GEOMETRIC MORPHOMETRICS ON SNAIL SHELLS

Warped Ideas: Geometric Morphometrics as a Complementary Technique for Studying Gastropod Shell Morphology

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ABSTRACT

Geometric morphometrics (GM) provides a complementary method for studying morphology. Snails have been analyzed in the field of morphometrics since the 1960s because their shells serve to record information about their life histories and environmental habitats. In this thesis, we present an annotated bibliography for advancements in GM, using applications to snail shells as a representative case study. We categorize 30 publications into four fields, morphology, ecology, taxonomy and evolution, and show that developments have been unequal among them. We conclude by predicting that GM applications on snail shells will increase, especially in hybrid fields, such as, ecotoxicology, which currently are underrepresented. As a demonstration, we describe an experiment wherein we applied GM as a complementary morphological method to study the garden snail species *Cepea nemoralis* in an organophosphate-pesticide, ecotoxicological setting. We conclude by showing that GM reveals subtle morphological differences among treatment groups with no relation to pesticide dosage.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AChE	Acetylcholinestrase
diM	Dimethoate
Wt	Weight
PCA	Principle Component Analysis
GM	Geometric Morphometrics
RW	Relative Warp
RWA	Relative Warp Analysis

DECLARATION OF ACADEMIC ACHIEVEMENT

Chapter 2 Manuscript: I declare that I have performed a literature search for this manuscript. It is formatted and ready for submission to the *Journal of Zoology*.

Chapter 3 Manuscript: I declare that I have conducted and performed the experiment for this manuscript. It is formatted and ready for submission to *Environmental Toxicology and Chemistry*.

1.0 CHAPTER ONE

1.1 INTRODUCTION

Snails are among the few animals that provide a directly measurable connection to their individual lives, even after death, through their shells. The characteristic spiral structures produced by snails conveniently suit them as bioindicators for a variety of environmental conditions. Shells provide surfaces for muscle attachment and protection from predators, mechanical damage, and desiccation. Modern studies in shell geometry started evolving with Moseley (1838), were adapted to a physical perspective by Thompson (1917), and radiated in a computational environment after Raup (1966). More recently, advancements in morphometrics have enabled researchers to analyze shells statistically while preserving the geometry in their forms. This geometric morphometrics (GM) revolution introduced new techniques that can be used to answer questions about structure, environments, classification, and biodiversity.

In this thesis, I investigated the effect of organophosphate (OP) contamination on nontarget organisms, using geometric morphometrics as a method of form analysis. I chose *Cepea nemoralis* as the nontarget organism species, as populations are native to southwestern Ontario and, so, could function as appropriate biondicators for OP application in local, domestic areas. Land snails constitute informative systems for studying "air-water-soil-flora-fauna" interrelations (Gomot *et al*., 2002). They can be used to conduct sublethal toxicity tests because they can be exposed to different contamination pathways, such as dietary intake through contaminated plants or soil and surface uptake through skin or breathing. They also can reveal contamination effects on food webs, because snails can transfer pollutants to predators (Gomot *et al.,* 2006).

Numerous studies have investigated sublethal and lethal effects from metal pollution on snails. A few studies were focused on the toxic role of pesticides (e.g., Rorke and Gardner 1974, Schuytema *et al.* 1994, and Gomot-de Vaufleury 2000). None examined the implementation of GM as a method for studying morphological changes when exposed to toxins. In this thesis, I review applications of GM to snail shells, separating publications into different categories, and describe an experiment that I conducted in which I applied geometric morphometrics to the shells of the snail species *Cepea nemoralis* after they had been exposed to dimethoate through diet.

2.0 CHAPTER TWO

ADVANCEMENTS IN GEOMETRIC MORPHOMETRICS USING SNAIL SHELLS AS A CASE STUDY

2.1 ABSTRACT

We compiled and enumerated papers published between 1998 and 2012 in which researchers applied geometric morphometrics (GM) as a statistical, biological form analysis method. As a detailed case-study, we concentrated on studies involving landmark-based data obtained from snail shells. Snail shells are unique, terminally elongating objects that result from accretionary growth and provide an ideal subject for form analysis. Our goal was to use the case-study to characterize different biological applications, and determine whether GM has been applied with equal frequency among different research fields. Among 30 publications, we categorized applications into the fields: *morphology* (n=12), *ecology* (n=8), *taxonomy* (n=5), and *evolution* (n=5). We implemented thin-plate splines in an analogy, to illustrate visually advancements in GM being heavily weighted in the fields of *morphology* and *ecology*.

Keywords: Coordinate grid transformation, Gastropod, Landmark points, Relative warp, Statistics

2.2 INTRODUCTION

We draw together in this annotated bibliography research that was conducted between 1998 and 2012 demonstrating the application of geometric morphometrics (GM) as a simple-to-use and novel method for studying morphology. Morphological form (i.e., size and shape) has been an important topic in many biological fields, such as anatomy, ecology, systematics, and phylogeny. During the early twentieth century, morphologists began a transition from adopting qualitative to quantitative approaches. The modern morphometrics age started when form was quantified using standard statistical analyses to describe variation within and among groups (Adams, Rohlf, and Slice, 2011).

The first advance in modern morphometrics research occurred when forms were described by reference to morphospaces. Each point in a morphospace represents an individual organism each axis corresponding to a parameter that describes a certain character about the organism. In 1917, the Scottish biologist and mathematician Sir D'Arcy Wentworth Thompson became the first modern morphometrician to attempt to apply geometry and statistics to biological form. He introduced coordinate grid transformations as a means for comparing developmental and evolutionary differences and, so, provided a glimpse into morphospace. In his classic work "*On Growth and Form*", he used mathematics to describe snail shell spiral shapes. He described shells as surfaces produced through revolutions around imaginary fixed axe by closed curves that remained similar to themselves. They increased geometrically while the rotation angle increased arithmetically, and the surfaces traced in space resembled equiangular spiral shells. In 1966, the American palaeontologist David Raup extended morphospace usage by developing a computational model to describe coiled shells and using the model to analyze coiled shells and situate them within a morphospace. Each point in that morphospace represented a shell form that was described by three parameters: translation rate, expansion rate, and displacement. Raup was among the first researchers to translate the mathematical language of Thompson into pictures. In the 1980s, modern geometric morphometrics emerged with a formal quantification of coordinate grid tranformations. Above all other fields in science, geometric morphometrics has preserved Thompson's legacy (Williams, 2009; morphospace analyzes preserve his spirit (Stone, 1997)).

2.2.1 A brief history of conchology

Geometry in molluscan morphology has been identified since 287 B.C.E, when the Greek philosopher Archimedes described the spirals that now bear his name ($r = a\ \theta^{n}$). Later, the English architect, Wren, investigated logarithmic spirals, remarking that distances between revolutions are nonconstant (as they are in Archimedian spirals); instead, they increase in a geometric progression, resembling shell spirals. Still later, the Swiss mathematician, Bernoulli, christened the logarithmic spiral *Spira mirabilis,* the "marvelous spiral". Next, the Dutch biologist, Swammerdam, found spiral patterns among different shell forms. Finally, the British Reverend Moseley (1838) described turbinate and discoid shells.

The twentieth century started with Thompson (1917) introducing structuralism and allometry. He proposed diminishing natural selection as a key principle in form studies, instead, emphasizing physical laws and mechanics as determining morphologies. From the early 1980s to the present, the field 'statistical morphometrics' has developed that Thompsonian perspective substantially. The quantitative analysis of form has transitioned from traditional multivariate statistics as they apply to biometric data to more refined techniques that use landmark points (J. Stone and D. Lindberg, pers. comm., 2010).

2.2.2 Traditional multivariate morphometrics

Shape analysis has been developed statistically over the past quarter century. Development in the 1960s and 1970s involved multivariate morphometrics, wherein data were analyzed using techniques such as canonical variates analysis (CVA), discriminate functions analysis (DFA), principal components analysis (PCA) (Appendix I), and other related factorial methods. Studies comprised applying these multivariate morphometric techniques to linear variables or sometimes ratios, counts, and angles. Problems arose with these techniques, notably size correlation with shape, or allometry. Methods were developed to address the problems, but at least three difficulties remained. First, among the many size-correction methods proposed, each yielded slightly different results. Second, the variables that were used involved nonhomologous points, and, therefore, correspondence between specimens was difficult to establish. Third, the same measurements could be obtained from two different shapes because the locations of points relative to each other were not recorded. And fourth, because the geometry yielding the variables was lost in analysis, generating visual representations for shapes posed challenges (Adams *et al.*, 2011).

2.2.3 Geometrics morphometrics revolution

Early in the 1980s, a shift occurred in how morphological data were analyzed, which would help to resolve the issues inherent in traditional morphmetrics. The new morphometrics was focused on the geometry of morphological structures and preserving organismal form throughout analysis. Even the nature of data collection changed fundamentally, becoming landmark points and the geometric properties about their relative positions. Rohlf and Marcus (1993) declared this development in geometric morphometrics as a “revolution”. Two approaches were developed under GM, outline and landmark. Bookstein (1996) referred to this development as the “morphometric synthesis” (Appendix I).

2.2.4 Outline approach

The outline approach involves digitizing points on curvatures delineating organism forms and then comparing the data using mathematical functions (*e.g.,* Fourier analysis). This approach is limited to simple forms; other, related approaches have been derived to analyze more complex forms, such as using changes in tangent angles at points along outlines, analysing changes in x and y directions as functions of distances along curves, or treating coordinates as series using complex numbers. Again, problems remained because each approach yielded different results (Adams *et al.*, 2011).

2.2.5 Landmark approach

The landmark method involves collecting 2D or 3D coordinates of anatomically, mathematically and topographically defined positions (Appendix I). Nonshape variation (position, orientation, and scale) first must be eliminated to conduct pure shape analyses on these data. Superimposition (Appendix I, Fig. 1) is the procedure whereby nonshape variation of landmark point configurations is minimized by overlapping them using Bookstein shape coordinates or, the preferred method, Generalized Procrustes Analysis (GPA). GPA uses least-squares estimates, in a three-step algorithm. First, the centroids among all landmark point configurations are translated to a common origin. Second, landmark point configurations are rescaled to a common centroid size of one. Third, configurations are rotated to maximize overlap. The algorithm is repeated until the least-squares criterion is satisfied, and then a reference configuration is determined (Adams *et al.*, 2011).

Geometric morphometics can be defined as a multivariate statistical method that involves landmark point configurations and in which shape variation is described using least-squares Procrustes distances and the geometry of form is preserved throughout analysis (Adams *et al.*, 2011). With GM, researchers can generate graphical representations for comparison, such as thin-plate splines, which are deformation grids that depict shape transformations, one form being 'warped' into another (Appendix I). To compare differences between landmark configurations, shape vectors are analyzed, yielding 'partial warp' scores (multivariate data representing shape). In the early 1990s, a novel, partial-warp-extending, shape analysis was introduced, relative warp analysis (RWA; i.e., a PCA conducted on partial warp scores) (Appendix I).

2.2.6 Advances in geometric morphometrics

By the 1990s, advancements GM had developed into a different method, indeed revolutionary, for analyzing morphological form. This maturation resulted more from better understanding of the mathematical foundations underlying landmark methods in the biological community than from theoretical innovations. According Adams *et al.* (2005: Fig. 1), the number of publications involving GM increased significantly from 1976 to 2001. We observed a similar increase in GM publications over the recent years (Fig. 2) (J. Stone and D. Lindberg, pers. comm., 2010).

Additionally, software has become available freely, and standard sets of procedures for analysing landmark points have been established. Most software packages use GPA (Appendix I) to establish reference configurations. In addition, outline methods also have evolved by implementing the use of sliding semilandmarks (Appendix I), which enable outlines to be combined with landmark point data in analysis.

In this annotated bibliography, we review the use of landmark based morphometric techniques in analyzing organism form and growth. We specifically focus on their application to snail shells because they provide convenient subjects for landmark analysis, and, most importantly, their accretionary spiral growth preserves growth records. Also, as snail specimens are easy to culture in laboratories, we expect landmark based snail shell studies to increase in number over time (Stone, 1998).

2.2.7 Shell morphology advantages

Shell morphology in gastropods might is amenable readily to GM analysis. Shell morphology involves growth from the beginning of postembryonic life, provides ontogenetic information, and is correlated with behaviours and habitats, for example, shells that have low spires are more stable in areas of heavy wave environments (Brusca and Brusca, 2003).

The arbitrary concept of a whorl is defined as the accretion of calcium carbonate through an angular revolution of 2π radians. Whorls provide a convenient metric for detailed morphometric analysis because sectioning shells transversely, into two half-shells, provides a convenient way for obtaining data. Alternatively, data can be obtained from radiographs of shells, which allow specimens to remain intact.

Spiral accretionary growth involves the addition of new layers of $CaCO_3$ onto older ones. As Hayes (1995) has remarked, “if one could unroll a shell and flatten it out into a rectangle, the pattern would form a space-time diagram, with position in space measured along one axis (left to right) and sequence in time recorded along the other axis (top to bottom)”. The landmark-based techniques of geometric morphometrics may be applied effectively to gastropod shells because they are accreted rather than grown, and, so, landmark points on shell surfaces accumulate in time. The aperture trajectory (the path of shell accretion) provides a record of shell accretion, from apex to aperture. Thus, landmark points on gastropod shells can supply developmental information as well as the end result of the developmental process. Therefore, shell growth, may be utilized to illustrate the advancements of GM.

2.2.8 Shell structure & production

Calcareous layers in a shell often compromise aragonite or an aragonite-calcite mixture, and the imaginary coiling axis often is realized as the columella. The turns of the spire, whorls, join at sutures (Brusca and Brusca, 2003). Each shell consists of an outer, thin, organic periostracum that is composed of conchins, which are complex proteins secreted by the mantle; and two or three calcerous layers (prismatic, middle lamelate, and inner nacreous layer). The prismatic and lamelate layers are composed mainly of calcium carbonate, while the inner nacreous layer is made up of calcerous lamellae intermingled with thin films of conchin (Brusca and Brusca, 2003). Calcium carbonate accretes through crystals that are surrounded by a thin protein matrix and other small amounts of inorganic material obtained from the surrounding environment (i.e., calcium sulphate and magnesium carbonate); all constitute the calcium carbonate framework (Brusca and Brusca, 2003).

In this annotated bibliography, we examine the growth of GM by focussing on applications to shell morphology. We subdivided 30 papers into 4 different categories *morphology*, *ecology*, *evolution* and *taxonomy*. If, starting in 1998 (as among the first publication of GM application on snail shells), advancements and publications among the 4 categories were to have expanded uniformly and equivalently over the next 14 years, then advancements could be represented as an expanding square, each corner representing a developing category (Fig. 3a). If, instead, expansion were unequal, increasing more than average in two categories and less in the two others, then an appropriate representation would be a trapezoid. Thus, if one were to consider the corners in a square as landmarks representing categories, then one could utilise a thin-plate spline to describe advancements in GM (Fig. 3b). We used this analogy for illustrative purposes herein.

2.3 GEOMETRIC MORPHOMETRICS ON SNAILS

2.3.1 Morphology

Stone (1998)

Stone (1998) illustrated differences between traditional multivariate morphometrics and geometric morphometrics in their application to gastropod shells. He proposed a hypothetical example involving the fictitious snail genus *Conchus*, with three species, two of which shared closer kinship. *C. conchus* exhibited a particular shell shape, while the two more-recent and more-closely-related species, respectively, exhibited loosely coiled shells with wide whorls and tightly coiled shells with narrow whorls. Stone applied traditional multivariate morphometrics on these snails by generating computationally linear measurements such as length, width, height, aperture width, and aperture height and subjecting them to a PCA. He compared that analysis to a landmark-based thin-plate spline relative warp analysis on the same shells. Principal components clustered specimens into distinctive groups on the basis of gross aspects of shell dimensions (*i.e.,* wide shells and apertures versus long shells and apertures). Relative warps distinguished among specimens using general ontogenetic trends (e.g., lateral expansion of the spire and contraction of the body whorl); changes in landmarks recorded individual ontogenetic trajectories instead of merely capturing shape. Thus, Stone showed how relative warp analysis provided an effective method for distinguishing between gastropod shells of different species.

Guralnick & Kurpius (2001)

Guralnick and Kurpius (2001) investigated ontogenetic allometry and whether the magnitude of shape change due to allometry is greater than the magnitude of shape change due to whorl offset. They collected a sample of 33 snails for each of 7 different lots of *Littorina saxatilis* from the British Museum of Natural History. The lots represented 7 different localities throughout the United States and Europe. The researchers implemented GM, particularly the use of RWA to examine the axes of shape variation. Analyses were conducted on spiral growth, itself, to test whether the starting point of whorl segments are the same between and within lots. Through those techniques, Guralnick and Kurpius were able to distinguish whether similarities or differences among starting points for growth in *L. saxatilis* individuals exist. Most of the *L. saxatilis* specimens that were examined exhibited the same end points in shape space. Some populations of *L. saxatilis* from different habitats exhibited different but fixed endpoints in their trajectories because they either deposited an extra 270° or lost 90° growth. For example, shells in a sheltered habitat comprised more whorls, in comparison to shells from nonsheltered habitats, which comprised fewer whorls. Data did not directly support environmental impact as a mechanism of shell shape change; instead, genetics and plasticity were suggested. Guralnick and Kurpius concluded that GM analysis was able to test shell shape variations for worn, indeterminate gastropod growth.

Nehm (2001)

Nehm (2001) investigated the relationship between developmental processes and the loss of defensive shell features in a clade of marginellid gastropods. Age, shape, and size were compared among juveniles and adults of “ancestors” and “descendants” using many morphometrics analyses, including GM. Nehm hypothesized that heterochronic change alone accounted for modifications in antipredatory morphologies. Different sources of morphometric analyses were applied on to specimens of *Prunum* species to evaluate patterns of ontogenetic and morphologic change. RWA was implemented to separate out size and shape variation and compare configurations for ancestors and descendants. And aperture views were compared between juvenile and adults in shape space. Based on the relative warp ordination plot, similar sizes and whorl numbers were measured at the protoconch-telechonch boundary for *P. latissium* and *P. maoense* species and, therefore, no change in growth onset time. A change in the offset time was suggested by the smaller size and whorl reduction in *P. maoense* compared to *P. latissium*. Also, adults of *P. maoense* overlaped in shape space with juveniles of *P. latissium*. Thus, Nehm concluded that the reduction in shell features in *P. maoense* resulted from paedomorphosis via progenesis (acceleration of sexual maturation relative to the rest of development).

<u>Langerhans and DeWitt (2002)</u>

Langerhans and DeWitt (2002) examined how the adaptive value of phenotypic plasticity depended upon the degree of match between environment and phenotype. The freshwater snail species *Physella virgata* was used to study the specificity with which organisms use cues to induce defensive phenotypes. Molluscivorous or nonmolluscivorous sunfish were used as 6 different predator species (*Lepomis cyanellus*, *L. gibbosus*, *L. macrochirus*, *L. megalotis*, *L. microlophus*, *Micropterus salmoides*) on *P. virgata*. Only two of the fish were considered to be molluscivorous. GM was used to obtain detailed shell shape differences by retaining spatial covariation between landmarks. Snails showed two responses, dependent on predator class: 1) reduced growth or 2) development of rotund shells. Fecundity, itself, is reduced by growth reduction and prevents snails from reaching a size favourable for most predators. Using deformation grids, induced shape changes in sunfish treatments occurred in apertural regions; a thin-plate spline analysis also was conducted on 19 apertural points, corroborating that outcome. Comparing deformation grids, the researchers observed that the control group accreted teardrop-shaped shells while the treatment group accreted arch-shaped shells (rotund shells). Rotund shells entailed a higher vulnerability for predators through shell entry and higher crush resistance and so increase handling time and rejection rates by molluscivorous sunfish. The predator-induced morphologies were beneficial, as they precluded predation, but, the authors pointed out, unreliable cues can lead to phenotypic errors. An inappropriate phenotype-environment match can impose constraints on the evolution of phenotypic plasticity, as long as benefits outweigh costs.

Hollander *et al.* (2006)

Hollander *et al.* (2006) studied how predictable environments might lead to fixed ontogenesis while phenotypic plasticity is favourable for organisms that inhabit unpredictable environments. Phenotypic plasticity was tested experimentally between the snail species *L. saxatilis*, released as miniature juveniles on a shore, and *L. littorea*, released as drifting larvae, settling on different shores. Three different sources of stimuli, crab rich, crushed conspecifics, and wave action, were imparted to juveniles, and, after three months, magnitudes of phenotypic differences were compared. Phenotypic plasticity in shell shape was analyzed using Cartesian coordinates of anatomical landmarks. Distinctions among various species and ecotypes were determined by the different regions occupied in a relative warp analysis shape space. Hollander *et al.* hypothesized that *L. littorea* would show more plasticity than would *L. saxatilis*. Surprisingly, with the help of deformation grids, the researchers found that both species showed similar ranges in plasticity, with small differences between ecotypes of nondispersing species, such as, *L. littorea* had a more moderate measure of phenotypic plasticity compared to *L. saxatilis.* This suggested that phenotypic plasticity is an adaptive trait in *L. saxatilis*.

Conde-Padín, Grahame, and Rolan-Alvarez (2007)

Conde-Padín *et al.* (2007) used geometric morphometrics to determine shape and size variation among three species of marine snails (*Littorina saxatilis*, *L. compressa*, and *L. arcana*). Characterizing the morphology of sympatric populations that are common in European intertidal zones (*i.e.*, rocky shores of northeastern England) was problematic. GM were conducted on two snail ecotypes that were adapted to different degrees of wave exposure (*i.e.*, H on the upper shore and M on the mid shore). Relative warp 1 (RW1) described variation of aperture shape relative to body whorl. With the aid of deformation grids, Conde-Padín *et al.* found that snails in the H ecotype were characterized by round shells with big apertures (35% of the total shell area); while snails in the M ecotype were characterized by flattened shells with smaller apertures (29% of the total shell area). The larger aperture characterizing H ecotype specimens was required to accommodate the larger foot, necessary for avoiding dislodgment by waves, while the smaller aperture of the M ecotype provided protection against crab predation. Conde-Padi *et al.* used GM to quantify morphometric variability in size and shape between populations and specimens of the sibling species of British rough periwinkles.

Schilthuizen and Haase (2010)

Schilthuizen and Haase (2010) are among the few researchers to have applied GM to land snails. Schilthuizen and Haase investigated differences between coiling shapes of *Amphidromus inversus*, by using relative warp analysis as a technique to distinguish between dextral (clockwise) and sinistral (counter-clockwise) coiling. In snails generally, both forms of coiling are present but dextral predominates, with direction determined by a single gene with delayed maternal inheritance. Without a pedigree, researchers cannot predict with certainty the relationship between an individual coiling genotype and coiling direction, and, therefore, Schilthuizen and Haase predicted that dextral and sinistral might be mirror images of each other. Usually, mating is not favoured in snails of individuals with oppositely coiled shells chirality, but, based on field and molecular studies, the researchers found that sexual selection favoured matings between opposite chirality in *A. inversus*, thereby maintaining dimorphism. Applying geometric morphometrics revealed surprising results because sinistral shells exhibited slight but significant widening and twisting of the shell near the palatal and parietal apertural areas. With the help of deformation grids, subtle differences between the two coiling morphs were revealed. The grids showed transformation of one coiling morph into another, with most distortion occurring near the top part of the aperture. Caliper measurements (*i.e.,* for traditional morphometric analyzes) did not generate data useful for differentiating coil chirality morphs, because manual measurement required repositioning each shell each time a measurement was taken, while GM required positioning each shell only once. Schilthuizen and Haase concluded that selection on shape differences is either weak or constrained by pleiotropic effects on chirality gene early in development only.

Teso, Signorelli, and Pastorino (2010)

Teso *et al.* (2010) confirmed phenotypic variation in shell size and shape for *Olivancillaria carcellesi* in 4 different localities, by the application of GM. These sea snail species live along subtidal soft bottoms, from Rio de Janeiro, Brazil, to Punta Pardelas, Argentina. GM were employed to describe size and shape variation of *O. carcellesi* among four representative localities. The first three relative warps accounted for the greatest variance (70.5%). Specimens from Punta Pardelas exhibited allometry between size and shape, which produced higher spires. The morphs also were analyzed using traditional morphometrics and revealed differences in columellar callus thickness, length, and width. Different environmental factors generally can induce variation in shell morphology. For example, a wide range of surface seawater temperatures exists in the region, with the highest in Cassino beach (Brazil) and lowest in Punta Pardelas (Argentina). As a result, $CaCO_3$ concentrations increase with increases in latitude, from tropics toward poles. These environmental conditions were used to explain why *O. carcellesi* from Punta Pardelas are small and from Cassino are large. Teso *et al.* proposed other ecological possibilities that could account for the observed variation in shell shape described by GM (*i.e.* effects of tributyltin contamination).

Cuña *et al.* (2011)

Cuña *et al.* (2011) examined the role of amplified fragment length polymorphism (AFLP) variation along with GM to investigate polymorphism in the gastropod species *Melarhaphe neritoides* in different geographical regions. For each specimen, size was measured by centroid size, and shape measured by relative warps. Analysis of variance revealed significant shape differences among three separate localities along Galician rocky shores. Thin-plate splines revealed that shells from wave exposed sites had more rounded shells with larger apertures compared to shells from protected sites. The RW1 representation of the two sympatric species, *Nucella lapillus* and *Littorina saxatilis*, and *Melarhaphe neritoides*, living in the same microhabitat, displayed convergent polymorphisms regarding size of the aperture. Cuña *et al.* showed that the high dispersal capabilities (planktotrophic stages) of marine organisms displayed direct adaptive polymorphisms, which are rare.

Minton *et al.* (2011)

Minton *et al.* (2011) investigated the plastic environmental response of the pleurocerid snail *Elimia potosiensis* from a spring and creek in Arkansas, at a scale thousands of times smaller than previously investigated. *E. potosiensis* populations exhibited plasticity over a span of hundreds of metres. Phenotypic plasticity was assessed using GM to quantify shell variation and to distinguish between individuals and populations at the species level. Regression of shell shape showed significant spatial relationships. Shells from upstream were longer and thinner than were shells from downstream. Based on the relationship between Procrustes distance and geographic distance, Minton *et al.* found that similar shell shapes were exhibited in sites close to each other, indicating a sequential shift in morphology from upstream to downstream.

Queiroga *et al.* (2011)

Queiroga *et al.* (2011) applied geometric morphometrics to identify spatial shell shape variation of *L. saxatilis* (an ovoviviparous species) and *Melarhaphe neritoide* (a species with planktonic eggs and larvae). Samples of each species were collected from 6 sites along the west coast of the Iberian Peninsula. Landmark based morphometrics were applied to reveal shape change between the two species. A clear segregation of sites was detected for both species but more evident for *L. saxatilis* than for *M. neritoides.* Based on RW1, shape changes were common to both species, especially variation in the height of apical whorls relative to the rest of the shells. As the height of apical whorls increased among specimens, apertures widened. Snails with higher spired shells, are able to accommodate a large body with a small foot. The *M. neritoides* populations live in regions of high intertidal and supratidal zones, while the *L.* s*axatilis* populations have a wider distribution, occurring from lower supratidal to lower intertidal zones. The researchers identified many causes of ecotypic variation of shells on rocky shores, such as wave action, crab predation, and desiccation. Any of these could have driven shape variation, allowing for adaptation to microhabitat characteristics. The morphological responses observed exclusively through deformation grids might have represented a plastic response, but more research is required to identify whether it was related to local adaptation, allometry, or nonallometric plasticity.

Schilthuizen *et al*. (2012)

Schilthuizen *et al.* (2012) tested the impact of environmental factors on dimorphism present in a population of *A. inversus*. As discussed previously with Schilthuizen and Haase (2010), populations in this species are unique because genetic dimorphism is influenced by sexual selection. Schilthuizen *et al.* used landmark-based measurements, but this time on hatchling snails, to detect if ecological differences at a small spatial scale (*i.e.,* forest plots) caused any changes in coiling morphs. Observations made from deformation grids did not show any significant difference between morphs. Deformation vectors that illustrated transformations from the mean sinistral to the mean dextral shell were perpendicular to the center of the shell. This was explained as a confounding systematic error in positioning shells. Schilthuizen *et al.* concluded that the data do not provide evidence for the role of environmental factors in maintaining coil dimorphism.

2.3.2 Ecology

Carvajal-Rodriguez, Conde-Padín, and Rolan-Alvarez (2005)

Carvajal-Rodriguez *et al.* (2005) studied two ecotypes of *L. saxatilis* and showed that traditional morphometrics were unable to reveal significant shell shape differences. Depending on the tidal reach, species like *L. saxatilis* were observed to live under different physical and ecological conditions. Environmental conditions differed in slope, wave energy, and wave exposures. Those local changes might have been small but impacted shell morphology, thereby making populations polymorphic. Shells with larger and more-sculptured shells are fared better against crab predation versus smaller and thinner shells. At the same time, smaller, thinner shells fared better against wave action. Carvajal-Rodrıguez *et al.* extended the use of simple linear measurements that might not reveal biological relevance by applying GM to ecotype research.

Conde-Padín *et al.* (2007)

Conde-Padín *et al.* (2007) applied GM to analyze a population of *L. saxatilis* from exposed shores of northwestern Spain to investigate reproductive isolation and adaptation in sympatric ecological speciation. The researchers studied the genetic differentiation between ecotypes for shell traits using GM. Conde-Padín *et al.* were able to unravel size and shape components of morphological variation between the ecotypes. Through GM analysis, they found that differences between ecotypes appear for the same shape components (*i.e.,* in the x-axis for uniform component, and the relative size of the aperture for the first non-uniform component, RW1). Using thin-plate spline visualizations, Conde-Padín *et al.* were able to differentiate shapes between and within ecotype localities. RW1 deformation accounted for changes in apertures of shells. At different localities, RW1 portrayed the highest differentiation between ecotypes and between populations of the same ecotype, especially for high wave action. Shell patterns caused by genetic variation were inspected and visually displayed by GM. In summary, researchers found that there are certain characteristics of shell morphology that don’t change and therefore, allowing its diagnosis.

Minton & Norwood (2008)

Minton & Norwood (2008) applied landmark-point-based morphometrics to freshwater snail shells in the genus *Lithasia* from Duck River, Tennessee, USA, which exhibited plasticity.

Relative warp analysis revealed shorter shells with narrower apertures and longer and narrower whorls for snails in regions upstream; shells downstream were longer with wider apertures and wider, inflated whorls. Relative warps showed shells exhibiting reduced inflation and sculpture, which could be easily dislodged under strong current flow but offer advantages in moderate but changing environmental conditions of headwaters. Minton & Norwood have found that shell phenotypic plasticity exists across freshwater gastropod in many forms.

Hollander & Butlin (2010)

Hollander & Butlin (2010) assessed the adaptive value that phenotypic plasticity had on the survival of *L. saxatilis*. Juveniles from two ecotypes, exposed (E) and sheltered (S), were subjected to different ecological treatments (crab, wave, control) in a laboratory environment to initiate plastic development in different ontogenetic directions. The survivors were transplanted into 5 natural habitats to test the adaptive value of any plastic response that has influenced phenotypic variation within and between ecotypes. Landmark-based geometric morphometrics were applied to capture shape variation in shells. The two ecotypes were differentiated significantly along RW1, and treatment groups were differentiated on RW2. Deformation grids for the S-ecotype illustrated a pointy, deformed apex compared to the E-ecotype. Hollander & Butlin conclude that the partial plastic response has a significant impact on fitness, although it remains small compared to the overall adaptive difference between ecotypes.

Marquez, Gonzalez-Jose and Bigatti (2011)

Marquez *et al.* (2011) combined GM with computed tomography (CT) and scanning electron microscopy (SEM) to study the impact of tributyltin (TBT) pollution on shell shape. TBT is used as an antifouling agent in marine environments but causes ‘imposex’ in gastropod females by either the neoformation of a vas deferens or a penis or by causing shell malformation in bivalves. Little research had been conducted to study the impact of TBT on gastropod shells. Snails in the species *Odontocymbiola magellanica* were selected from a polluted site (P) and non-polluted site (NP) for shell shape comparison using 3D GM. PCA was conducted on the variance-covariance matrix of Procrustes coordinates. The first ten PCs explained 87% variance, but only the first one was required to reveal differences between the two zones. Based on thin-plate splines, snails from the P site possessed more-slender and narrower shells, with upward-pointing orientated apices and a narrower aperture, than did individuals in the NP site. Shell structure and density were analyzed further by the other two methods, CT and ESM scans. The P individuals showed a very fragile shell compared to thicker shells of NP individuals. Marquez *et al.* showed that GM can be a used as combined morphological technique to detect shell shape variation when exposed to different environmental variables.

Torres *et al.* (2011)

Torres *et al.* (2011) analyzed morphological variation within and among golden apple snail populations (*Pomacea canaliculata*) through landmark based methods. By distinguishing species found in different regions, Torres *et al.* (2011) were able to study the role of geographical isolation in evolution. Deformation plots showed significant changes in shape that geography alone could not explain. RW1 accounted for 32.6% variance in female samples, which comprised changes to apertures. Samples that scored positively on RW1 had shells with a narrower opening relative to the body whorl, while those with negative scores contained shells with a tapered posterior margin of the outer lip and highly elevated whorls. In terms of male samples, RW1 accounted for 40.19% variance and revealed changes in the breadth of apertures. Torres *et al.* illustrated how geography contributes to the population variability, and GM techniques were capable of describing those geographic differences by quantifying shape variation.

Torres *et al.* (2011)

Torres *et al.* (2001) also studied the golden apple snail, *P. canaliculata*, this time in its role as a pest for rice agriculture in the Philippines. Because different management approaches had been taken and risked populations benefiting from selection, the researchers decided to investigate inter- and intra-populational differentiation in 6 populations from the region of Agusan del Norte. Geometric morphometrics were applied to study spires, using landmark data to capture the dorsal and aperture views of shells. Based on five relative warps for both genders, overall trends in size and shape variation of shells among the geographical areas were similar. Therefore, Torres *et al.* were able to conclude that geographic distance is not a factor for differentiation of shells (*e.g.,* spires or apertures).

Dunithan, Jacquemin and Pyron (2012)

Dunithan *et al.* (2012) studied covariations between morphology and different environmental variables at local and regional scales in the North American freshwater gastropod species *Elimia livescens.* Relative warp analysis using Procrustes superimposition was applied to investigate shape variation. Shape differed significantly between flowing and non flowing habitats, drainage areas, latitude and longitude, water temperatures, conductivities, substrate types, and the abundance of woody debris. At the positive extreme along RW1, shells with a shorter aperture width, shorter body whorl, and elongated apex length were represented. The actual specimens are located in southeastern Indiana, in regions of a small drainage subbasin with lower conductivity, higher dissolved oxygen, and coarse and less-complex substratum. Individuals represented by such loadings are found commonly in lake habitats. At the positive extreme along RW2, shorter and wider shells, robust and thicker overall, were represented. Actual specimens are located in regions of higher water temperature and lower frequency of coarse substrate. Dunithan *et al.* (2012) proposed that morphological variation for aquatic gastropods occurring in variety of environments is a key feature for maintaining wide distribution and maximizing local success.

2.3.3 Taxonomy

Cunha *et al.*, (2008)

Cunha *et al.* (2008) reconstructed phylogenetic relationships among large-shelled marine venomous snail species in the genus *Conus* based on mitochondrial *cox1* and *nad4* haplotype sequences. The researchers applied geometric morphometrics to characterize shell and radula variability among different clades. Shells were analyzed using semilandmarks and landmarks to ensure a good characterization over entire specimens. The researchers found significant differences in radular tooth shape between *C.pseudonivifer/C.trochulus* and *C.venulatus/C.ateralbus* groups. Northwestern Boavista and Maio specimens clustered together to the exclusion of eastern Boavista specimens. Furthermore, large-shelled *Conus* have similar biogeographical patterns of diversification with those of small-shelled *Conus*. Cunha *et al.* uses GM as a complementary morphological technique to study radular teeth shape variation as predictors for species taxonomic status.

Urra, Oliva and Sepulveda (2007)

Urra *et al.* (2007) applied geometric morphometrics to distinguish between populations of the two species *Adelomelon ancilla* and *Odontocymbiola magellanica* in southern Chile. These snail species are important to the fishery of Chile's southern zone because they act as a potential resource for artisanal fishery. Their external morphologies are very similar and they both are categorized under *A. ancilla* in the fisheries national statistics. Thus, to discriminate between the taxa, radular morphology and shell body characteristics were analyzed using traditional multivariate morphometrics and landmark based measurements. No significant differences were identified on the basis of the traditional multivariate measurements shell thickness or weight; nevertheless, researchers were able to distinguish between the two species using GM, which revealed differences in shell shape. Shape differences depicted by thin-plate splines showed larger apertures with low spires for *O. magellancia* and smaller apertures with higher spires for *A. ancilla.* No historical records on populations for these species are recorded, so determining whether differential fishing pressures are being exerted on any of them is important for local ecosystem conservation. Urra *et al.* have implemented the use of GM to detect differences in shape between these two species.

Moneva *et al.* (2012)

Moneva *et al.* (2012) used GM to detect subtle differences in sexual shell dimorphism for *Pomacea canaliculata*, which has caused a lot of considerable taxonomic confusion. High RW1 scores indicated less-pronounced and narrow shell apertures, while low RW1 scores indicated bigger shell apertures and more-globose shells. Gender variation in *P. canaliculata* could be distinguished by differences in height of the spire and aperture. Females were characterized by more-concave opercula while males were characterized by convex opercula. Moneva *et al.* emphasized how geometric morphometrics were able to detect subtle differences between sexes.

Cruz, Pante and Rohlf (2012)

Cruz *et al.* (2012) quantified shape variation in *Conus*, which is known for its peptide-based toxin, and it enables it to be significant tropical reef predator. Landmark-based morphometrics were applied to five species: *C. consors*, *C. miles*, *C. stercusmuscarum*, *C. striatus*, and *C.* textile; a phenogram was generated from morphological distances acquired from landmark coordinates digitized on shells. RW1 and RW2 eigenvector loadings accounted for

81.06% variance and showed clear separation among groups, especially for *C. miles*, with some overlap amongst others (*e.g.*, *C. stercusmuscarum* and *C. striatus*). Thin-plate splines revealed differences in shape. *C. miles* is characterized by a conical body whorl, *C. consors* by a cylindrical body whorl, and *C. stercusmuscarum*, *C. striatus*, and *C. textile* by ovate body whorls. Cruz *et al.* proposed utilizing of GM on gastropod specimens for species-recognition programs.

Perez (2011)

Perez (2011) analyzed the new polygyrid land snail genus *Praticolella*, found in northeastern Mexico using mitochondril 16S rDNA and cytochrome c oxidase subunit-I-sequences along with geometric morphometrics. Invasive populations have been established in the USA and Caribbean, but confusion between *P. griseola* and *P. berlandieriana* was problematic. Shell shape was analyzed by GM to differentiate between the two taxa. GM was carried out using an "Integrated Morphometrics Package" (IMP software suite: Sheets 2003). Shape variation was revealed through deformation grids, showing changes in shape from *Praticolella* n.sp. to *P. berlandieriana* and from *Praticolella* new species to *P. griseola.*

2.3.4 *Evolution*

Hollander, Adams, and Jonhannesson (2006)

Hollander *et al.* (2006) analyzed polymorphic shell shape in *L. saxatilis* to study evolutionary changes as a result of different environmental factors, life-stages, or genders. To understand different causes of morphological variation, Hollander *et al.* (2006) compared ontogenetic trajectories by applying geometric morphometrics to analyze shell shape. Ontogenetic trajectories varied depending on habitat, age, and gender. For example, adult males found on rocky cliffs grew isometrically (growth that occurs at the same rate for all parts of an organism) through their lifetime, while females found in the same habitat switched from isometric juvenile to allometric (growth that occurs at different rates) adult growth. Contrastingly, male and female snails found in another habitat, characterized by boulders, were found to grow allometrically as juveniles, and isometrically as adults. Deformation grids were used to determine growth pattern differences among different regions for each gender and showed no changes from juvenile to adult life stages, indicating isometric growth. Thin-plate spline deformation grids revealed that most allometric growth occurred around apertures and at apices, which could have been observed only with a technique that preserved geometry throughout analysis. Hollander *et al.* (2006) concluded that constraints to plasticity can effectively impede the production of an optimal phenotype through plastic development, and limits to evolve plasticity might even preclude adaptation to potential environment.

Guerra-Varela *et al.* (2009)

Guerra-Varela *et al.* (2009) used GM to analyze the species *Nucella lapillus* and investigate differences between size and shape of shelled embryos collected from the wild. Different ecotypes of *N. lapillus* in northwestern Spain can occur either allopatrically or sympatrically depending on the degree of wave exposure and microhabitat. Landmark based techniques were applied to capture morphological differences that classical statistical methods (e.g., distances and ratios) were unable to. Landmark based methods also proved to be convenient for embryo shell analysis because specimens could be digitized to record coordinate positions. GM characterized morphology of exposed and sheltered ecotypes. RW1 revealed that shells of the exposed ecotype were squatter with relatively larger apertures. This suggested that the relatively large shell aperture increased the ability to resist strong swells, while squatter shells had lowered the risk of dislodgement because they produced less drag. Contrastingly, shells with relatively smaller apertures provided sheltered ecotype specimens with defense against predators. Quantifying morphological change, Guerra-Varela *et al.* (2009) proposed that the two forms were distinctly adapted to differences in shore level and exposure to wave action.

Sepulveda & Ibanez (2012)

Sepulveda & Ibanez (2012) analyzed clinal variation in shell morphology for the species *Acanthina monodon*, on the Chilean coast. Sepulveda and Ibanez (2012) found that size and shape scores decreased in the southern region, revealing large biogeographical and latitudinal variation, because of the thickness of the lip and spire length. Multivariate morphometric analysis illustrated two major morphotypes: northern populations contained robust shells with short spires and thick, smooth lips, and southern populations contained weaker shells with longer spires and thinner, ornamented lips. Relative warps were generated for the data using TPS software (version 1.49) with the parameter $\alpha=1$, which weights more heavily more-remote landmarks. The variance for the first two relative warps accounted for 78% variance revealing decreasing clinal variation with latitude. With the aid of GM, Sepulveda and Ibanez concluded that morphotypes were generated as a result of local variation, producing site-specific eco-phenotypes. They employed a geometric model to study shape variation of *A. monodon* due to latitudinal differences, using landmark coordinates.

Hayes, Minton and Perez (2007)

Hayes *et al.* (2007) used the GM approach (landmarks) and mtDNA to investigate whether genetic divergence among isolated *Elimia* populations related to shape. CVAs for each population were conducted but showed a lot of overlap between populations, with no geographic pattern. When these drainage populations were viewed in pairs, a moderate amount of morphological separation was revealed, such as between Brazos and Rio Grande (Gulf of

Mexico) populations, which are at the extreme eastern and western ends of the range of *E. comalensis*. GM was used to analyze phenotypic plasticity of shell form to determine slight and subtle differences in shape between populations that arose due to factors unique in local environments. With the help of phylogenetic analysis of COI sequences, Hayes *et al.* were able to conclude that *E. comalensis* is a single species with no genetic divergence among widely separated populations; thus, it can be considered endemic to one area and might have been dispersed accidently to new springs by humans.

Mariani, Peijnenburg, and Weetman (2012)

Mariani *et al.* (2012) discussed differences between neutral and adaptive evolutionary processes in shaping spatial structures of natural populations in field of ecology and conservation biology. They focused on marine gastropods known as ‘common whelks’, *Buccinum undatum*, because the species lacks a planktonic larval stage, which might promote isolation of local populations and lead to a phenotypic divergence by either genetic drift or local adaptation. Geometric morphometric techniques were applied to 10 whelk populations in coastal waters around Ireland, along with other statistical approaches, such as microsatellite and shell thickness analyzes, to study the evolutionary dynamics behind their spatial variation. The first three relative warps accounted for 65.7% variance. RW1 represented a range of broad, round, and short shells to more narrow, slender, and longer shells. RW2 represented a range of shells exhibiting narrow shell apertures to wider shell apertures. Finally, RW3 represented either smaller apertures and tighter and elongated siphonal ends or truncated and wide open ends. The results from GM were not easily interpretable to relate to specific ecological factors, but RW3 offered some clues. For example, shells with wider apertures and tighter sphional ends could represent an adaptation against ‘handling’ by clawed predators such as crabs, which are also more abundant in Atlantic areas than near Ireland coastal waters. Mariani *et al.* concluded that the multidimensional descriptors of shell shape obtained through GM are less easy to link to specific ecological processes but some clues are offered by the pattern of relative warps.

2.4 CONCLUSION

The role of geometry in studying organism form in modern biology originated with D'Arcy Thompson, including his model for snail shells. Snail shells have been analyzed computationally since the 1960s and have functioned as model organisms in developing morphospace methods. Advancements in morphometrics since the 1980s, especially with landmark data and related analytical techniques, suited shells as ideal subjects for morphological studies. As a result, with geometric morphometrics advancing as a complementary morphological method, its application on gastropod snail shells is expected to be increased in the future.

Geometric morphometrics holds promise as a tool in many fields, as techniques can reveal shape changes that are too subtle to be identified by other, traditional methods. Herein, we have shown researchers that were able to implement GM as a technique in morphological, ecological, taxonomical, and evolutionary type of research. Conde-Padín *et al.* (2007) have used GM to test hypotheses about phenotypic adaptation as an example of morphological studies. Additionally, GM as a technique for morphometric analysis was implemented by Sepulveda & Ibanez (2012) to investigate the impact of environmental conditions on shell shape. GM also has been applied to study ontogenetic development or taxonomic classification. Finally, rresearchers like Hollander *et al.* (2006) and Cruz *et al.* (2012) illustrate how shells can be a significant biomarker, providing ontogenetic records for growth, and how GM can be utilised as a shape analysis method for species classification.

One hybrid field, spanning ecology and evolution, that holds promise for GM but currently is underrepresented is ecotoxicology. Torres *el al.* (2011) and Marquez *et al.* (2011) studied shape changes in the pest organism *P. canaliculata* can become a critical component to study when examining pest management. Geometric morphometrics is beneficial for detecting changing populations of pests and managing the impact of environmental pollution on shell structure. Furthermore, Urra *et al.* (2007) reported that GM can be applied to distinguish between species and, thereby, become an important application in establishing fisheries statistics and management strategies.

Geometric morphometics applied on gastropod shells can be implemented as a complementary morphological tool that serves to describe shape variation. It has proven itself to be an outstanding method compared to traditional multivariate statistics, as GM quantifies shape based on ontogenetic trends rather than gross aspects of shell dimensions. Thus, with continued advancements in geometric mophometrics, I predict that the expanding representation of GM publications in the next 10 years will continue to increase with equal representations in all categories and perhaps new fields (e.g., ecotoxicology).

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2.6 APPENDIX I: TERMS AND CONCEPTS

Principal Components Analysis: A traditional morphometric method that reduces complexity of data and reveals underlying morphological variables. It generates components, vectors, that account for variation in the data. Components are defined as linear combinations of variables that quantify patterns among the linear measurements. For snail shells, if all loadings on PC1 are unidirectional (i.e., same sign), then it represents size, and, if loadings are bidirectional (eigenvectors carry opposite signs), then it represents shape.

Geometric Morphometrics: A relatively new morphometric technique that uses landmark-points on coordinate grids to measure morphological similarities and differences among specimens. It preserves geometry of organisms and represents morphological change through deformation grids.

Landmarks: Points defined anatomically or according to other criteria. Landmarks are applied to represent shape, and each point must be homologous from one specimen to another.

Semi-landmarks: Also known as 'sliding landmarks', these are used when a unique landmark cannot be identified on all specimens (*e.g.,* a landmark on a curvature). A tangent is estimated as the line parallel to the segment connecting adjacent landmarks. Each semi-landmark is confined to "slide" along the line tangent to the curve.

Superimposition: A procedure for fitting all the configurations (specimens) together using least squares (the sum of distances between homologous landmarks is minimized). It separates non-shape components by performing three procedures: translating to a common grid, rescaling to a common size, and rotating to a common configuration.

Procrustes Distance: The square root of the sum of squared distances between homologous landmarks of corresponding configurations.

Relative Warp Analysis: Essentially a PCA of scores obtained from a partial warp analysis, which may be visualized with a grid deformation.

Thin-plate splines: Visual representation of relative warp, where rectangular grids are superimposed on a drawing representing a specimen, revealing the required "warping" from the mean shape to the specified configuration.

2.7 APPENDIX II: FIGURES

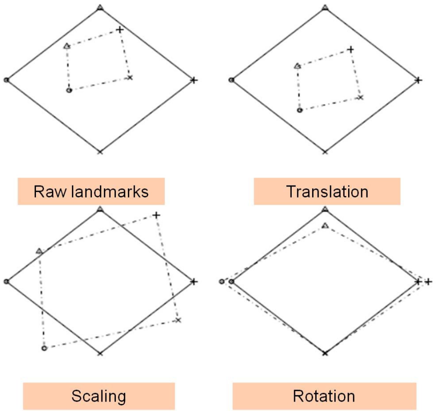

Figure 1. Example of the superimposition method applied to two differently shaped quadrilaterals.

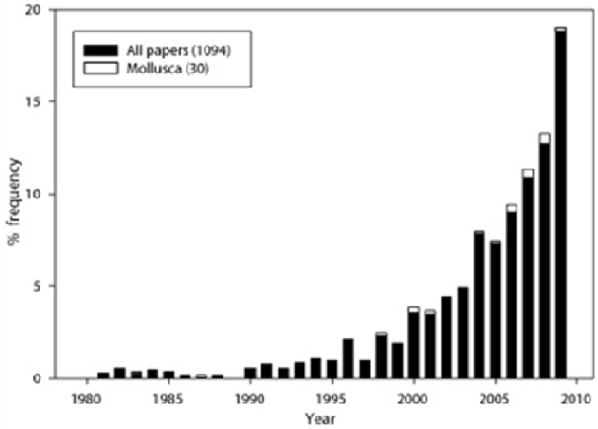

Figure 2. Geometric morphometrics papers from 1980-2010. The graph represents percent frequency of all GM research papers and on molluscan research.

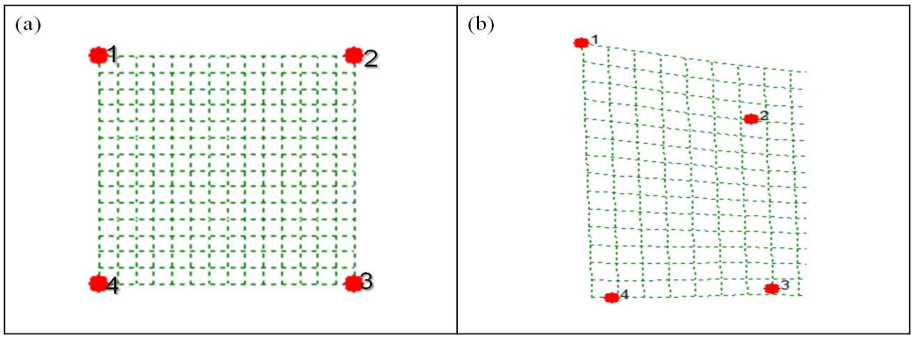

Figure 3. Thin-plate spline representing research distribution for 30 publications. Landmark points represent categories: 1) *morphology*, 2) *ecology*, 3) *evolution*, 4) *taxonomy*. (a) consensus configuration, which would represent publication frequencies if all 4 categories had expanded equally (b) deformed configuration, which represents actual publication frequencies for the 4 categories and shows that expansion was greatest in morphology and ecology.

3.0 CHAPTER THREE

GEOMETRIC MORPHOMETRICS APPLIED TO THE GARDEN SNAIL *CEPEA NEMORALIS* AS A MORPHOLOGICAL METHOD IMPLEMENTED IN AN ECOTOXICOLOGICAL EXPERIMENT

3.1 ABSTRACT

We explored whether geometric morphometrics could be utilised as a morphometric tool to identify effects of organophosphate pesticides on nontarget organisms, using dimethoate (diM) and shells of the snail species *Cepaea nemoralis*. diM exposure was achieved through dietary uptake for 8 weeks. Survivorship decreased with greater diM concentration but nonsignificantly. Feces production as a proxy for food intake decreased significantly as concentrations increased. AChE activity was almost nil at concentrations 2000 ug/g diM and greater. Dry soft body weight increased significantly with concentration exposure. Principal Components Analysis (PCA) plots revealed that shape change was most pronounced during the last week but otherwise were difficult to interpret. Consequently, we applied geometric morphometrics (GM) to analyze shell shape. A thin-plate spline relative warp analysis for specific concentrations revealed shell deformations, with compressed apertures or compressed apical whorls and expanded body whorls. GM enabled us to analyze as bioindicators, land snail shells, which already had been shown to be informative in size change terms, and utilize an overlooked, complementary aspect, shape change. Because GM involves simple data collection, rapid analysis, and graphical visualization, it holds promise as a new assessment technique for application in toxic environmental management.

Keywords—Dimethoate, Land snail, Relative Warp Analysis, Shell Morphology, Thin-plate Spline

3.2 INTRODUCTION

After World War II, scientific knowledge that had been used militarily was redeployed to wage war against pests. Chemicals pesticides were developed, and agricultural productivity increased. Chemical pesticide application has become common but still presents a risk to aquatic and terrestrial ecosystems [1]. A variety of methods are implemented today, with significant impacts on the environment [2]. In Canada, pesticides are applied through techniques such as aerial and ground-based application [3]. Consequently, a considerable amount of chemical reaches water surfaces and soil; chemical residues affect other, nontarget organisms in surrounding ecosystems, which often are more susceptible to chemical toxification [2].

Organophosphates (OPs) are chemicals that are applied as therapeutic agents, plasticisers, lubricants, flame retardants, and fuel additives, but they are utilized most-commonly as insecticides. They are preferred over organochlorines because OPs are less persistent and tend to degrade faster [4]. Even though OPs comprise a wide range of chemical structures with diverse physicochemical properties, their main toxicological action is to inhibit acetylcholinesterase enzyme (AChE) in central and peripheral nervous systems [5]. According to Pope *et al.* (1990), all OPs share a common mechanism of toxicity, by phosphorylating AChE [5]. Chemical interactions occur between the toxicant and AChE, where the serine residue found on the active site is organophosphrylated, resulting in a nonfunctional enzyme. Communication between nerve cells is mediated chemically by the release of ACh from the axon terminal of a presynaptic neuron into the synaptic cleft. Once ACh binds to the postsynaptic membrane, a chemical signal is converted to an electrical one until AChE is removed from the receptor by AChE. Normally, the AChE in a synapse degrades acetylchoine into acetate and choline; but, when AChE is inhibited by 50% or greater, acetylcholine accumulates, and cholinergic receptors become overstimulated, leading to clinical signs of cholinergic toxicity, such as disrupted nerve function and eventually mortality. The reactivation of AChE is very slow and considered irreversible [5].

3.2.1 Dimethoate (diM)

Dimethoate ($C_5H_{12}NO_3PS_2$; diM), is among the most-widely used oraganophosphorous insecticides [3]. diM was introduced in 1956 and has continued to be used widely since, due to its molecular composition, which exempts it from the many restrictions imposed on ogranochlorines [8]. Agricultural applications in Canada involve terminating a wide variety of insects and mites that are considered to endanger fruits, vegetables, and a wide range of crops and forests [6]. diM also targets pests such as aphids, grasshoppers, leafhoppers, mites, and plant bugs [7]. As diM lacks selectivity, it also can affect nontarget organisms [4]. Thus, it has been chosen as a reference pesticide by the European Community R&D project of testing systems, to assess sublethal effects of chemicals in soil environments [4].

The Pest Management Regulatory Agency (PMRA) has labelled diM as a systemic group 1B insecticide, categorized for AChE inhibitors, which are responsible for normal nerve impulse transmission [3]. When diM is released into environments, it has a low affinity for soils and, therefore, is subject to leaching [9]. The main route of degradation is decay in soil, with a half-life between 4 and 16 days [11]. According to Coeurdassier *et al.* (2002), the DT_{50} (degradation time for 50% of a compound) of diM in soil is 21 days. At the same time, evaporation and biodegradation enable diM to be lost from the soil. It can contaminate nearby lakes and rivers due to irrigation or runoff. As a result, AChE inhibition through pesticide/diM application affects growth, feeding rate, and activity levels of different aquatic and nontarget organisms [9]. diM penetrates digestive and epithelial tracks and biotransforms into omethoate and other oxidative analogues, which are 1000 times more inhibitory [1]. Based on previous literature, such as the

Environmental Response Management Application (2001), diM is toxic to organisms that live in aquatic environments, such as crustaceans, fishes, and birds, with EC_{50}=0.4 mg/L, LC_{50}=1 mg/L, LD_{50} =5.4 mg/kg, respectively.

Canada uses approximately 100000 kg diM per year [10], and, in 2002, diM was detected at the Rennie Street Landfill in Hamilton, Ontario, at levels below 0.5 mg/kg of soil. Cygon 480, an emulsifiable concentrate containing diM, is employed through aerial or ground application to different agricultural crops [3]. Peas provide an example of the lowest concentration utilized, 132-204 g/Ha, whereas strawberries provide an example of the highest, 1.32 kg/Ha. Each agricultural crop has a specific application rate, for example, ground application of diM is applied to mature asparagus in the summer after harvest and continued at three to four week intervals until defoliation [3].

3.*2.2 Geometric morphometrics*

GM is a relatively new technique that is used in many fields, such as morphology, ecology, taxonomy, and evolution but is under-represented in hybrid fields such as ecotoxicology [13-16]. GM, as a complementary technique, enables researchers to analyze subtle differences in morphology in a biologically relevant manner [12]. The major advantage of GM over other methods is that GM avoids reducing biological form into series of linear or angular measures that exclude information about the geometry of specimens and, therefore, preserves the physical integrity of specimens [14]. Morphological transformations between organisms are depicted on the basis of deformed Cartesian coordinates representing landmark points [12].

GM analyses start with landmark data that represent a spatial map of the relative location of precisely identifiable points on specimens. Landmarks should be homologous, meaning they should correspond one-to-one, among the specimens under study [12]. Different types of landmarks can be identified. Type I landmarks are defined as anatomical features that meet at a biologically relevant location (e.g., intersections of suture lines). Type II landmarks are identified by the maximum or minimum curvature of structures (e.g., curvature of shell whorls). Finally, type III landmarks are classified as extreme points (e.g., bottom of the shell). Landmarks that cannot be defined as definite points among individuals are called semilandmarks, usually representing geometric features such as curvatures. Semilandmarks are allowed to 'slide' along a line tangent to a curve, so they can be used to minimize the bending energy (described subconsequently) [17]. Therefore, based on the coordinate location of these landmarks, we can accurately obtain the geometry or form of biological objects [18].

Allometric relations describe the association between size and shape in populations of organisms; only rarely do organisms grow larger while maintaining shape (*i.e.,* exhibit isometric relations) [18]. The core purpose of using GM is to ensure that only shape is compared among different objects, extraneous information regarding position, orientation, and scale is eliminated [12, 19]. The three operations involved in Procrustes superimposition transform landmarks over all specimens into a set of maximally overlapping points [18]. Homologous landmarks are rescaled to a standard size, translated to a standard position, and finally rotated to define a standard centroid orientation. Rescaling involves taking the square root of the sum of squared distances of a set of landmarks from their centroid [20]. Once all the specimen landmark configurations have been rescaled to a centroid size of one, translation shifts the centroid to lie along the coordinates (0, 0), and (1, 0). Finally, rotation moves a landmark configuration around its centroid until overall least-squares 'best fit' is achieved [12].

These adjustments constitute a necessary first step in GM, and researchers ultimately use them to specify divergences from a reference [18]. Generalized Procrustes analysis quantifies differences among forms using the aforementioned superimposition method, which involves that reference [12]. All of the landmark configurations are rescaled, translated, and rotated so that they match the reference, and, finally, the magnitude and direction of difference between forms at each landmark are determined [18]. Algorithmically, the reference is established by taking one configuration (first one in data set) as a target and fitting all other configurations to this initial target configuration, using the least squares criterion. A consensus configuration is computed by averaging landmark coordinates among all configurations, including the one that was used as the target. Each landmark configuration is fitted to this new consensus configuration. At the end of a cycle, a new reference configuration is computed by averaging the corresponding coordinates of the superimposed configurations (this average no longer includes the target configuration, because it is the average from the previous round and not a landmark configuration from the sample). Replicates stop when the consensus configuration no longer changes (within a prescribed error range) [12].

GM uses a deformation method that visualizes shape differences through a coordinate grid, to associate morphologies of forms to the reference [20]. The thin-plate spline is one visualization technique that we used to map the location of landmark configurations for specimens to the reference. Uniform changes (affine transformations) represent geometric transformations that are identical in all parts of landmark configurations (i.e., parallel lines remain parallel), whereas non-uniform changes (non-affine transformation) characterize geometric transformations that differ among parts (i.e., straight lines are transformed into curves) [12].

To describe the mathematics underlying shape change descriptions, we herein focus on the three components of transformation grids: principal warps, partial warps, and relative warps.

The first step in obtaining these components involves deriving a bending energy matrix (BEM). Bending energy represents the degree of deformation, in which high bending energy reflects highly localized landmarks. The bending energy is minimized in transforming landmarks among all specimens to the reference configuration [18]. Only nonuniform components of change are considered in the BEM. Principal warps are extracted from the BEM by conducting a principal components analysis (PCA). The eigenvectors of the PCA enable decomposing shape and are known as principal warps. Principal warps provide orthogonal vectors that minimize the bending energy required for transforming, a description for the relative movement of landmarks without referring to a specific direction such as their x and y coordinates [12]. For each principal warp, one can derive partial warps, projections in x and y directions. Partial warp scores, representing weights of each partial warp, can be combined with principal warps to describe the actual shape change that represents the transformation from a starting to a particular target configuration [20]. Finally, a PCA can be performed on partial warp scores to yield relative warps, a summary of shape variation for a sample study [12]. Relative warps may be used to provide animated plots that show the reference object deformed as a thin-plate splines, in positive and negative directions along a selected relative warp axis [12]. Relative warps are very useful for visualizing overall shape change, as they demonstrate how displacement vectors would affect each of the landmarks [12].

Due to the promising features of GM, such as the preservation of geometric features and easy visualizations using thin-plate spline and convenient properties of mollusk shells, GM has been implemented as a morphological tool for shape variation in gastropod shells. In addition, considering the under representation of GM in the ecotoxological research, we believe that GM can serve as a complementary method that can assess the impact of OP contaminants on nontarget organisms.

3.3 MATERIALS AND METHODS

3.3.1 Chemicals

O,O-dimethyl S-(N-methylcarbamoyl-methyl) phosphorodithioate (DIM technical; CAS 60-51-5, 96–98% pure) was provided generously by Cheminova Agro (Windsor, Ontario, Canada).

3.3.2 Exposure conditions

Snails (*Cepea nemoralis)* were purchased (Ward's Natural Science, product number 6700800; St. Catherine's, Ontario, Canada). The supplier collected individuals from the wild (age approximately 1 year old, mean weight 2.7 ± 0.2g). After their arrival to the laboratory, snails were reared under controlled conditions for one month. Individuals were distributed by manually placing them into eight different aquaria. Each aquarium was provided with food and cleaned continuously.

One-hundred and forty four snails were placed into 24 transparent, 12 L, glass aquariums (Top Fin Aquarium, Product number 11164156; Petsmart, Burlington, Ontario, Canada), 6 snails x 3 replicates x 8 groups. The floor of each aquarium was covered with washing cloths (3 Ultra cloths, No Name brand, product number 6038365728, Fortino's; Burlington, Ontario, Canada), newsprint (Newsprint Scrapbook Fierro Paper Industries, product number 2745500290, Walmart; Burlington, Ontario, Canada), and a sponge (Hero-All purpose sponges, product number 6652235108, Canadian Tire; Burlington, Ontario, Canada) was included to control humidity. Ground Supreme Pet Foods Reggie Rat Original Food (Product number 2754947, Petsmart; Burlington, Ontario, Canada) was provided in a Petri dish. The diM was mixed into the food along with 1% $CaCO_3$ in the form of ground chalk (Crayola nontoxic white chalk, product number 636521406; Toronto, Ontario, Canada).

Snails were exposed to diM concentrations of 100, 250, 500, 1000, 2000, 4000, and 6000 ug/g feed for eight weeks. Each concentration treatment involved three replicates containing six snails in each.

3.3.3 Food preparation

Food containers were cleaned and food mix was renewed three times a week. Snails were reared under a light-dark photoperiod 18:6 h, using three fluorescent indoor lights (GE linkable fluorescent under-cabinet fixture, product number 750481, Home Depot; Burlington, Ontario, Canada), at 20 ± 2°C. Relative humidity 80-95% was maintained using an indoor hygrometer (Natural Geographic, product number 5747525543, Walmart; Burlington, Ontario, Canada). The food mix for each concentration was premade and stored until use in a -20°C refrigerator, to prevent diM from degradation. Each Petri dish containing the food mixture was sealed with parafilm (Bemis, product number 54956; Toronto, Ontario, Canada).

3.3.4 Chronic toxicity

Dead animals were removed every 2 days. EC_{50} values were determined after exposure by interpolating fitted curves (Table 1). Tidepool scientific software (5.0.32) also was used to generate EC_{50} values, in which an 'additive model' for the effects caused by diM was applied and data were rescaled so that the control group represented no effect and the 6000 ug/g group represented 100% effect. We report both interpolated and software-generated EC_{50} values but prefer those interpolated according to equations because Tidepool constrains data using built-in assumptions and functions. For instances, Tidepool implements by default an arc-sin transformation to stretch out both tails of distribution and compresses the middle because the data are proportions; to fit a logistic or sigmoid function to data, users must choose a model involving parameters for predetermined sets of species; we chose "bivalves-blue mussels" as the closest to snail species.

An attempt was made to assess activity levels, by measuring how long snails took to approach food, but this was aborted because snails took a long time to respond. Instead, at week eight, images containing visible cues, such as drooping eyestalks (DE) [4], were captured using a Canon Rebel T1i (500D DS126231) DSLR digital camera attached to a Nikon microscope SMZ 1000 with an EOS utility.

3.3.5 Reproductive Assessment

Reproductive activity was measured by analysing egg laying. The number of times each snail laid eggs was recorded for each group over the eight weeks. The numbers of eggs laid by each snail was not recorded.

3.3.6 Food Intake

Food intake was assessed indirectly by measuring feces consumption every 2 days. Feces were collected manually from each aquarium and allowed to air dry for weighing. Results are expressed as [mg of feces (dry wt)]/[g of snail (wet wt)] and [mg of feces (dry wt)]/[snail per 2 days].

3.3.7 Measurement of AChE activity

Snails were terminated by deep-freezing at -80°C. The foot and viscera were dissected from each individual and stored until required. A Bradford protein assay was conducted to determine the amounts of sample protein per 1 mL of incubate [4]. All procedures were carried out at 4°C. Each snail foot was homogenized for 1 minute in Tris buffer (100mM, pH 7.5) at a 1:10 (w/v) ratio using homogenizer at 12000 rev min $^{-1}$. The homogenate was centrifuged at

9000 *g* for 40 min to yield a postmitochondrial (supernatant S9) fraction. A soup solution was prepared with 95 mM tris buffer (pH 7.5) and DTNB 0.4 mM. The acetylthiocholine (AthCh) (2.25 mM) was hydrolyzed by AChE, producing thiocholine and acetic acid. The reaction was followed by the interaction between thiocholine and 5,59-dithio-bis-2-nitrobenzoate (DTNB), which produces 5-thio-2-nitro-benzoate (TNB), a yellow compound that absorbs light at 412 nm (absorbance, 13.6 mM/cm).

3.3.8 Growth Assessment

At the end of each week, total weight (shell and soft body) of each snail was calculated. To calculate weekly mean soft body dry weights, regression analysis was performed; an additional sample of snails (n=24) was ordered to generate a relations between total weight (shell and soft body) and wet and dry soft body weight. For each snail, the soft body was dissected, weighed, and allowed to air dry for one day; then dry soft body was weighed.

Growth Efficiency Coefficients (GEC) for each concentration were calculated for the three growth variables: wet and dry soft body weight and maximum shell diameter. GEC was calculated according to the following formula:

$$\mathrm{GEC} = (W_t/W_0)*100,$$

where, w_t was the mean soft wet body weight of snails exposed to C ug/g of diM for *t* days, w_0 is the mean soft wet body weight of snails exposed to C ug/g of diM at t=0, and C is the concentration of diM. Based on the above equation, we derived the following, to express growth coefficient change explicitly as a difference:

$$Y\text{-}100=100\ [\ (W_t\text{-}W_0)/W_0\ ],$$

where the right side is relative change in weight, presented as a percentage.

3.3.9 Bivariate statistical analysis

Regression relations were used to examine the dependence on total weight by wet/ dry soft body weight using Microsoft Office Excel 2007.

3.3.10 Principal component analysis (PCA)

Linear shell variables such as length (d), width (w), aperture length (ad), and aperture width (aw) were measured in aperture view of the shell, using Mathematica 4.0.1.0 (Fig. 1A). A PCA was conducted to observe size and shape changes during the eight weeks in each of the diM concentrations.

3.3.11 Geometric morphometrics

All (n=112) snails were used for morphometric data acquisition. TPS software, TPSDig2 (version 2.16), TPSUtil (version 1.47), and TPSRewl (version 1.49) [21], were used to generate digitized images, obtain landmark data, and relative warp plots [22]. Snail shells accrete calcium carbonate in a spiral form, starting from the larval protoconch and ending at the teleoconch. During shell ontogeny, new shell material is appended to a previously accreted shell surface, so homologous landmarks can be determined from the apex toward the body whorl [23]. Shells were mounted along the columella axis and rotated to present the aperture in frontal view. A photograph in aperture view of each specimen was taken. Each shell was photographed and digitized twice using TPSDig2 to assess measurement error. Each shell yielded ten landmarks on anatomical features (type I) (apex and sutures) and 2 landmarks on the curvature of body whorl (type II) (Fig. 1B). These landmarks were selected because they: i) are located on distinct anatomical features, ii) represent a good approximation of the body whorl shape and shell elongation, and, iii) reveal shape variation of the aperture. In TPSUtil, we created a slider file for the sliding landmarks. The landmark and sliding landmark folders constituting the 12 landmarks for the 112 shells were input into tpsRelw to generate relative warp visualization plots. Each concentration group in the relative warp plot was colour coded using Mathematica 4.0.1.0, and a 95% confidence interval ellipse was drawn around each concentration [24].

We also considered assessing the repeatability and precision of the relative warps. Variation between shapes has to be distinguished from measurement error [20]. Precision is important to avoid additional noise that disrupts the true direction of the biological variation in data. We therefore captured images and digitized each specimen twice [12].

To test whether outliers in the dataset introduced undue variance, variation in the direction of the difference of each observation from the average was generated in MorphoJ (1.03d). The resulting sigmoid curves were shifted slightly because the distribution was 'heavy-tailed,' which is common with morphometric data. The importance of allometry in structuring spatial variation was detected through a regression between RW1 and centroid size, which generated no relationship, indicating that size does not have an effect on shape [12].

3.4 RESULTS

3.4.1 Survivorship

After the 8-week exposure, the EC_{50} fitted according to the exponential equation of survivorship was interpolated to be 2608 µg/g diM; according to Tidepool, it was 1650 ug/g diM. The total number of snail survivors in each diM concentration group decreased with higher diM exposures but nonsignificantly (Two-way ANOVA: P=0.08693) (Fig. 2A).

3.4.2 Food intake

Feces production was measured as a proxy for food consumption. Feces production differed significantly among groups (two-way ANOVA, P=1.35E-16). At concentrations beyond 2000 ug/g diM, feces production (mg of feces/g snail) decreased (Fig. 2B). No difference in feces production among replicates within groups was detected (two-way ANOVA, p=0.85). EC_{50} fitted according to the exponential equation of food intake was interpolated to be 221 μg/g diM; according to Tidepool, it was 98.3 ug/g diM.

3.4.3 Chronic toxicity and clinical signs

Response to feeding was measured as a form of physical activity. Unfortunately, the time required for snails to approach food was extensive and unreliable. As a result, clinical signs of lethargic behaviour were attempted, following Coeurdassier *et al.* (2001). Images of drooping eyestalks (DE) were compared at week 8 and no distinctive difference among groups was observed. Furthermore, snails showed no unusual behaviour of rapid withdrawal into shells when handled. Thus, only direct physiological measurements are considered hereafter.

3.4.4 Variation in AChE activity

Acetylcholinestrase activity in the foot tissue decreased significantly with exposure concentration, starting from 500 μg/g diM (Two-way ANOVA: P=3.62E-15). Activity was almost nil at concentrations 2000 μg/g and greater. EC_{50} values dteremined according to the exponential equation of AChE activity was interpolated to be 484 μg/g diM; according to Tidepool, it was 636 ug/g diM (Fig. 2C).

3.4.5 Growth assessment

Based on linear regression relations between wet and dry soft body against total weight (r^2=0.69 and r^2=0.88, respectively), mean wet and dry soft body over time were generated. Mean soft body dry weight differed significantly among groups at time 0 (P=0.03122) and no significant difference among replicates within groups were detected (P=0.65797). The repeated measures ANOVA revealed significant differences over the 8-week exposure period for five concentrations: 100 (P=0.01126), 1000 (P= 0.00026), 2000 (P=0.00054), 4000 (P=0.00706) and

6000 (P=0.00007) ug/g diM. A positive correlation between GEC dry soft body weight and concentration was established (r^2=0.82), with significantly higher GECs for higher concentrations (P= 1.3E-15) (Fig. 2D). The EC_{50} fitted according to the exponential equation GEC dry soft body weight was interpolated to be 3680 ug/g diM; according to Tidepool, it was 2600 ug/g diM. Finally, shell diameter increased gradually over the study period for all groups, but changes were small and statistically nonsignificant.

3.4.6 Principal components analysis

PCA was used to describe the complex patterns of shell growth. A variety of PCA plots were generated, including all concentration groups plotted at each week (e.g., control-6000 ug/g at t_0) (Fig. 3A) and each concentration group plotted over all 8 weeks (e.g., control at t_0-t_8). Principal component 1 (PC1) generally represented size, and principal component 2 (PC2) represented shape. A PCA plot for all groups at t_0 is compared to the PCA at t_8 (Fig. 3B). For the PCA plot of all groups at week 8, PC1 and PC2 represented shape axes, explaining 59% and 30% of variance, respectively. At t_0, PC1 represented size and PC2 represented shells that have long and wide shells with smaller apertures. All ellipses are scattered with no distinct pattern. At t_8, PC1 represented long and wide shells with wide apertures and PC2 represented long and wide shells with narrow apertures opening. PCA plots emphasized that shape deformations occurred, but no clear distinction among concentration exposure groups was identified.

3.4.7 Relative warp analysis

Over 76.85% of the variance in the data was accounted for by the first two relative warps, 63.69%, and 13.15% respectively. The respective eigenvalues, obtained as squared singular values, were 0.5005, and 0.1034, respectively. Plots for relative warp 1 versus 2 (Fig.4) revealed morphological variation among treatment groups. Points did not cluster distinctly according to concentrations.

Visualization plots assisted in the interpolation of shape variation. For shells that scored positively on RW1, thin-plate spline deformations revealed compression of landmarks 9-11 of the body whorl (Fig. 4). For shells that scored positively on RW2, thin-plate spline deformations revealed compression of landmarks 1-8 toward apical whorls and expansion of the body whorl (Fig. 4).

3.5 DISCUSSION

3.5.1 Survivorship

Snail survivorship decreased with increases in diM concentration, with EC_{50}=2608 μg/g diM (Fig. 2A). This median lethal concentration was smaller than that reported for *Helix aspersa* by Coeurdassier *et al.* (2001), which might indicate that *Cepea nemoralis* is more sensitive to OP insecticides. However, snails exhibited rapid withdrawal into their shell when handled, so one cannot conclude that diM imparted clinically observable signs of lethargic behaviour. Additional studies should be conducted to examine diM effects on survival in *Cepea nemoralis.*

3.5.2 Food intake

Replicates within exposure concentration groups consumed the same amount of food (see RESULTS), but snails in exposure concentration groups greater than 2000 μg/g consumed less (Fig. 2B). As observed by Schuytema *et al.* (1993), snails exposed to metal contaminants exhibited decreased food consumption and net growth. Snails in the higher exposure concentration groups could have developed a taste aversion toward contaminated food. Unlike the significant decrease in snail weight observed by Schuytema *et al.* (1993) on *Helix aspersa*, in our study, appetite loss due to toxication did not generate any decrease in weight loss. Therefore, starvation does not explain observed deaths, as mortality increased with as exposure time, as observed by Schuytema *et al.* (1993). Additionally, lower food consumption might have resulted from impeding effects on metabolism. According to Sharaf *et al.* (1975), OP insecticides can reduce levels of glycogen, oxygen consumption, and glucose metabolism in aquatic gastropods [4]. Additional studies should be conducted to examine diM effects on food consumption in *Cepea nemoralis*.

3.5.3 AChE activity measurement

As noted by Coeurdassier *et al.* (2001), AChE activity is much higher in the foot than in the viscera, and therefore, we decided to restrict activity analysis to the foot. Exposure inhibited AChE activity at concentrations 2000 µg/g diM and greater (Fig. 2C). We obtained an AChE activity level for the control group that was similar to that reported for *H. aspersa* by Coeurdassier *et al.* (2001). Activity was decreased at concentrations greater than 500 µg/g diM, but, unlike the patterns reported by Coeurdassier *et al.* (2001), exposure concentrations lower than 500 µg/g of diM did not inhibit AChE activity appreciably. These findings lead to two explanations for observed mortality: 1) activity inhibition at concentrations greater than 2000 ug/g diM was severe enough to be lethal or 2) lethal negative effects were imparted by diM on metabolism, as reported by Sharaf *et al.* (1975). As a result, we explored complementary biomarkers to assess the effects of OP contamination.

3.5.4 Growth inhibition

Three variables were examined to measure growth inhibition. Growth efficiency coefficient (GEC) increased with increased exposure concentration. As described previously by Coeurdassier *et al.* (2001), the use of wet soft body weight tends to underestimate the toxic effects of diM, due to water retention by tissues. Mucus is produced in mucocytes in the foot and digestive system, leading to retention [4]. Dry weight is a more sensitive variable, but its use has few drawbacks. At the end of the experiment, extra snails were terminated to estimate weekly dry weight. As exposure concentrations increased, GEC of dry soft body weight increased, with a median effective concentration 3680 μg/g (Fig. 2D). Finally, shell diameter increased but in a statistically nonsignificant manner over the 8 week exposure period.

None of these variables yielded a dose-dependent relationship; no growth inhibition was observed as exposure concentration was increased. Snails exposed to lower diM concentrations showed greater survivorship, higher food intake, and higher AChE activity levels, as expected, but they exhibited lower soft body growth. Snails in the 2000 μg/g diM and higher groups showed lower survivorship, lower food intake, and lower AChE activity levels, as expected, but they exhibited the highest soft body growth. As suggested by Maurer *et al.* (2009), constraints of different habitats induce resource allocation differently in different species [26]. We suggest that diM exposure poses a severe energetic challenge to *C. nemoralis* snails, diverting energy toward essential processes for survival. According to Tracer *et al.* (2002), energy allocation must occur in ways to optimize fitness. Because energy is finite and limited, investment in one element or stage of life history makes less energy available for investment into other elements or stages [27]. Energy input is first distributed toward metabolism (i.e., for maintenance, immune system response, and basal activities), growth, and reproduction. Metabolism is a necessary energy expenditure for survival; once that requirement has been accommodated, growth and reproduction follow. As described in *Tracer et al.* (2002), a crisis emerges among life history stages compete when energy is limited, and, therefore, tradeoffs have been identified in energy expenditure. We hypothesize that *C. nemoralis* snails exposed to different diM concentrations experienced tradeoffs between energy allocations for metabolism vs. growth and reproduction, as follows:

a) at lower diM concentrations, snails possessed greater energy, sufficient to allot to metabolic activities responsible for maintenance, immune system response, and basal activities and, therefore, greater survivorship; leftover energy was allotted to growth and reproduction; thus, snails in lower concentrations were more active and lived longer but grew the least.

b) at higher diM concentrations, snails possessed lower energy and little was left after allotting to metabolism what was available; snails in the highest exposure groups eshibited lower activity, and these snails invested leftover energy mainly toward soft body and shell diameter growth (which was statistically nonsignificant) [27, 28].

Therefore, the relationship between dimethoate exposure and energy allocation can be categorized into expected relations (energy increase diverted toward metabolism for survival) and unexpected relations (energy decrease insufficient for metabolism and survival and diverted toward growth).

An alternative and the preferred explanation for the increase in dry soft body weight with increased exposure concentrations can be formulated using results reported by *Coeurdassier et al.* (2001), wherein diM concentrations 500 µg/g or greater produced water retention in tissues that was significantly greater in exposed specimens compared to controls [4].

Another possible alternative is that the feces production as a proxy for food consumption might be underrepresenting food intake. Dimethoate might be impacting feces production for snails in the higher concentrations; so, food intake was measured as low, when, in fact, they were consuming the food and growing.

3.5.5 Geometric morphometrics as a complementary morphometric tool

Quantifying phenotypic change is a critical assay for understanding the ecology of species [23]. Because PCA plots are able to allocate patterns of high dimensional data, they can reveal shell shape changes. However, GM is able to extract information beyond traditional linear measurements and reveal detailed shape variation. GM presents the ecological field with a tool that enables easy visualization of shell morphology while avoiding the reduction of specimen geometry. RW plots in ordination space reveal shape differences compared to the control group. Thus, GM serves as a complementary morphological method that has been used rarely for environmental evaluation against perturbations.

Thin-plate splines allow visual representation of shape variation [29]. The thin-plate splines for RW1 and RW2 reveal shape changes that elude other morphometric analyzes, like PCA. Specimens that scored positively on RW1 possessed shells with aperture expansion toward the outer shell wall (compressed landmarks, 9-11), while specimens that scored negatively on RW1 possessed shells with aperture compression (compressed landmarks, 8, 9, 10 and 12). Specimens that scored positively on RW2 possessed shells with compressed apical whorl, (compressed landmarks, 1-8) and body whorl expansion, while specimens that scored negatively on RW2 possessed shells with expanded apical whorls and a compressed body whorl with a flat aperture (compressed landmarks, 9, 10, and 12). These relative warps convey shape variation of the aperture relative to apical whorls (Fig. 4).

Unfortunately, no discrete pattern was discernible among the 8 exposure concentration treatments in ordination plots (RW1 vs. RW2; Fig. 4) and no obvious clustering of groups that would relate shape and diM concentration in a dose-dependent manner. Changing exposure concentrations possibly led to morphological variation related to shell proportions. Ellipses with

95% confidence intervals were plotted for each group, and revealed that, once the dimethoate insecticide had been applied, shell shape in some treatment groups diverged from shell shape in the control group. For example, the 100 ug/g diM group scored slightly more negatively on RW1 than did the control group. The aperture underwent a slight compression along x-component and expansion along y-component. The 250 ug/g diM group scored more positively on RW2, indicating more-compressed apical whorls. The 500 ug/g diM group scored more positively on RW1 and RW2, indicating bigger apertures and more-compressed apical whorls. The 1000 ug/g diM group scored slightly more negatively on RW1 and positively on RW2, indicating slight compression in the x-component and expansion in the y-component and slightly compressed apical whorls. The 2000 ug/g diM group scored slightly more negatively on RW1, indicating slight compression in the x-component and expansion in the y-component. The 4000 ug/g diM group showed no shape change relative to control. Finally, the 6000 ug/g diM group scored slightly more positively and negatively on RW2, indicating slight compression or expansion of apical whorls. We speculate that the similar pattern observed for the ellipses representing the highest diM concentration groups, 4000 and 6000 ug/g, and the control group resulted from small sample size due to deaths in the highest concentration groups; but, as only the 250 and 500 ug/g diM groups showed more-than-slight shape changes (*i.e.,* the other 3 groups are similar to the control group), sample size cannot be the sole cause.

We are puzzled that the lowest concentration groups (250 and 500 ug/g) expressed the greatest shape change compared to the higher concentration groups. GM was able to detect these small biological variations through relative warps. This along with the weight gain observed with increased concentrations constitute ‘mysteries’ requiring explanation.

3.5.6 Impact of morphological change

Several studies have examined the impact of pesticides on morphological variation in nontarget invertebrate organisms. Investigating the role of morphology in invertebrate life history is a critical feature. For example, Masaki *et al.* (2008) found that the impact of pesticides on freshwater cladocerans (crustaceans) can enhance or inhibit the development of antipredator morphologies. The insecticide carbaryl hindered defensive morphology, as revealed by decreased body sizes compared to control groups during the presence of predators [30]. The significance of such an inhibitory action is increased predation risk due to chemical disturbance and this, in turn, will affect the mortality and, thereby, population dynamics. Additionally, according to Cain *et al.* (1980), elongated shells with greater number of body whorls are characterized by greater structural strength. Variation in aperture area is a critical factor in snail fitness. Aperture area is a predominant factor in preventing predation or regulating water loss [31]. The morphological variation accompanying snails experiencing environmental perturbations might impart negative effects on fitness. Previous researchers (e.g., Krist *et al.* 2002) have shown that variation in spire height and aperture size in freshwater snails may reduce predation in headwaters, while low spires and large apertures may reduce dislodging and damage during tumbling [32]. These morphological variations enable GM analysis to be considered as an important asset in pesticide contamination monitoring.

Morphological changes in a natural setting like the change in the size of the spire and the aperture often are a consequence of selection by predators; we can not conclude that diM imparted the same effects as predators or other environmental variables. We speculate that OPs can cause *C. nemoralis* snails to retract in their shells; this would necessitate desiccation minimization over long time periods, leading snails to accrete contracted apertures.

3.5.7 Cepea nemoralis vs. Helix aspersa

Our study focused on *Cepea nemoralis* as a host organism of OP contamination, while the study by Coeurdassier *et al.* (2001) involved *Helix aspersa*. We remark on several differences between the studies, which might explain differences in results obtained. First, specimens were different in age. When our experiment began, snails already had reached adulthood, whereas, in the study by Coeurdassier *et al.* (2001), snails were only one month old. Therefore, growth rates differed appreciably. The growth rate in young snails is greater than in adults [34]. Therefore, growth rates for *C. nemoralis* in our study might have been subtle; nevertheless, they were significant. Second, snails in our study were collected from the wild rather than descended from a laboratory strain, which led to greater morphological variance among our specimens variant. Third, a different type of food was used to expose *C. nemoralis* to pesticide (Reggie rat food compared to Helixal in Coeurdassier *et al.* 2001, which was designed specifically for *Helix aspersa*). Dietary exposure is important because it is the main source of contamination. Using different types of food for exposure might have led to differences in assessment of food intake.

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3.7 APPENDIX I: TABLES

Tables

Variables	Test
Survivorship	$y = 17.523e^{-0.0001x}$ $r^2 = 0.94$ EC_{50}=2608 ug/g
Food Intake	$y=30.174e^{-0.0002x}$ $r^2=0.81$ EC_{50}=221 ug/g
AChE Analysis	$y = 18.293e^{-0.000004x}$ $r^2 = 0.63$ EC_{50}=484 ug/g
Growth Efficiency Coefficient (GEC)	$y = 101.46e^{0.00004x}$ $r^2=0.83$ EC_{50}=3680 ug/g

Table 1. Effects of progressive concentrations of dimethoate on four variables of *Cepea nemoralis*. Each equation is based on an exponential fitted model (x=[dimethoate] (ug/g); Y=variables). EC_{50} calculated using equation.

3.8 APPENDIX II: FIGURES

Figures

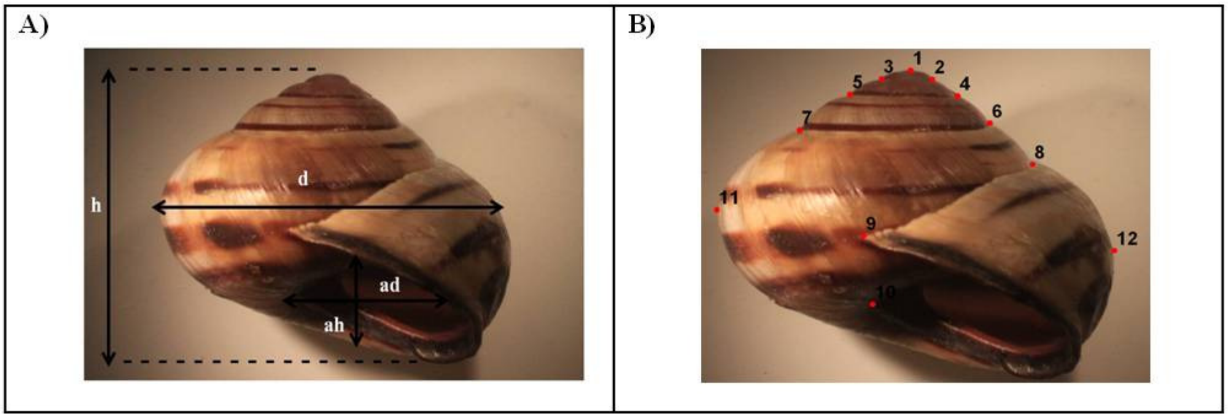

Figure 1. Image of a *Cepea nemoralis* snail shell with labeled traditional variables and landmark points (A) Measurements used in traditional morphometrics, h=length, d=width, ah=aperture length, ad=aperture width (B) Landmark based morphometrics, involving twelve digitized landmarks.

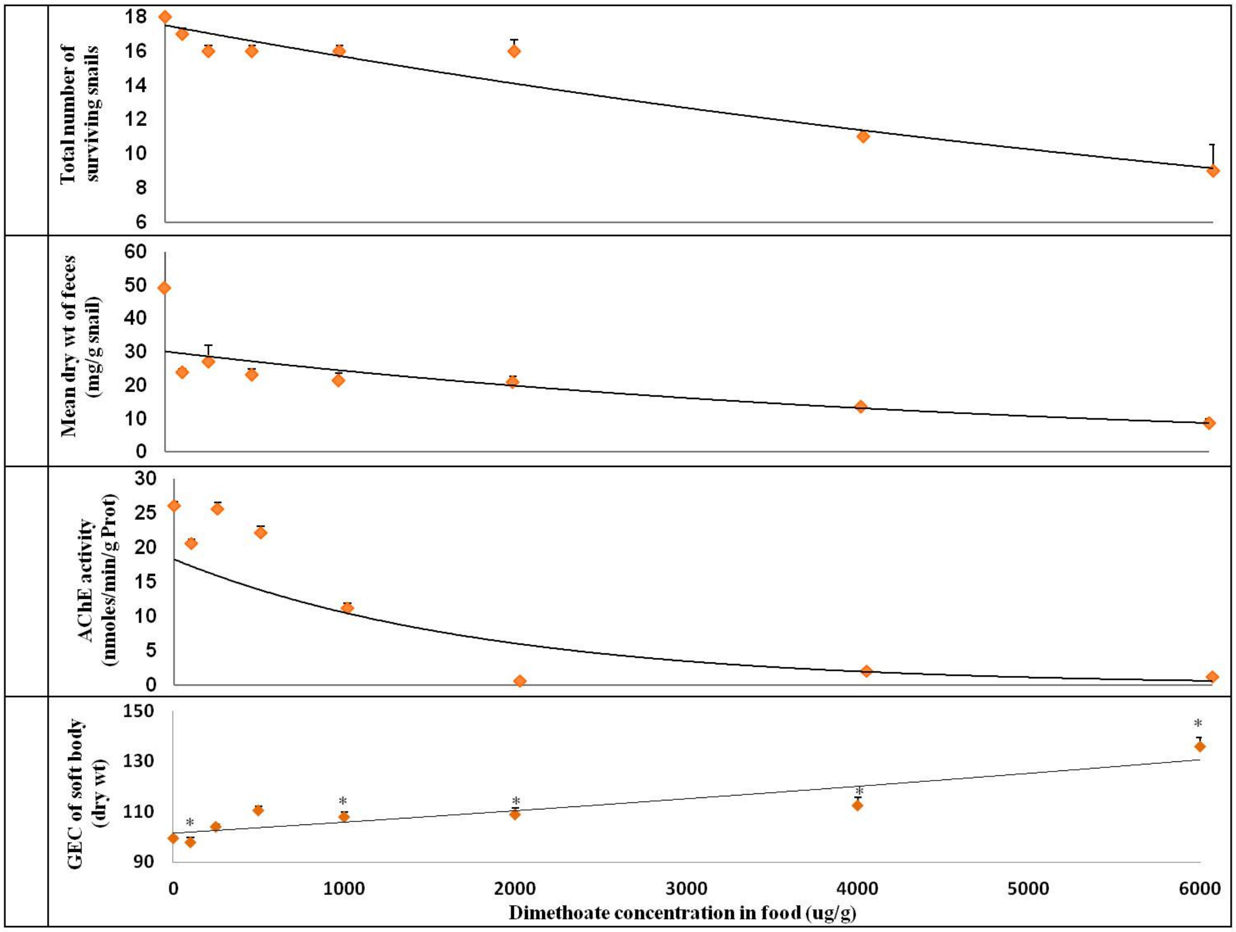

Figure 2. Graphs showing response variables as a function of dimethoate concentration. (A)Total number of survivors ($y = 17.523e^{-0.0001x}$,$r^2 = 0.94$, EC_{50}=2608 ug/g), (B) Mean dry weight of feces excreted ($y=30.174e^{-0.0002x}$,$r^2=0.81$,EC_{50}=221 ug/g), (C) Acetylcholinesterase (AChE) activity in the foot ($y = 18.293e^{-0.000004x}$, $r^2 = 0.63$, EC_{50}=484 ug/g), (D) Growth coefficient of dry weight of soft body ($y = 101.46e^{0.00004x}$,$r^2=0.83$, EC_{50}=3680 ug/g). Results are expressed as the mean + standard error. Means were compared with controls by two-way ANOVA or repeated measures ANOVA. *$p<0.05$.

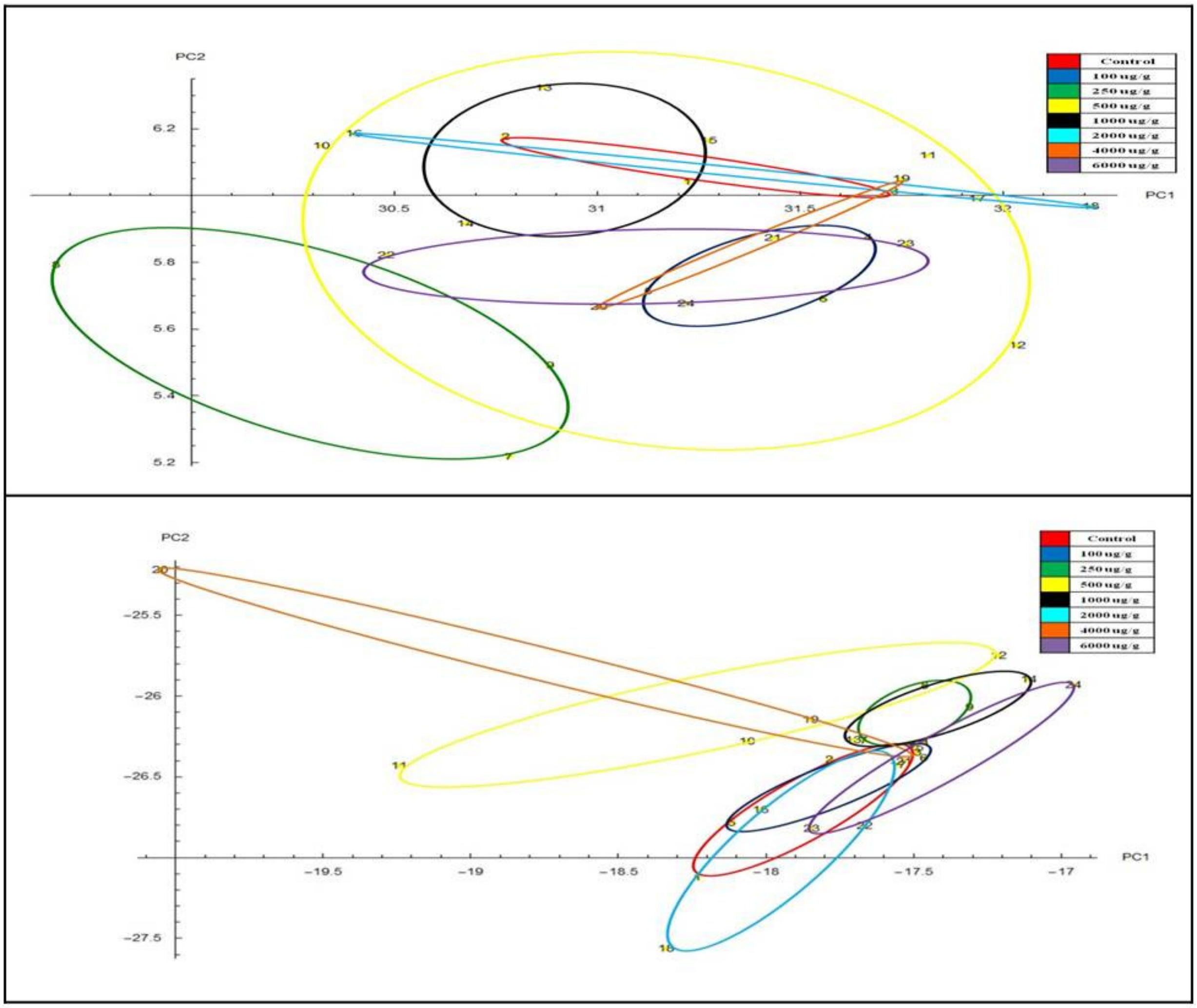

Figure 3. Principle Component Analysis (PCA). (A) PCA for T_0, showing PC1 as size axis (76% variance) and PC2 as shape axis (13% variance), (B) PCA for T_8, showing PC1 and PC2 as shape variables (56% and 30% variance respectively). Each ellipse represents average shell dimensions for the three replicates for each concentration.

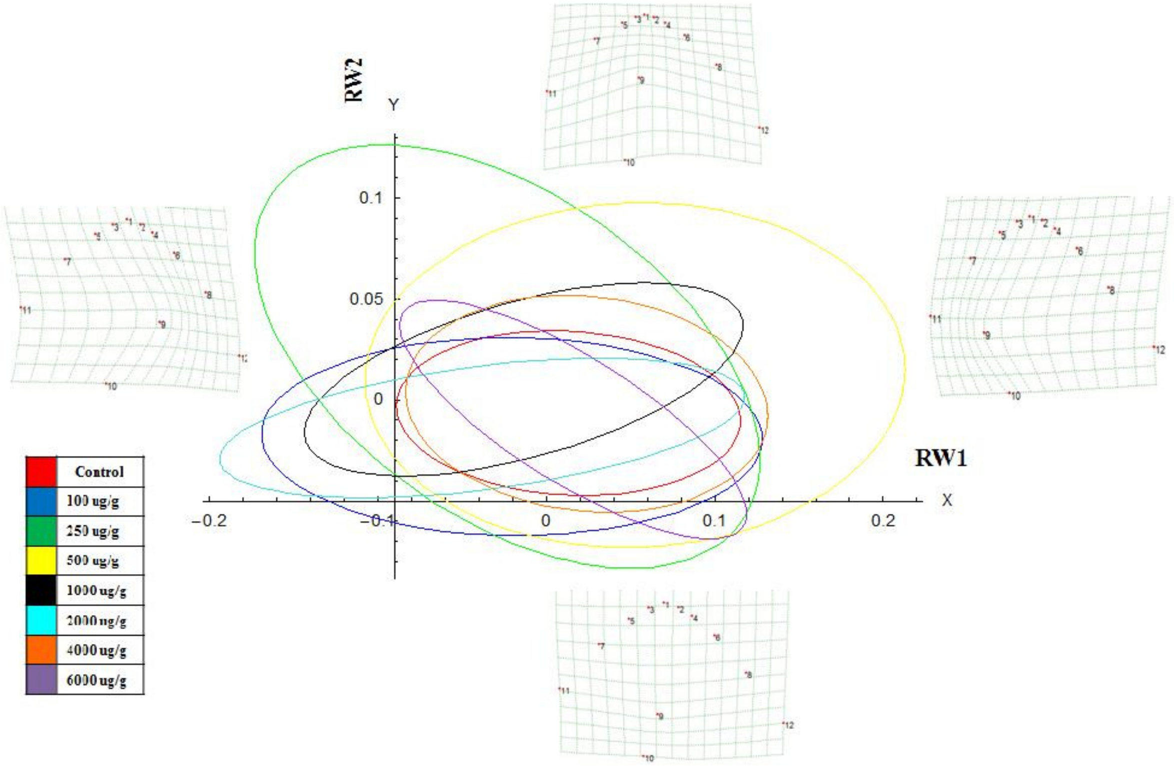

Figure 4. Ordination plot of relative warps with landmark points representing most extreme positive and most extreme negative deformation of the landmarks in *x* and *y* components, α=0. Relative warp 1 (RW1) (63.7% variance) against relative warp 2 (RW2) (13.2% variance), including 95% confidence ellipses.

4.0 CHAPTER FOUR

4.1 CONCLUSION

Snails served as a nutrient source for early hominins, but, more recently, their shells have provided biologists with an important source of information about their environments. Shells provide a feature that can be analyzed in morphological studies because they reveal information from the past at the individual and taxonomic levels. These calcium carbonate structures are accreted in a spiral growth pattern that records ontogenetic development of the individual producing it and, therefore, serve as informative models for morphological research. As I have shown, geometric morphometrics provides powerful techniques for analyzing snail shells, as it has been applied to analyze morphology in different research fields. Herein, I applied GM for the first time as a morphological technique to measure OP contamination. I have shown through deformation grids that GM is capable of detecting subtle shape differences that other, traditional techniques are unable to reveal. In relative warp space, a lot of overlap among the different concentration groups was observed and, therefore, I was unable to generate a dose-dependent relation between concentrations and morphological impact. Future studies should implement weekly analysis of geometric morphometrics on shells to account for the change in morphology throughout ontogeny and maintain an equivalent shell sample size for each concentration group. I suggest that GM should and predict that it will be implemented with greater frequency in an ecotoxological setting, as a complementary morphological technique, in future studies.