```
h2<-function(anova_table)
{
  s<-anova_table$Df[1]+1
  d<-(anova_table$Df[2]/s)+1
  k=(anova_table$Df[3]/(s*d))+1

  sigma2_S<-(anova_table$`Mean Sq`[1] - anova_table$`Mean Sq`[2]) / (d*k)
  sigma2_D<-(anova_table$`Mean Sq`[2] - anova_table$`Mean Sq`[3]) / k
  sigma2_W<-anova_table$`Mean Sq`[3]
  Va<-4*sigma2_S
  Vp<-sigma2_S + sigma2_D + sigma2_W
  return(Va/Vp)
}

m3<-lm(log(ML.norm) ~ as.factor(Sire)/as.factor(Clutch), data=Heri)
(aov_m3<-anova(m3))

hist(m3$residuals) #check residuals

h2(aov_m3)
```

R-Script 2: Narrow-Sense Heritability and BLUP Script using MCMCglmm Method

```
install.packages("MCMCglmm")

require(MCMCglmm)

Heri <- read.csv("HSD.csv", header=T)

Heri <- Heri[-c(490, 489, 488),]

tail(Heri)

length(unique(Heri$Individual))


ped<-Heri[,c(1,2,4)]

ped$Sire<-paste0("S",ped$Sire)

ped$Clutch<-paste0("D",ped$Clutch)

Heri$animal=Heri$Individual

parents<-data.frame(animal=c(unique(ped$Sire),unique(ped$Clutch), ped$Individual),

Sire=c(rep(NA,20), ped$Sire), Clutch=c(rep(NA,20),ped$Clutch))

install.packages("nadiv")

require(nadiv)


listD<- makeD(parents)

Dinv<-listD$Dinv
```

```
Heri$dom<-Heri$animal
prior_ext<-list(R=list(V=1,nu=1), G=list(G1=list(V=1,nu=1,alpha.mu=0,alpha.V=1000),
                                         G2=list(V=1,nu=1,alpha.mu=0,alpha.V=1000)))

modeldom2<-MCMCglmm(log(ML.norm)~1, random=~animal+dom, ginverse=list(dom=Dinv),
                    family="gaussian", prior=prior_ext, pedigree=parents,
                    data=Heri, nitt=500000, burnin=10000, thin=50, pr=TRUE)

autocorr.diag(modeldom2$VCV)
plot(modeldom2[["VCV"]])

#Define heritability
herit2<- modeldom2$VCV[, "animal"]/(modeldom2$VCV[, "animal"] + modeldom2$VCV[, "dom"] +
modeldom2$VCV[, "units"])

#Take mean of posterior distribution
mean(herit2)
HPDinterval(herit2)
#Check posterior distributions
plot(herit2)
summary(modeldom2)

#Preparing BLUPs (50,000 iterations for each individual)
View(modeldom2$Sol)
#Adjusting the BLUPs using the intercept for each iteration
BLUPadj <- BLUP[,2:ncol(BLUP)]+BLUP[,1]
#Checking how many additive BLUPs there are (sires, dams, offspring total), only including the
#Additive plots, not the dominance plots
BLUPadj<-BLUPadj[,1:507]
#Taking means for each individual's adjusted BLUPs for a final, single BLUP value
BLUPadj<-colMeans(BLUPadj)
```

R-Script 3: Calculation of Correlation Coefficients and Creating Scatterplots

```
#Calculating correlation coefficient for sire BV vs MLc
```

```
cor(Sires$MLc, Sires$BLUP, use = "complete.obs")
#r = 0.9357918

#Calculating correlation coefficient for dam BV vs MLc
cor(Dams$MLc, Dams$BLUP, use= "complete.obs" )
#r = -0.007655092

#This method above did not give me a p value for dams or sires, so I am going to use another
#method and check that the r values match

#Using ggscatter to create a scatterplot showing sire BV vs MLc
sireplot <- ggscatter(Sires, x = "MLc", y = "BLUP",
                add = "reg.line",  # Add regression line
                add.params = list(color = "blue", fill = "lightgray"), # Customize reg. line
                conf.int = TRUE # Add confidence interval
)

#Calculating correlation coefficient and p values for sires
sireplot + stat_cor(method = "pearson", label.x = -0.4, label.y = -2)
#r= 0.94; p= 0.019
#r value is consistent

#Using ggplot to make a nicer looking version of the ggscatter graph above with sire r and p
#values annotated in
ggplot(Sires, aes(x=MLc, y=BLUP)) + geom_point()  +
  scale_color_continuous() + ggtitle("Correlation between Sire Phenotype and Breeding Value") +
  theme(plot.title = element_text(hjust = 0.5)) + xlab("Corrected Mandible Length (mm)") +
ylab("Predicted Breeding Value") +
  scale_y_continuous(n.breaks=5) + geom_smooth(method='lm') +
  theme(plot.title = element_text(face = "bold"))  +
  annotate("text", x = 0.085, y = -2.5, label = "r= 0.935  p= 0.019")

#Using ggscatter to create a scatterplot showing dam BV vs MLc
damplot <- ggscatter(Dams, x = "MLc", y = "BLUP",
                     add = "reg.line",  # Add regression line
```

```
                      add.params = list(color = "blue", fill = "lightgray"), # Customize reg.
line
                      conf.int = TRUE # Add confidence interval
)



#Calculating correlation coefficient and p values for dams
damplot + stat_cor(method = "pearson", label.x = -0.4, label.y = -2)
#r= -0.0077; p= 0.98
#r value is consistent

#Using ggplot to make a nicer looking version of the ggscatter graph above with dam r and p
#values annotated in
ggplot(Dams, aes(x=MLc, y=BLUP)) + geom_point()  +
  scale_color_continuous() + ggtitle("Correlation between Dam Phenotype and Breeding Value") +
  theme(plot.title = element_text(hjust = 0.5)) + xlab("Corrected Mandible Length (mm)") +
ylab("Predicted Breeding Value") +
  scale_y_continuous(n.breaks=5) + geom_smooth(method='lm') +
  theme(plot.title = element_text(face = "bold"))
annotate("text", x = 0.085, y = -2.5, label = "r= -0.0077  p= 0.98")
```