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<u>**I, Kimberly M Wyatt , hereby submit this original work as part of the requirements for the degree of Master of Science in Biological Sciences.**</u>

It is entitled:

Rapid morphological divergence among subpopulations of the introduced common wall lizard, Podarcis muralis

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Rapid morphological divergence among subpopulations of the introduced common wall lizard,

Podarcis muralis

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Abstract

The evolutionary dynamics of invasion and range expansion are of considerable interest to biologists. Novel environments place selection pressures on invaders, and response to such pressures may determine the success of invasive species. Theory suggests the process of range expansion may also facilitate evolution and adaptation, but empirical evidence for this is limited. We investigated the extent of morphological and genetic variation among introduced subpopulations of the common wall lizard *Podarcis muralis* in the Greater Cincinnati area. A single introduction in 1951 of a very small number of founders gave rise to several subpopulations that show pronounced genetic structure. We found evidence of significant variation among subpopulations for several morphological traits, including hind foot length, ventral coloration, ventral mottling, cephalic scale shape, and centroid size. We confirmed previous reports of significant differences in the genetic structure of subpopulations. No relationships between genetic structure, geographic distribution, and morphological variation were identified. The morphological differences have developed over very small spatial scales ($<$ 10 km) and over short time periods of time ($<$ 63 years). Based on their population history, we suspect that genetic drift due to serial founder effects is the most likely cause of the observed morphological divergence, although we have not ruled out developmental causes. Studies of invasive species during range expansion may reveal evidence of rapid evolutionary processes that in turn, could facilitate adaptation to a novel environment. Future research should seek to identify the factors driving variation among the Greater Cincinnati subpopulations of *Podarcis muralis.*

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Introduction

Neutral and Adaptive Evolution

One of the major goals of evolutionary biology is to understand the roles of neutral and adaptive forces in influencing population differentiation (Mariani *et al.* 2012). Neutral evolution is driven by genetic drift. Alternately, adaptive evolution is driven by natural selection and is shaped by interactions of population genetics and underlying network constraints (Olson-Manning *et al*. 2012). Adaptive evolution can significantly impact the ability of an invasive species to perform well in its new environment (Prentis *et al*. 2008, Clements & DiTommaso 2011, Chahal *et al*. 2013) by enhancing fitness (Taddei *et al*. 1997), and is critical for organisms experiencing rapid climatic changes (Lavergne *et al*. 2010). Though the traditional understanding of evolution focuses on gradual changes that occured over large temporal scales (Saylo *et al*. 2011), recent studies have found examples of rapid evolution in a number of introduced species, including cane toads (*Bufo marinus*; Phillips *et al*. 2006), reed canarygrass (*Phalaris arundinacea L*; Lavergne & Molofsky 2007), and St. John's wort (*Hypericum perforatum*; Maron *et al.* 2004). Rapid evolution is defined as, "genetic change occurring rapidly enough to have a measurable impact on simultaneous ecological change" (Hairston *et al*. 2005). It is attributed mostly to non-adaptive processes such as genetic drift, and can be potentiated by phenotypic plasticity (Behera & Nanjundiah 2004).

Invasive Species

Invasive species can serve as excellent models by which to study the dynamics of adaptation and evolution. Rapid evolutionary changes are associated with factors related to invasion potential, and invasion may reflect evolution driven by either non-adaptive or adaptive processes**.** Reproductive and growth rates, adaptation to novel environments, dispersal ability,

and size have undergone evolutionary changes in a variety of taxa over timescales much shorter than what is typically noted in studies of evolution (Sakai *et al.* 2001, Lee 2002, Dlugosch & Parker 2008).

The significant impact of evolutionary change on invasive species has been recognized for decades (Baker 1974). However, the association between rapid adaptive evolution and its effects on invasion has only begun to receive attention in recent years. When species arrive in novel environments, they often face a variety of selection pressures (Novak 2007) to which they may be poorly suited (Facon *et al.* 2006, Richardson and Pysĕk 2006, Prentis *et al.* 2008). Rapid adaptation may enable non-native species to overcome ecological and genetic barriers in order to establish, reproduce, and spread in new environments (Maron *et al.* 2004, Bossdorf *et al.* 2005). For example, invasive California poppies (*Eschscholzia californica*) in Chile have been shown in common garden experiments to rapidly adapt to abiotic gradients and reestablished phenotypic associations with landscape found within the native range (including smaller and later flowering plants in coastal areas compared to inland sites (Leger & Rice 2007).

A less-commonly studied aspect of invasions is adaptive evolutionary divergence within an invasive population. Phillips *et al.* (2006) found that invasive cane toads (*Bufo marinus*) in Australia had evolved longer legs compared to cane toads from near the center of the introduced range. This adaptation presumably functions to facilitate dispersal during range expansion. A recent study of three invasive brown trout (*Salmo trutta*) populations found evidence of local adaptation to rivers that lie a few kilometers apart (Westley *et al.* 2013).

In addition to adaptation, there are multiple other processes associated with colonization success in invasive species. One factor contributing to colonization success is phenotypic plasticity (Marshall & Jain 1968, Kaufman & Smouse 2001, Parker *et al.* 2003, Knop & Reusser

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2012). Phenotypic plasticity is frequently associated with variation within and between populations of invasive species (Bradshaw 1965, Whitman & Agrawal 2009, Losos *et al*. 2000, Pfennig *et al*. 2010, Davidson *et al*. 2011), and appears to have a strongly positive effect on the survival of populations in new environments (Price *et al*. 2003). Environmental factors can influence phenotypic variation independently of genetics. Competitors, predators, pathogens, and habitat may all differ from the native range (Elton 1958, Gillett 1962, Crawley 1987), and phenotypic plasticity may enhance an individual's ability to use novel environmental conditions to its advantage (Kolbe & Losos 2005, Clements & Ditommaso 2011).

Plasticity is not necessarily a symptom of genetic variation, but genetic structure may select for or against plasticity based on the number of plastic versus nonplastic loci. Limited genetic diversity may result in a lower frequency of nonplastic loci, which can enable invasive populations to evolve increased trait plasticity (Davidson *et al*. 2011). Increased dispersal rates favor plasticity because during range expansion, phenotypic plasticity may function as a form of bet-hedging (Scheiner & Holt 2012). During dispersal, members of a population often encounter a variety of environments. Plasticity therefore can increase the likelihood that at least some individuals will experience high fitness in a novel environment.

Genetic Factors in Invasions

When non-native species arrive in new areas, they are often thought to experience genetic bottlenecks (Golani *et al*. 2007, Franks *et al*. 2011). Typically, the number of individuals present at the start of an invasion is limited, which places immediate constraints on the gene pool. In reduced populations, the role of genetic drift is increased, which may lead to the reduction of already rare alleles.

There are methods by which genetic drift can influence invasion success. Low-frequency alleles can "surf on the wave of advance of a population range expansion" (Excoffier and Ray 2008, Klopfstein *et al.* 2006). If dispersal is limited during range expansion, genetic drift can be strong in the low-density populations at the forefront of the expansion. If low-frequency alleles are present at the edge of a range expansion, random sampling can quickly and significantly increase their frequencies. According to this model, in areas colonized by surfing, sharp allele frequency gradients should distinguish between sectors of low genetic diversity. This process could augment adaptation (Hofer *et al.* 2009, Short & Petren 2011). Other studies have shown evidence of species exhibiting high phenotypic variability in the presence of low genetic diversity (Kinsey *et al.* 1993, Richards 2000).

By combining an understanding of genotypic and phenotypic variations, and the interaction between the two, it is possible to begin to identify the rate at which evolution takes place and the evolutionary processes underlying patterns of differentiation within invasive species. In this way it is possible to distinguish between processes such as genetic drift, phenotypic plasticity, and adaptive evolution (Mariani *et al.* 2012, Zalewski & Bartoszewicz 2012).

The Common Wall Lizard, Podarcis muralis

Introduced populations of the common wall lizard, *Podarcis muralis,* provide excellent models for studying the dynamics of adaptation and range expansion. It is a relatively recent invader (~60 years), and there is strong evidence to support a single introduction event in Greater Cincinnati (Deichsel & Gist 2001). A significant collection of historical records pertaining to the establishment of this species makes it possible to follow its spread and precisely correlate temporal, spatial, environmental, morphological, and genetic information.

Podarcis muralis is a small lacertid lizard native to southern and central Europe (Oliverio *et al*. 2000). *Podarcis muralis* is insectivorous and oviparous and commonly found in urban areas around rock walls and railroad ties (Draud & Ferner 1994, Brown *et al*. 1995, Burke & Deichsel 2008). Around 1951, approximately ten lizards were collected near Lake Garda in northern Italy and brought back to the United States. These individuals were subsequently released at a private residence on Torrence Ct. in Cincinnati, Ohio (Deichsel & Gist 2001, Ferner & Ferner 2002). Since their initial introduction, *P. muralis* have established in many parts of Cincinnati and northern Kentucky, and have also appeared in Indiana (Brown *et al*. 1995, Burke & Deichsel 2008). These lizards have been extremely successful invaders, with densities of some Greater Cincinnati subpopulations estimated to be as high as 1,500 individuals/acre (Kwiat & Gist 1987).

In the introduced range, *Podarcis muralis* has accumulated pronounced genetic structure in a short period of time that is most likely caused by sequential founder effects upon range expansion (Lescano & Petren, unpublished data). Here we investigate whether the genetic differences are coordinated with phenotypic variance. By sampling a variety of morphological traits and genotypes for a number of distinct but geographically-close study sites, we will elucidate any morphological differences in subpopulations that have evolved post-introduction.

Much of the current research pertaining to rapid evolution of invasive species involves a geographic component. Successful invaders are capable of dramatic range expansion within a short time frame (Sakai et al. 2001, Peterson 2003, Blumler 2006), but not all invasive species undergo range expansion over large geographic regions. Few studies have examined the possibility of rapid evolution in a population of an introduced species occupying a very small spatial scale (but see Tsutsui *et al*. 2000, Grosholz 2001). Within their native range, some species

show significant genetic structuring occurring over small spatial scales (tens of km: Weetman *et al.* 2006; < 1m: Ledoux *et al*. 2010). Fine-scale genetic structure may also exist within invasive populations (Short & Petren 2011). The current introduced range of *P. muralis* in Cincinnati is quite small compared to the range of many invasive species (tens of km^2). Because differences in climatic conditions are negligible at such small distances, many broad environmental factors may be able to be ruled out as contributing to variation among Greater Cincinnati *P. muralis* subpopulations. However, microhabitat differences may function as limiting environmental factors (Galland 2011) which could influence morphology.

Research Goals

The goals of this study were (1) to test whether significant phenotypic variation exists among introduced subpopulations of *Podarcis muralis* in Greater Cincinnati and (2) to determine whether observed genetic divergence, which is likely due to drift, is correlated with morphological divergence. To accomplish these goals, we employed standard measures of linear morphology and geometric morphometrics and also looked at color variation. Standard linear measurements can be good predictors of morphological differences (Stockley *et al*. 2013), but are limited in their ability to accurately portray variations in shape. Alternately, geometric morphometrics can detect small degrees of shape variation using discrete, repeatable landmarks on an organism (Monteiro 1999). The intersections of craniodorsal scales serve as clear and consistent Type 1 landmarks (Bookstein 1991) and are good candidates for geometric morphometric analysis (Stayton 2005, Bruner & Constantini 2007, Kaliontzopoulou *et al.* 2010). Differences in shape and size are potentially under natural or sexual selection, or may be the result of plasticity or developmental instability (Richtsmeier *et al*. 2002, Kaliontzopoulou *et al*. 2008). Quantifying morphological divergences within the Greater Cincinnati population of

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Podarcis muralis is necessary in order for neutral or adaptive factors driving phenotypic variation to be identified.

Methods

Sampling Protocol and Morphology

During the months of July to November, 2011 and May to November, 2012, a total of 167 adult *Podarcis muralis* lizards were collected within a 14 kilometer-wide area in Greater Cincinnati, from three locations in Cincinnati, Ohio and two locations in northern Kentucky (Table 1). Figure 1 provides a visual representation of the study sites, which were selected based on their history of large *P. muralis* population size, proximity to the initial site of introduction (Torrence Court, Cincinnati, OH), and pronounced genetic differentiation, as indicated by previous research (Lescano & Petren, unpublished data).

Lizards were noosed (Brown *et al.* 1995) or captured by hand, and were brought back to the University of Cincinnati for measuring and imaging. To aid in identification of recaptured individuals and for use in geometric morphometric analysis, dorsal, ventral, and side photographs were taken of the head and body of each specimen. Measurements to the nearest 0.5 mm were taken for snout-vent length (SVL), hind limb span (HLS, measured as the linear distance between the tips of the fourth digits on the hind limbs), tail length (TL), head length (HL), and hind foot length (HFL, measured from the heel to the tip of the fourth toe on the right foot). Mass was measured to the nearest 0.1 g using a spring scale. Approximately 1 cm of tail tissue was collected and stored in 70% ethanol for genetic sampling (Le Galliard *et al*. 2004). Lizards were released at the location they were captured on the same day they were collected. If any specimen was injured or deformed (*i.e.* missing toes or with a broken or regenerated tail), it was excluded from standard linear morphological analyses.

Linear regressions were performed for each morphological measure using snout-vent length as the independent variable. The remaining five morphological measures were log-transformed before analysis. Although sexes differed by size (determined by grouping sexes separately on the same linear regressions of each morphological measure), Student t-tests of residuals grouped by sex were not significantly different. Thus, we pooled sexes for measures of SVL, HLS, TL, HFL, HL, and mass. Pearson's correlation, ANCOVA and MANOVA were performed in XLSTAT (Addinsoft SARL 2013, Tables 3, 4).

Coloration

Podarcis muralis exhibit unique patterns of blue scales on their sides and shoulders (Deichsel & Gist 2001). Per Gracceva *et al.* (2008), images of the sides and shoulders of each lizard were collected (Figure 2). Blue outer ventral scales (located on the sides along the abdomen) and blue ocelli scales (located on the shoulders) were counted for each specimen. Variations in scale counts between sexes and sites were examined via ANOVA.

Three discrete color morphs exist in *P. muralis* and are commonly used as phenotypic measures in scientific research (Sacchi *et al.* 2009). Additionally, individuals of all color morphs can exhibit differing proportions of black scales (henceforth referred to as "mottling") on their abdomens and throats, although this has not previously been treated as an independent variable. Ventral color was classified as white, yellow, or red (Sacchi *et al.* 2009, Figure 3). The degree of mottling on the ventral scales of each lizard was visually scored by one researcher to reduce measurement error: no mottling = 0; light mottling = 1; intermediate mottling = 2; and heavy mottling = 3 (Figure 3).

The occurrence of different color morphs is known to vary widely among populations of *Podarcis muralis*. However, Sacchi *et al*. report the mean relative frequencies of ventral

coloration among *P. muralis* in northern Italy not far from the location of our source population (2007). We compared these data from Sacchi *et al.* to counts of color morphs in our Cincinnati subpopulations with chi-square (χ^2) tests to look for differences between native and invasive populations (see Sacchi *et al.* 2007 for more information). Variation in coloration among and within the Greater Cincinnati subpopulations was further examined by dividing the data by sex and performing likelihood ratio tests to reveal any correlations between color and sex. Frequencies of ventral mottling by study site and sex and the interaction of color and mottling were examined in JMP v. 10 (JMP 2013).

Geometric Morphometrics

Geometric morphometric studies require larger sample sizes than other types of morphological analysis (Cardini & Elton 2007). We collected a minimum of 30 individuals per site (Table 1). High-definition video was taken of the cephalic scales of each lizard using a Canon Vixia HFR10 video camera mounted on a tripod. Still frames were taken from the videos for use in geometric morphometric analysis. All images were collected on a common plane with a scale bar for reference.

Based on a previous *Podarcis* morphometric study, 14 landmarks located at the boundaries of the frontal, frontoparietal, interparietal, and occipital scales were sampled (Figure 4; see Bruner & Constantini 2007 for additional information). All imaging procedures were performed by the same researcher in order to reduce measurement error. The software program ImageJ (Abramoff *et al.* 2004) was used to collect landmark coordinates. As measurement error can be particularly problematic for morphometric studies (Palmer & Strobeck 1986), two still photographs were independently sampled and scored for each lizard and five individuals from each study site were randomly selected to be re-measured 10 times.

Shape analyses were performed in MorphoJ (Klingenberg 2011). The selected landmarks exhibited bilateral object symmetry, and unless otherwise specified, the data were treated as symmetric. Landmark coordinates were superimposed using generalized Procrustes superimposition, which removes variation due to non-shape components by translating each individual's coordinates to a common centroid, scaling to a common centroid size, and rotating each set of coordinates to ensure the best fit. Centroid size was used as a measure of size for geometric morphometric analyses, and was calculated by taking the square root of the summed squared distances of the full set of landmarks to the centroid.

Standard statistical methods require data to be in a flat Euclidian space (Jones *et al.* 2007, Viscosi & Cardini 2011), but Procrustes shape space is actually multidimensional. Therefore, prior to subjecting geometric morphometric data to statistical analyses, shape space was approximated to a tangent Euclidean space using *tpsSmall* v. 1.20 (Rohlf 2003). Euclidean distances in Euclidean space were regressed through the origin onto a set of Procrustes shape distances. Our regression yielded a slope of 0.996 and a correlation of 1.0, confirming that linear models could be used for statistical analyses.

Following the Procrustes superimposition, a covariance matrix was generated in MorphoJ prior to any further analyses. The degree of variation within shape space for each study site was quantified via principal component analysis (PCA), and a canonical variate analysis (CVA) was performed to graphically examine the shape differences among *a priori* defined groups. Procrustes ANOVA and MANOVA tests were used to assess the significance of symmetry and directional asymmetry. Thin-plate spline (TPS) deformation grids were generated to aid in describing shape variation. TPS grids allow for interpolation of differences between landmarks

by depicting the change necessary for one shape to be warped into another as a deformation of shape space.

Variation in centroid size among populations was examined via ANOVA. To explore the relationship between size and shape we performed a multivariate regression of the shape coordinates on log centroid size.

Genetics

In order to compare the genetic structure of Cincinnati and northern Kentucky *P. muralis* subpopulations, total genomic DNA was extracted from 117 tail clippings via a guanidine-based method (Petren 1998). A multiplex polymerase chain reaction (Qiagen Multiplex PCR Kit) using four fluorescent labeled primers (FAM, NED, VIC, PET) was performed to amplify eight microsatellite loci. We selected loci *Lv-3-19, Lv-47-2, Lv-alpha, B6, B7, A7, B3,* and *D1* due to the fact that they are some of the most variable loci in *Podarcis* (Boudjemadi *et al.* 1999; Nembrini & Oppliger 2003; Lescano & Petren, unpublished data).

A total of 15 µL was used for each PCR. Conditions for the polymerase chain reaction were: 95°C for 15 minutes, 33 cycles of 94 °C for 30 seconds, 52 °C for 1.5 minutes, 72°C for 60 seconds, and final extension at 60 °C for 30 minutes (Lescano & Petren, unpublished data). Fragment analysis was performed at the Cornell University Biotechnology Resource Center via capillary gel electrophoresis (Applied Biosystems 3730xl DNA Analyzer). Genotyping and allele sizing estimation were completed using GENEMAPPER v. 3.7 (Applied Biosystems).

Descriptive statistics for the Greater Cincinnati *Podarcis* population and for each sampling site were calculated using GenAlEx v. 6.5 (Peakall & Smouse 2012). The average number of alleles per locus (A), the effective number of alleles (A_E), the expected proportion of heterozygotes (H_e), the observed proportion of heterozygotes (H_o), and deviation from Hardy-

Weinberg Equilibrium, measured as Wright's fixation index (F) were calculated for each study site.

Pairwise Weir & Cockerham's θ (1984) were calculated for the five study sites using ARLEQUIN v. 3.11 (Excoffier *et al*. 2005) at 1000 permulations. IBDWS (Jensen *et al*. 2005) was used to detect a pattern of geographic isolation by performing a Mantel test using genetic distance θ (Weir & Cockerham 1984) and log-transformed geographic distance.

Finally, we examined the association of genetic structure (characterized by genetic distances) and phenotypic variation. XLSTAT-Pro (Addinsoft SARL 2013) was used to generate a principal component analysis of the standard morphological data. Using these principal components and those from the geometric morphometric analysis of cephalic scale shape, Euclidian distances matrices were constructed (Gizaw *et al*. 2007). Mantel tests were performed using a genetic distance matrix generated from θ values and the Euclidian distance matrices for the two types of morphological data.

Results

Standard Morphology

The means for each standard morphological trait by site are shown in Figure 5. Linear regressions of log-transformed hind limb span (HLS), tail length (TL), hind foot length (HFL), head length (HL), and mass against snout-vent length (SVL) were all significant ($P < 0.0001$). Pairwise correlations among variables are presented in Table 2.

The effects of study site on morphology were analyzed via multivariate analysis of variance (MANOVA) and analysis of covariance (ANCOVA). A MANOVA using study site as the main effect and SVL as a covariate was not significant ($P = 0.2933$), while the effect of SVL

was significant ($P < 0.0001$). In individual ANCOVA analyses of each variable, only foot length was significantly different among locations ($P = 0.001$, Table 3).

Tukey pairwise comparisons by site found Rose Circle and House of Tropicals differed significantly in hind limb span ($P = 0.0333$) and hind foot length ($P = 0.0003$), and Rose Circle and Fairview differed in hind foot length ($P = 0.0409$).

Coloration

The sides and shoulders of *Podarcis* are marked with a discrete, unique pattern of blue scales (Figure 2). ANOVA showed there was not a significant difference between the numbers of blue scales on the right or left side or shoulder, so only counts from the right sides were used in subsequent analyses.

Males had more blue on the sides than females across all sites ($P = 0.0045$), as well as more blue on the shoulders ($P = 0.0011$). The minimum number of blue outer ventral scales for all females was 0; the maximum count for females was 7. Across all males the range was from 1 to 14. The number of blue ocelli scales in females ranged from 0 to 17, and in males ranged from 0 to 106. ANOVA results indicated the amount of blue scales did not differ among locations.

Sacchi *et al.* report that relative frequency of color morphs is highly variable among locations in Italy (2007). Ventral coloration in Greater Cincinnati *Podarcis muralis* shows some differences from a polymorphic analysis of European lizards. Mean coloration among populations of *P. muralis* in northern Italy was found to be 56.6% white, 28.7% yellow, and 14.7% red (Sacchi *et al.* 2007). Greater Cincinnati lizards were 57.8% white, 41.0% yellow, and 1.2% red. These proportions are significantly different (chi-square, $P = 0.001$) and the relative frequency of red morphs alone was also significantly different ($P = 0.0007$).

Proportions of ventral color for all study sites in the Greater Cincinnati population were significantly different. The most different locations were Fairview and Rose Circle ($P = 0.0001$), and the least different were Beechmont and Ft. Thomas ($P = 0.041$). In comparisons of proportions of each color morph by population, most sites showed no differences between abundances except: (1) Fairview and Rose Circle were significantly different in terms of the proportion of white morphs ($P = 0.037$), (2) Fairview was significantly different from all other Greater Cincinnati study sites in the proportion of yellow morphs (BM: $P = 0.035$; FT: $P = 0.034$; HT: $P = 0.019$; RC: $P = 0.010$), and (3) Ft. Thomas was significantly different than all other sites in the proportion of red morphs ($P < 0.0001$). In addition, the overall proportion of red morphs was significantly different among sites ($P < 0.0001$).

Within- and between-site comparisons of sex revealed significant differences in ventral color frequencies. When all Greater Cincinnati sites were pooled, males had a significantly greater frequency of yellow morphs ($P = 0.001$), and females had a greater proportion of white ($P = 0.0011$). Red morphs did not differ significantly by sex across pooled samples. Beechmont, House of Tropicals, and Rose Circle had significant differences in the proportion of white females and males (BM: $P = 0.023$; HT: $P < 0.0001$; RC: $P = 0.035$). House of Tropicals and Rose Circle differed significantly in their proportions of yellow by sex (HT: $P < 0.0001$; RC: $P = 0.017$), and Ft. Thomas differed in the proportion of male and female red morphs ($P < 0.0001$). Likelihood ratios for both sex and location by color morph were significant ($P < 0.0001$).

We found that mottling differed significantly among color morphs, study sites, and sexes. The frequencies of types of mottling between sites (scored 0, 1, 2, 3) did not differ significantly for white and red morphs, but was significantly different among yellow morphs ($P = 0.023$). The frequency of no mottling was significantly different between all color morphs ($P = 0.001$), and

was most commonly observed in white morphs. The frequency of no mottling also differed among all study sites ($P = 0.029$), being most common in the Fairview subpopulation and least common at House of Tropicals.

Across all study sites, mottling frequencies were significantly different among sexes ($P < 0.0001$). No mottling was more prevalent in females ($P = 0.0001$), whereas intermediate and heavy mottling were more common in males ($P = 0.001$, $P = 0.001$).

When color and mottling were treated together, frequencies were significantly different for males and females ($P = 0.0001$). The frequency of white morphs with no mottling was significantly higher in females than males ($P = 0.002$). The frequency of yellow morphs with intermediate mottling was higher in males than females ($P < 0.0001$), as was the frequency of yellow morphs with heavy mottling ($P = 0.001$). Figure 6 shows a breakdown of color and mottling by site and sex.

Geometric Morphometrics

A repeated measures ANOVA found measurement error for individual specimens was completely negligible when using different photographs ($P = 0.999$). Thus, digitization of landmarks is assumed to not contribute to variation seen between individuals.

Procrustes analysis of variance (ANOVA) for centroid size was significantly different between study sites when sexes were pooled and when the data was analyzed separately by sex (all samples: $P = 0.031$; females: $P < 0.0001$; males: $P = 0.007$). Procrustes ANOVA for shape was also significantly different between study sites when sexes were pooled, and when the data was analyzed separately by sex (all samples: $P = 0.025$; females: $P < 0.0001$; males: $P = 0.0004$). There were also significant differences in shape between the left and right hemispheres of the

head (termed "side", all samples: $P < 0.0001$; females: $P < 0.0001$; $P < 0.0001$). Interaction was significant when sexes were pooled ($P = 0.0015$).

Multivariate analysis of variance (MANOVA) found significant difference for the symmetric component of shape variation ($P < 0.0001$), but did not find significant variation resulting from interactions between study site and side for the asymmetry component of shape.

A principal component analysis (PCA) of cranial shape indicates that the morphospace, a multidimensional representation of the variables associated with shape, is largely characterized by the first two axes. 29% of shape variation was attributable to PC1, and an additional 18% of variation was explained by PC2. Subsequent principal components explained 15% or less of the morphological variance. The first principal component is associated primarily with head length, and indicates a posterior reduction of frontal scales, lengthening of frontoparietal scales, and posterior elongation of occipital scales. The second principal component is associated with an overall widening of the cephalic scales and an elongation of interparietal scales (Figure 7).

Canonical variate analysis was able to successfully categorize 83% of individuals into their proper study sites. CV1 accounted for 61% of the variation, and CV2 accounted for an additional 22% of the variation (Figure 8). A permutation test (10,000 permutation rounds) for Procrustes distances between groups found significant differences between all pairwise study sites, with the exception of pairings House of Tropicals and Ft. Thomas, and House of Tropicals and Fairview. Average shape differences of each site from the Greater Cincinnati mean can be seen in Figure 9.

Centroid size is the most commonly used estimator of size in geometric morphometrics. In the absence of allometry, centroid size should be uncorrelated with shape variables. Log centroid size differed significantly among the study sites (Figure 10, $P < 0.0001$). However, a

pooled within-group regression of shape onto log-transformed centroid size found that size accounted for only 2.26% of shape variation, and PC scores do not strongly correlate with log centroid size ($P = 0.0019$). This indicates that allometry is very modest and probably negligible.

Genetic Diversity among Subpopulations

For the five Greater Cincinnati study sites, the mean number of alleles per locus was $A = 3.30$, and the mean expected heterozygosity was $H_e = 0.493$ (Table 4). These values were notably lower than those found in European populations of *Podarcis muralis* ($A = 12$, $H_e = 0.90$, Boudjemadi *et al.* 1999). The average genetic distance was $\theta = 0.0454$.

Spatial Genetic Analyses

Population structure was found to be significant in all but one pairwise site comparison (House of Tropicals and Fairview) and in six after Bonferroni correction (results and significance values, Table 5). Significant pairwise θ values ranged from $\theta = 0.038$ to $\theta = 0.079$. The least differentiated sites were Rose Circle and House of Tropicals (3.68 km apart), while the most differentiated were Rose Circle and Ft. Thomas (5.71 km apart). Geographic distance was not correlated with genetic distance (Table 5, Figure 11). The Rose Circle subpopulation showed high values for θ across all pairwise comparisons except for House of Tropicals.

Principal components for standard morphological data (Figure 12) and for geometric morphometric shape data (Figure 7) were used in the creation of Euclidian distance matrices. No significant associations were indicated for genetic, standard morphometric, or geometric morphometric data.

Discussion

Our results indicate that significant morphological variation exists between subpopulations of introduced *Podarcis muralis* within the Greater Cincinnati region in terms of

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both body shape and coloration. Subpopulations differed in hind limb span, hind foot length, color morph and mottling frequency, centroid size, cranial shape, and asymmetry. Although genetic structuring and morphological variation both differed significantly among study sites, patterns of morphological differentiation were not correlated with genetic diversity or genetic divergence. These results confirm the prediction that range expansion with limited amounts of gene flow between populations can lead to both genetic differentiation and morphological divergence. In the introduced Greater Cincinnati *Podarcis muralis* population, these differences have accrued in less than 65 years and at spatial scales of less than 10 km.

Morphological Variation

Hind foot length has been shown to vary among lizards in relation to habitat substrate and locomotor performance (Miles *et al.* 1995, Melville & Swain 2000, Herrel *et al*. 2001, Kohlsdorf *et al*. 2001). The subpopulations in this project were located within a very small spatial area and were therefore exposed to the same weather conditions and other broad environmental patterns. However, the five study sites did exhibit clear differences in microhabitat. The study sites consisted of a blasted hillside with a large retaining wall and sparse vegetation (Beechmont), a grassy landscaped park with a low rock wall and adjacent forest (Fairview), a retaining wall against a grassy hillside (Ft. Thomas), the parking lot of an abandoned retail building with a retaining wall adjacent to a wooded hillside (House of Tropicals), and a low decorative rock wall in a suburban neighborhood (Rose Circle). In light of these differences in microhabitat, our results of morphological variation between sites highlight an area of future study: investigating morphometrics and landscape for *Podarcis muralis* using a larger dataset with repeated categorical sampling sites.

Rose Circle differed from two other study sites, Fairview and House of Tropicals, in hind foot length and coloration. Rose Circle and House of Tropicals also differed significantly in hind limb span. Differences in microhabitat among these sites have not yet been examined, and should be addressed in more detail in future research as a possible influence on phenotype. At a glance, Rose Circle and Fairview actually appear to be the most similar of the sites. Both share relatively level, mowed lawns and low natural rock walls, and both sites are adjacent to fairly quiet roads with sidewalks. Alternately, House of Tropicals is in a much more highly trafficked part of the city, and consists mostly of brick and pavement. It is possible that small differences in the landscapes could be influencing morphological variation. Despite being the most dissimilar study sites in terms of morphological variation, Rose Circle and House of Tropicals were actually the least genetically differentiated of all significant pairs of study sites. These sites are located 3.68 km apart, which is the second-shortest distance between any two study sites. Rose Circle's location in Kentucky seems as though it should pose significant challenges to the possibility of migration by individuals from other parts of Greater Cincinnati, because it is separated from Ohio populations by the Ohio River. However, it is believed that human-mitigated jump dispersal happens fairly regularly (Draud and Ferner 1994, Deichsel and Gist 2001), so it is possible that migration across the river may be occurring.

Coloration

Our analysis of coloration focused on three phenotypic traits: blue scales, ventral color, and ventral mottling. Though counts of blue outer ventral scales and blue ocelli scales were significantly different between males and females at all five study sites, scale counts did not significantly differ. This is unsurprising, as counts were extremely varied between individuals across all study sites, especially in the number of scales in blue ocelli. Males saw a more than

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six-fold increase in the range of their ocelli scale counts compared to females. This corresponds

to reports of sexual dichromatism in lizards (Vitt & Cooper 1985, Galán 2008). A number of

studies have looked into the role and effects of blue scales in lacertid lizards. Outer ventral scales

and blue ocelli in *P. muralis* have been shown to reflect ultraviolet light (Arribas 2001, Thorpe &

Richard 2001, Arribas 2012). Possessing these scales could be seen as a trade-off as these scales

may aid in intraspecific communication by increasing visual prominence, while putting more

conspicuous individuals at higher risk of predation (Cooper & Vitt 1993). Male *Iberolacerta*

lizards, a lacertid genus that is sympatric with *Podarcis* in its native range, appear to engage in

anti-predatory behaviors in order to compensate for negative effects of conspicuous coloration on

predation risk (Cabido *et al*. 2008). The fact that this trait varies by sex suggests that it could be

either an intersexual or an intrasexual signal, and therefore open to sexual selection. In many

species, it is common for males to have more vibrant coloration than females (Rand 1992).

Recent research by Sacchi *et al*. (2013) supports our findings that morph coloration is

significantly different between males and females. Studies in other lizard species in which the

appearance of ventral color in males is associated with reaching maturity have found that

androgenic sexual hormones may be involved in coloration (Abell 1998, Bauwens & Castilla

1998).

Ventral coloration is widely-studied in *Podarcis muralis*, and color polymorphism is

well-described (Sacchi *et al.* 2007, Calsbeek *et al.* 2010). Relative frequency of color morph is

known to be highly variable (Sacchi *et al*. 2007), so the significant differences in the frequency

of morphs found between Italy and Greater Cincinnati could be expected by chance. The most

notable difference between the native and nonnative populations was the relative frequency of

red morphs. These were much less common in our population than in Italy. There are a few

possible explanations for the pronounced decrease in red morphs in the Greater Cincinnati

population. The most likely reason for this difference is genetic drift, which can result in a

reduced frequency of certain alleles, particularly rare ones such as the red allele in Italian

populations (Maruyama & Fuerst 1985). In Greater Cincinnati, the small founding population

and subsequent genetic bottleneck experienced after introduction may have played a role in

decreasing the frequency of the rarest color morph.

Other possible factors that could be driving differences in morph frequencies are parasite

loads and immune system function (Galán 2008). Research on European populations of *P.

muralis* has revealed morphological differences associated with ventral coloration. Sacchi *et al*.

(2007) and Calsbeek *et al*. (2010) reported variations in body size between morphs. Homing

behavior (Scali *et al*. 2012), response to stress (Galeotti *et al*. 2010), and stamina (Calsbeek *et al*.

2010) have all been found to be associated with color.

Cephalic Scale Shape

Geometric morphometric analysis of cephalic scale shape in *Podarcis muralis* revealed

shape differences between our five study sites. Variation in scale shape patterns often directly

relates to changes in the morphology of the head. Bruner & Constantini (2009) state that "an

intra-specific covariation pattern is supposed to be the result of a true biological factor creating

functional and structural relationships between the anatomical elements." It is possible that novel

environmental pressures at each invasive subpopulation are contributing to the variation seen in

P. muralis scale shape. Exposure to novel food sources has been shown to drive rapid adaptation

in structures of the head in an invasive population of a different *Podarcis* species (Herrel *et al.*

2008), and the functional relationship between skeletal musculature, diet composition, and prey

capture is considered to be the major driver of lizard skull morphology (Verwaijen *et al*. 2002,

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Stayton 2005). If food sources vary by site, there would be strong selective pressure to adjust the head morphology to feed more efficiently (Herrel *et al*. 2008). To further investigate this possible correlation in the introduced Cincinnati populations, gut-flushing could be used to determine whether there are dietary differences among invasive *Podarcis muralis* subpopulations.

Differences in shape between the left and right sides of the head were consistently driven by a landmark at the anterior edge of the frontal scale, located near the eye. This consistency points to directional asymmetry, with the lizard's left hemisphere being more varied than the right.

Genetics

Our results confirm the prediction that range expansion with limited amounts of gene flow between populations can lead to both genetic differentiation and morphological divergence. The fact that a correlation between genetic and morphological differentiation was not found could be due to the limited genetic sampling that was performed. More comprehensive genetic research may help to reveal the mechanisms by which variation occurs.

Conclusions

Theory suggests that under some conditions, the process of range expansion can actually promote evolution and adaptation (Prentis *et al*. 2008). Many different subpopulations, each with a different subset of genetic variation, provide many different opportunities for selection to act as the population spreads through a novel environment. Our results suggest that expanding populations with limited gene flow can not only build genetic structure, but they can be accompanied by morphological divergence, further strengthening empirical support for conditions that may promote evolution during range expansion. Serial founder effects that

decrease genetic diversity may result in developmental instability, and thus may limit population growth and range expansion, at least in some subpopulations (Băncilă *et al*. 2010). However, despite the potential negative consequences of genetic bottlenecks, *P. muralis* is succeeding as an invasive species in Cincinnati, as evidenced by its rapidly expanding population.

In this study, several possible methods by which genetic, biotic, and abiotic factors could be influencing *Podarcis muralis* in Greater Cincinnati were proposed. Despite extensive analyses, the exact causes of morphological variation (genetic, developmental, or environmental) and the extent to which they represent or will lead to adaptations are still unknown. Though differentiation between these populations may be due to genetic drift and/or phenotypic plasticity, recent research involving *Podarcis* and other lizards gives some support to the possibility that *P. muralis* may be undergoing rapid adaptation to its invasive habitat (Herrel *et al.* 2008). Further research will be necessary to attempt to identify specific heritable genetic, developmental, and environmental factors contributing to morphological variation in these lizards.

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Table 1. Five sampling sites in southwestern Ohio and northern Kentucky. *N* is the number of individuals sampled.

Study Site	*N*	State	Distance to point of introduction (km)
Beechmont (BM)	32	Ohio	3.86
Fairview (FV)	42	Ohio	8.46
Fort Thomas (FT)	31	Kentucky	5.96
House of Tropicals (HT)	31	Ohio	8.65
Rose Circle (RC)	31	Kentucky	6.12
Total	**167**	-	-

Table 2. Two-tailed test of correlation between quantitative morphological variables. Numbers above the diagonal indicate Pearson's correlation coefficient. *Indicates significant result ($P < 0.0001$)

Variables	Snout-Vent Length, SVL (mm)	Hind Limb Span, HLS (mm)	Tail Length, TL (mm)	Hind Foot Length, HFL (mm)	Weight (g)	Head Length (mm)
Snout-Vent Length, SVL (mm)	-	0.684*	0.605*	0.606*	0.832*	0.946*
Hind Limb Span, HLS (mm)		-	0.564*	0.785*	0.766*	0.646*
Tail Length, TL (mm)			-	0.569*	0.715*	0.594*
Hind Foot Length, HFL (mm)				-	0.698*	0.552*
Weight (g)					-	0.782*
Head Length, HL (mm)						-

Table 3. ANCOVA for standard morphological measurements. Location was the main effect, and SVL was used as a covariate. SVL was highly significant for all tests ($P < 0.0001$). *Indicates significance in relation to study site ($P < 0.05$)

	df	F	P
HLS	4	2.370	0.0587
HFL	4	4.9639	0.0012*
TL	4	0.7001	0.594
Head	4	0.3221	0.8624
Weight	4	0.5873	0.6727

Table 4. Descriptive statistics of *Podarcis muralis* from Cincinnati subpopulations. *A* = mean number of alleles per locus; *AE* = effective number of alleles per locus; *He* = unbiased expected heterozygosity; *Ho* = observed heterozygosity; *F* = Wright's fixation index for each population.

Study Site	*A*	*Ae*	*He*	*Ho*	*F*
Beechmont	3.375	2.268	0.433	0.565	0.239
Fairview	2.875	2.238	0.474	0.542	0.101
Ft. Thomas	3.500	2.276	0.478	0.547	0.099
House of Tropicals	3.750	2.505	0.509	0.603	0.143
Rose Circle	3.000	2.429	0.569	0.594	0.011
Mean	**3.300**	**2.343**	**0.493**	**0.570**	**0.118**

Table 5. Pairwise comparisons of genetic distance (Weir and Cockerham's θ, 1984) and geographic distance (above the diagonal). Geographic distances between Greater Cincinnati subpopulations range from 3.47 (FV & HT) to 13.92 (BM & HT). The average distance between all Ohio and Kentucky study sites is 8 km. *Indicates genetic distances are significant ($P < 0.05$) **Indicates significance after Bonferroni correction

	BM	FV	FT	HT	RC
BM		11.87	5.50	13.92	12.40
FV	0.041*		7.46	3.47	5.71
FT	0.047**	0.055**		8.74	6.90
HT	0.024*	0.021	0.020*		3.68
RC	0.077**	0.079**	0.052**	0.038**	
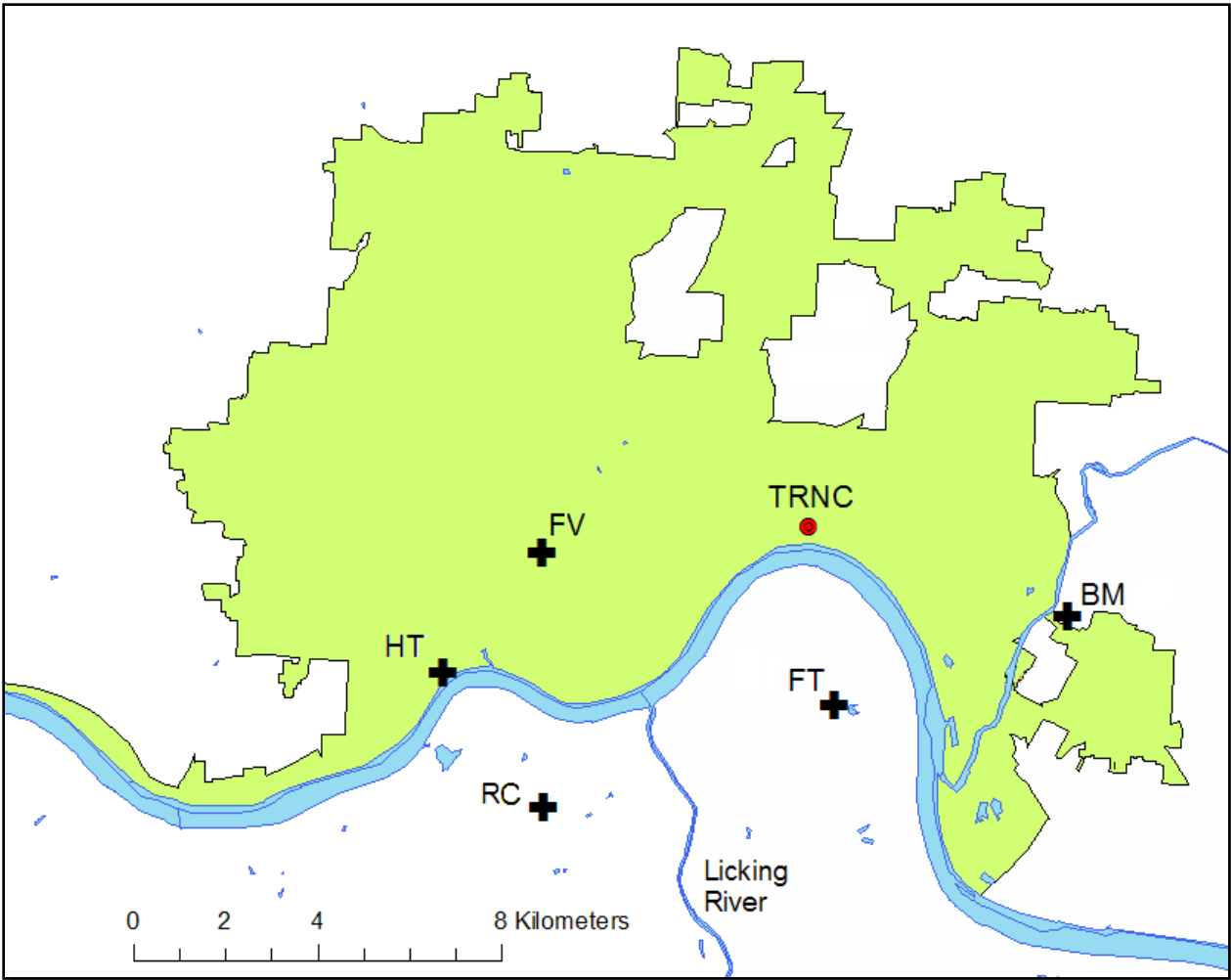
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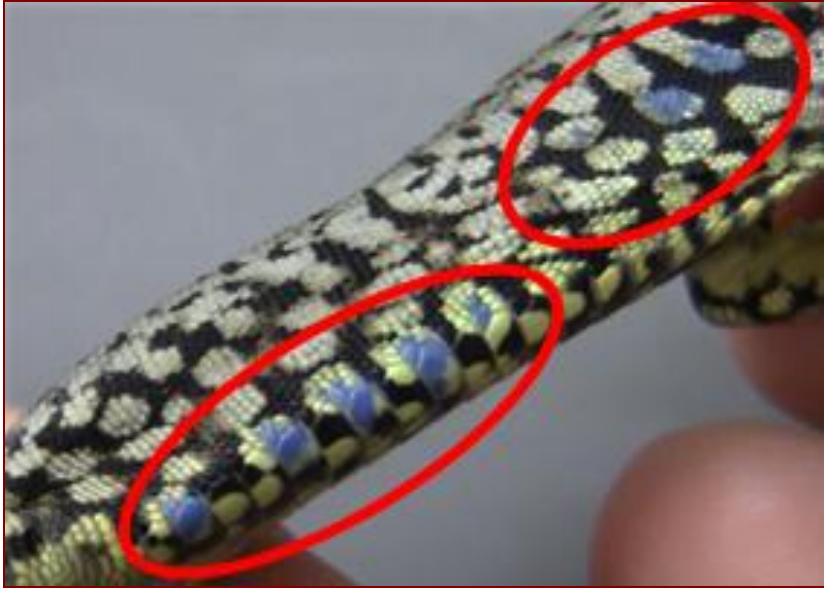
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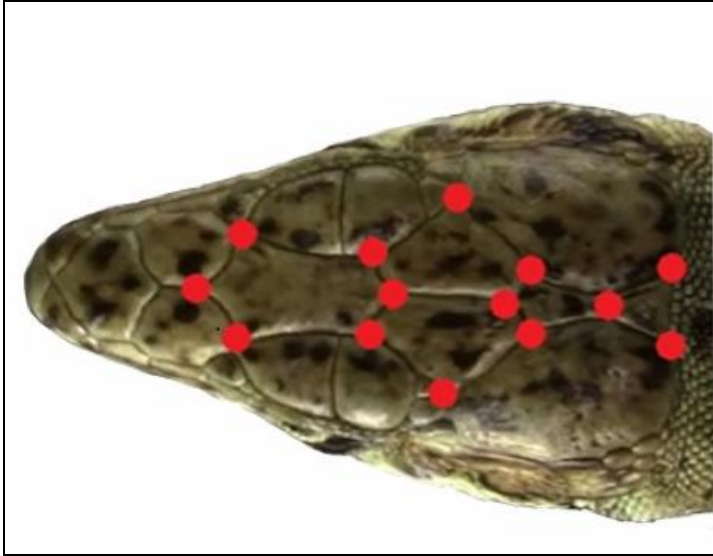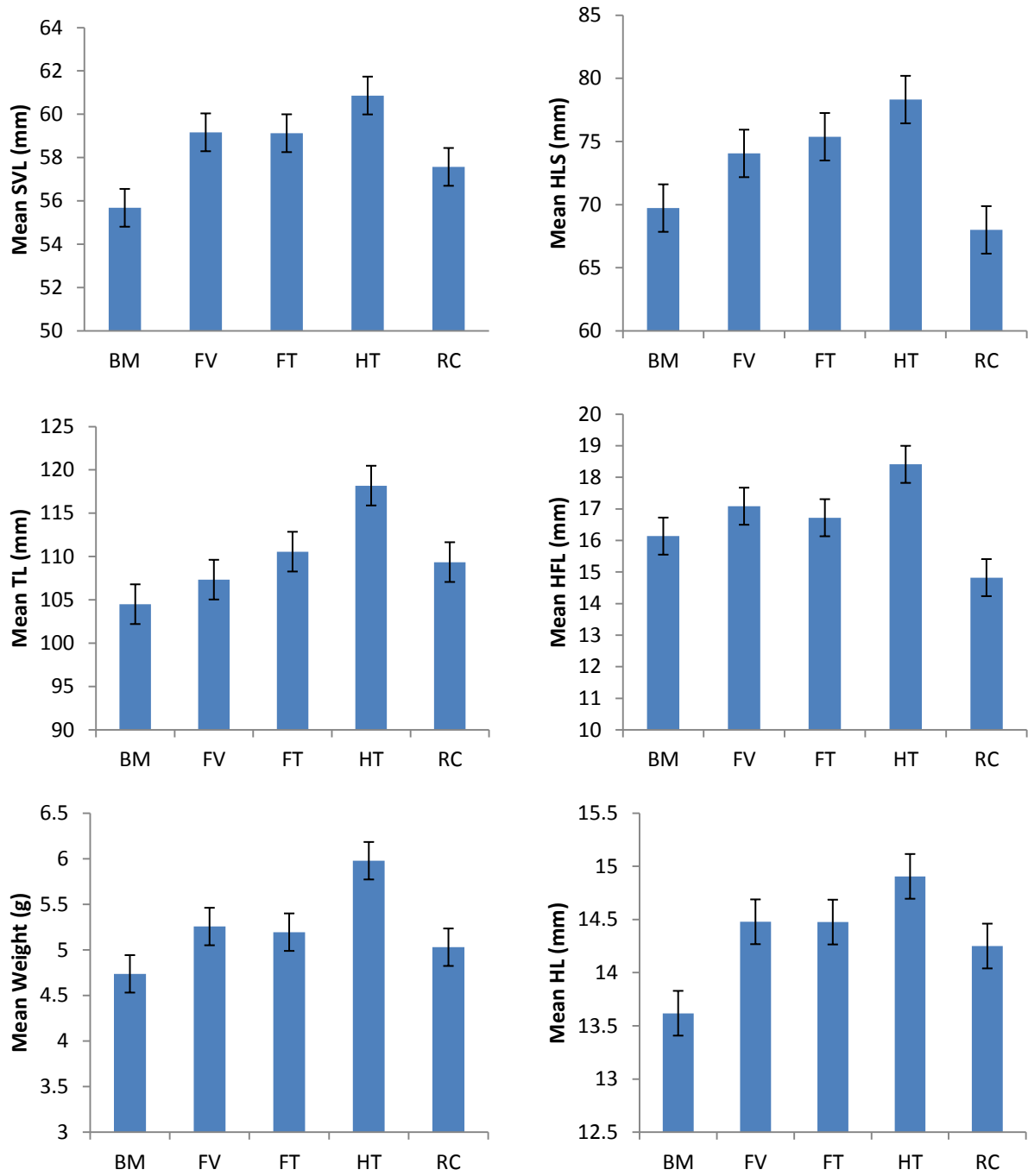
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Figure 4.

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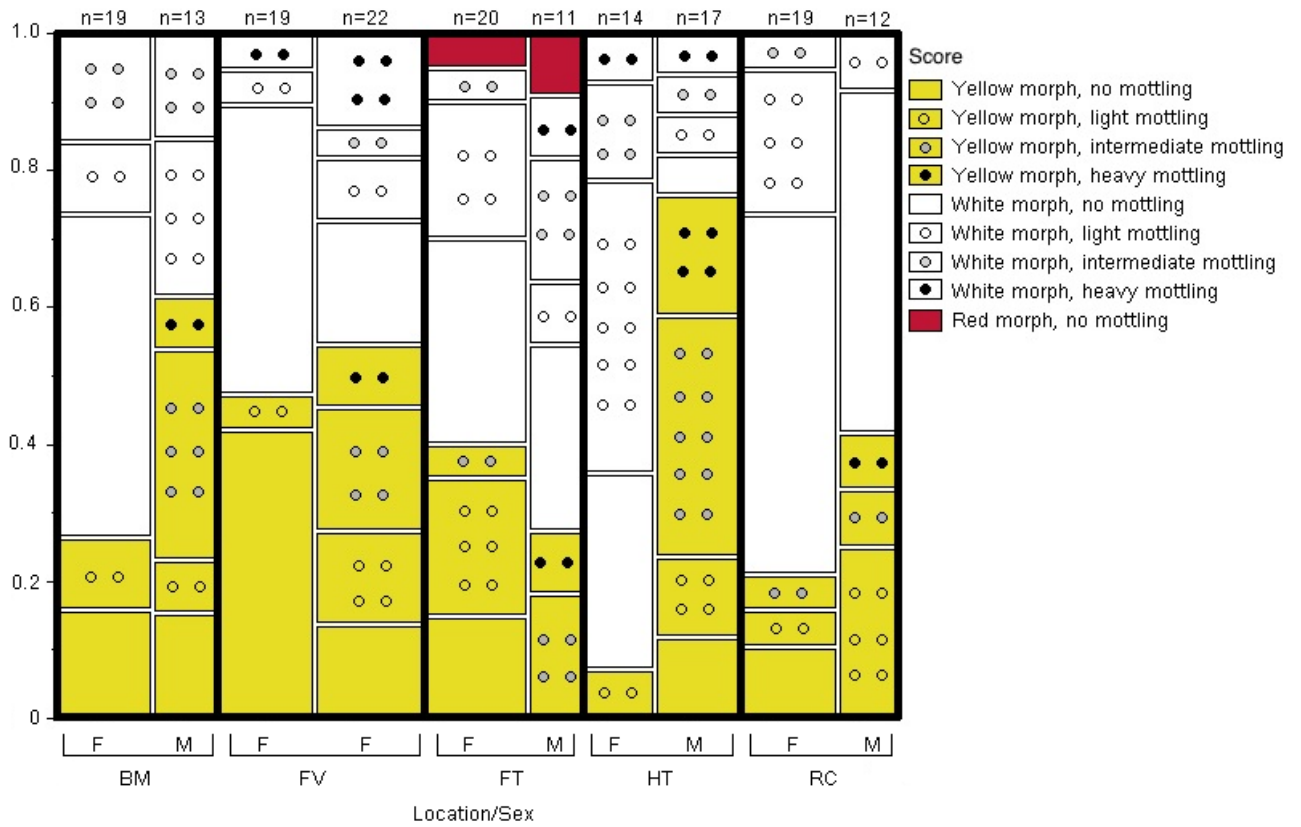
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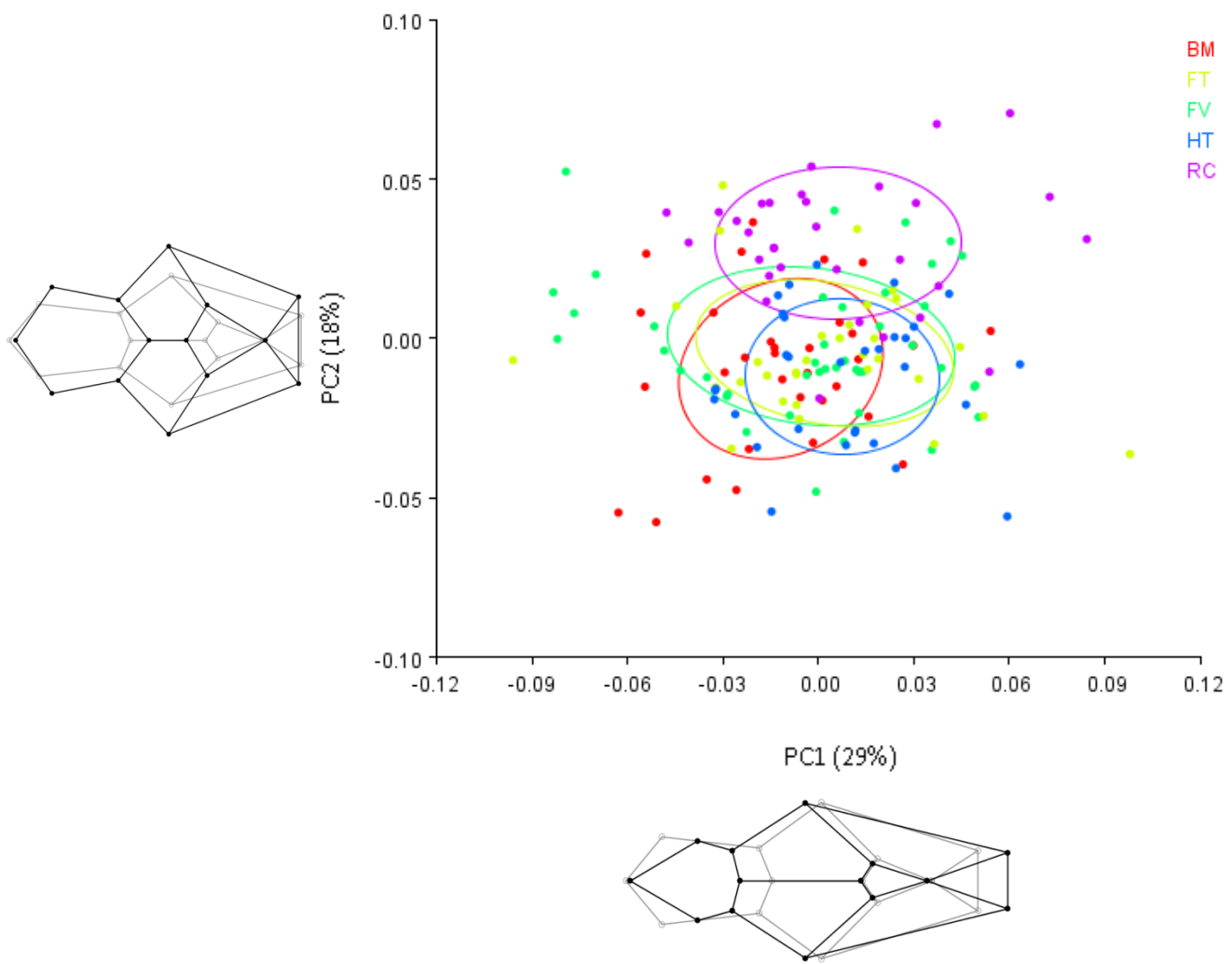
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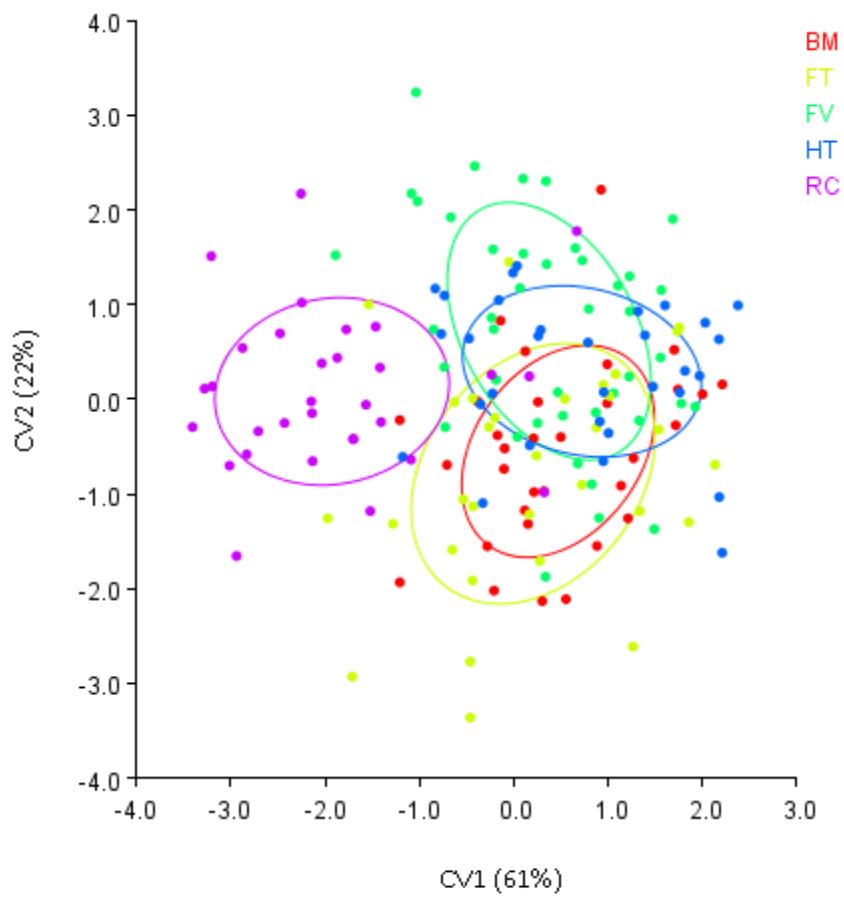
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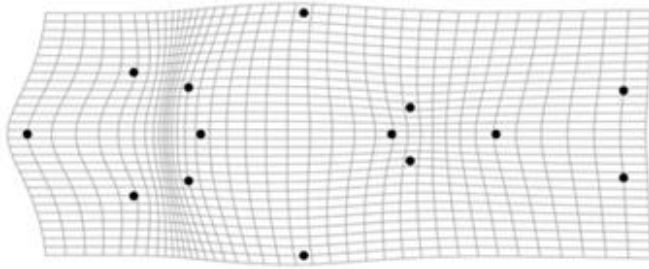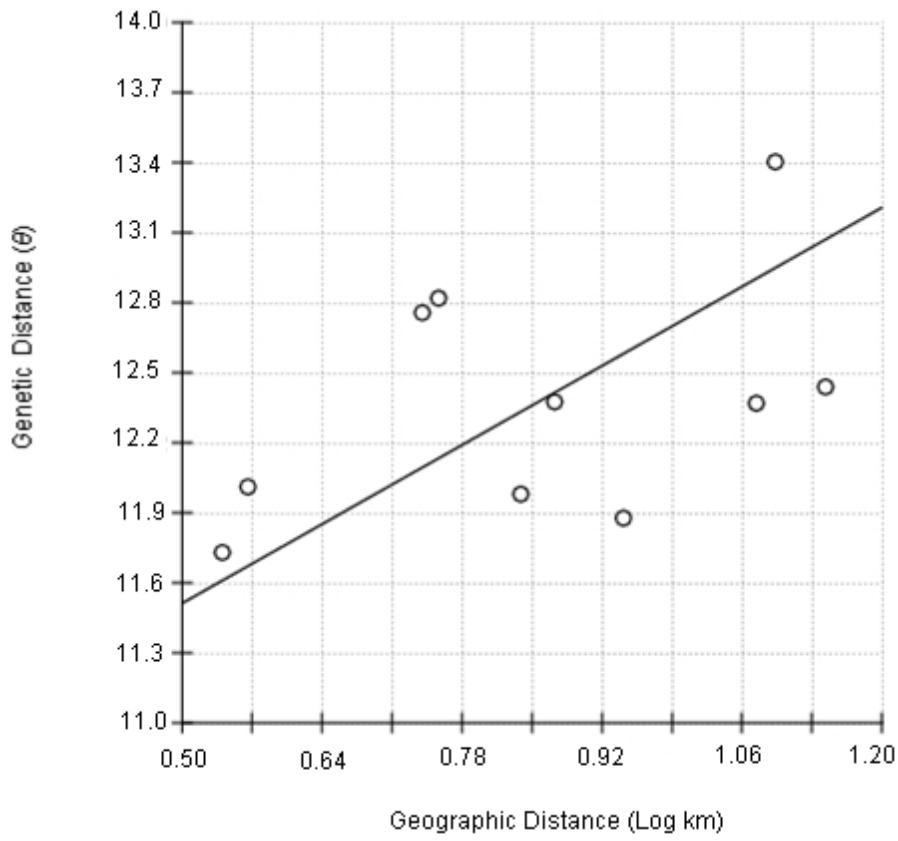
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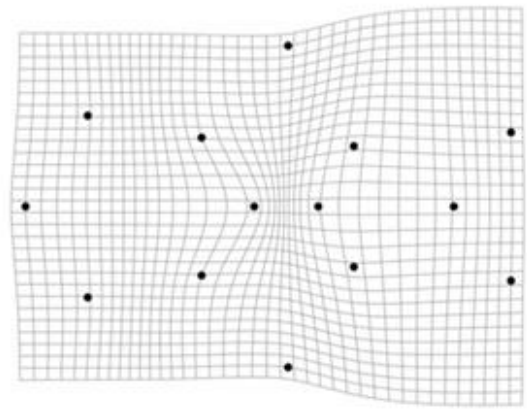
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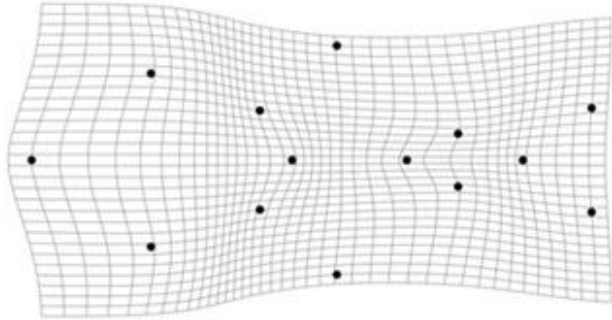
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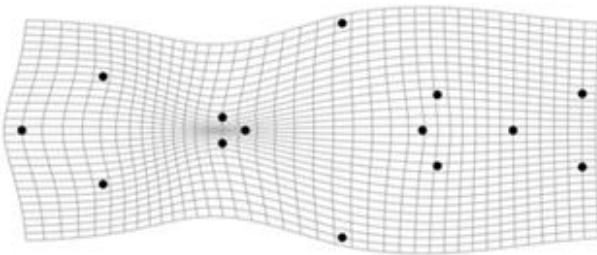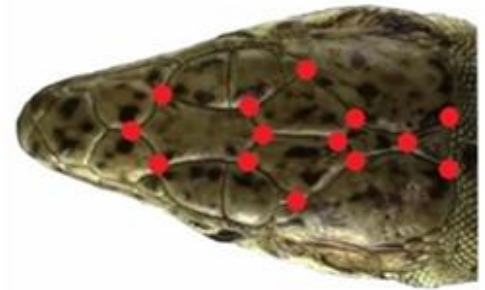
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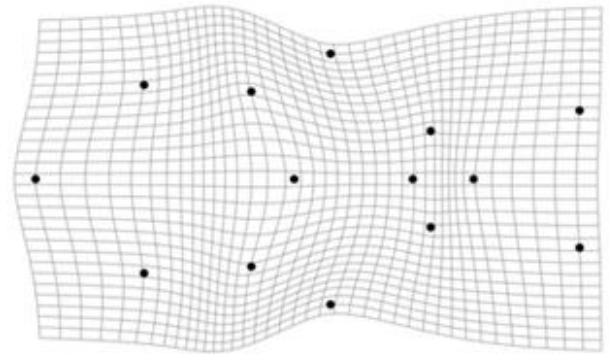
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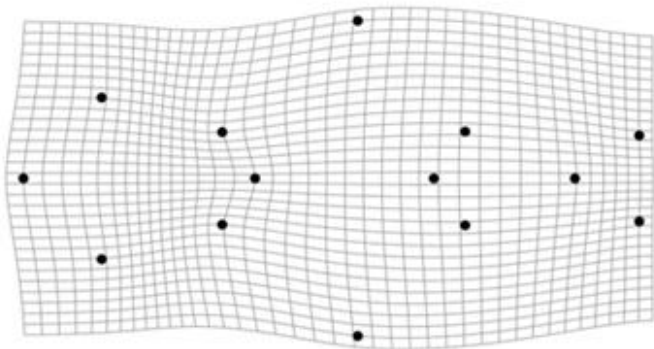
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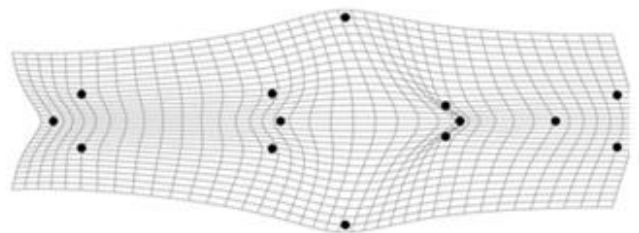
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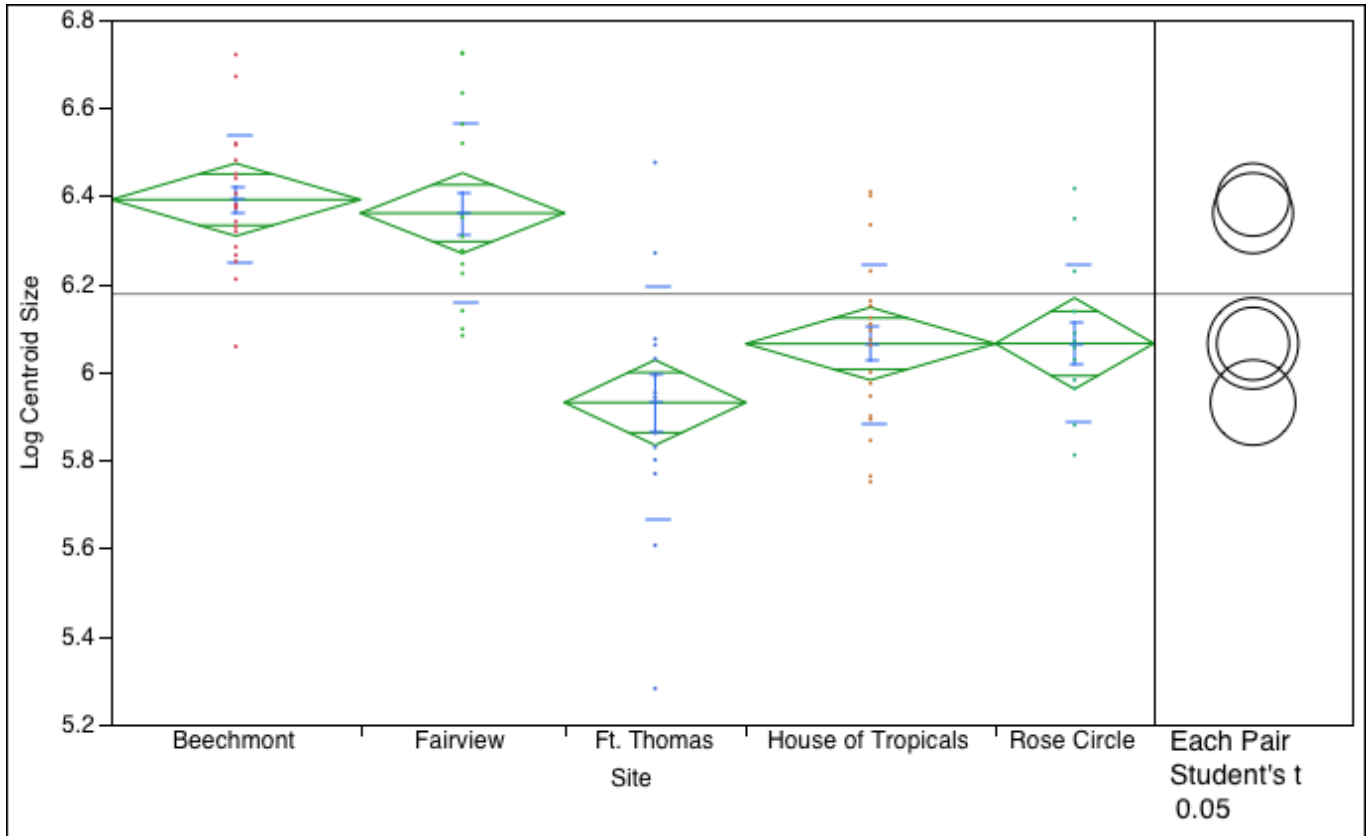
Figure 10.

Figure 11.

Figure 12.