# MORPHOLOGICAL EVOLUTION OF THE FEEDING MECHANISM IN STINGRAYS

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

> Ecology and Evolutionary Biology University of Toronto

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## Abstract

Evolutionary transitions between ecosystems are rare, and yet in lineages that have made these transitions we observe drastic changes in their diversity and richness as they adapt to novel habitats and novel resources. The main goal of my dissertation is to examine how transitions among diets and habitats have molded the evolution of predator traits. To test hypotheses regarding how prey materials have shaped the evolution of predators, I examined predator feeding biomechanics using experimental methods and deep-time approaches. Using 3D printing, computational milling, and computed tomography scanning, I compared the biomechanical performance of jaw shape for durophagous stingrays which prey on mollusks with shells of drastically different material and structural properties. I found that all of these jaw morphologies were equally well-suited to crushing the entire breadth of prey mollusk diversity, establishing equifinality of anatomical form stemming from convergent feeding mechanics. Insectivory has evolved only once within modern sharks and rays, found within the enigmatic freshwater stingrays of South America. Using high-speed videography and computed tomography scanning I found that these freshwater stingrays use chewing motions of the jaws when feeding on prey, facilitated by a highly kinetic cranial skeleton, and that these motions are exaggerated for tougher prey like insects. Loose jaw joints, transverse jaw kinesis, passive tooth reorientation, and a hydrodynamic tongue allow freshwater *Potamotrygon* rays to chew in a manner similar to

many mammals. I investigated how the evolution of novel feeding modes such as insectivory and molluscivory in these freshwater rays has altered the tempo of their evolution by generating a molecular phylogeny for the family and analyzing feeding trait adaptation across 40 million years of evolution. The evolution of molluscivores and insectivores in this stingray clade are relatively modern innovations, coincident with the changing nature of the Amazon basin and repeated colonization of new riverine habitats. These dietary strategies, in addition to piscivory, are representative of truly novel adaptive peaks and have contributed to shifts in the rate of both lineage and morphological evolution in these remarkable freshwater stingrays.

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## 1 General Introduction

In asking why some groups of related animals are more diverse in appearance and ecology than others, the nature of the resources these organisms use is frequently mentioned (Simpson, 1953; Schluter, 2000; Losos, 2010). Evolution along a resource gradient is frequently associated with notions of adaptive radiation, where related taxa specialize along resource axes in order to stave off competitive interactions (Connell, 1980; Schluter, 1996). The impetuses for novel resource use are thought to be catalyzed by several potential phenomena: (1) loss of a competitor or predator, (2) key innovations, (3) habitat transitions, or some combination of these occurrences, all providing some ecological opportunity (Schluter, 2000; Glor, 2010). These ideas into the conditions permitting adaptive radiation (ecological opportunity; e.g. habitat shift, key innovations), the substrate upon which selection occurs (morphology, physiology), potential mechanisms (e.g. character displacement), and the corresponding ecological covariate (the resource gradient, e.g. habitat, diet) form the majority of what we know about the ecological theory of adaptive radiation (Brown and Wilson, 1956; Grant, 1972; Schluter, 1994; 1996). One of the primary difficulties in discussing character evolution in the context of adaptation is demonstrating the fitness advantage of the focal trait. Wainwright (1989, 1994) proposed a research program that posited study of ecomorphological performance as the foundation for critically analyzing trait evolution in an ecological context. Many studies have measured general shape change or shifts in number of meristic characters, but the functional relevance of these traits is often unfounded. In order to illustrate that traits are adaptive and play a significant role in the ecology of an animal, these traits must first be demonstrated to advantageously affect performance (and indirectly, overall fitness) at a given task (Arnold, 1983). Adaptive traits do not develop in a vacuum and depend on ecological context. Ecological opportunity is one of the many cited tenets of adaptive radiation, whether it can be clearly detected or not (Glor, 2010;

Yoder et al., 2010). From the perspective of adaptive traits, ecological opportunity can be considered an expanded niche volume in which species are now freer to move across the adaptive landscape. Ecological opportunity is presumed to reduce or weaken selection on phenotypes within the adaptive landscape, either by reducing competition for resources through a key innovation or movement into a new habitat, or the elimination of predators and competitors. Ecological opportunity can then either be the catalyst or the removal of barriers which permit species to range farther from ancestral adaptive peaks, allowing the 'jump' to new peaks or a shift to a broader adaptive plateau (Simpson, 1953).

A critical aspect of ecological theories of trait evolution is the amenability of a taxon to adaptation along a resource gradient (Schluter, 2000). This implies that traits are able to overcome phylogenetic, developmental, and constructional constraints in order to adapt to a changing fitness paradigm (Arnold, 1983; Barel et al., 1989; Wainwright, 1994). This critical aspect of trait evolution is frequently discussed, and until recently, difficult to address due to limits on knowledge of a clade's phylogenetic history, particularly its fossil record. The disparity (and sometimes notions of ecological "success" or species richness of a given clade) has been attributed to the plasticity of the focal clade given ecological opportunity (Liem, 1973; Seehausen and Bouton, 1997).

A confounding factor for measuring the performance-efficacy of a structure is the concept of equifinality, popularly known as 'many-to-one mapping.' This concept maintains that multiple anatomical configurations can and do lead to similar performance outcomes (Young et al., 2007; Wainwright et al., 2005, 2007). This allows animals to circumvent phylogenetic constraints on form to find novel means of generating performance outcomes which are convergent with distantly-related, and typically very disparate, taxa. This notion is intertwined with the idea of phylogenetic "baggage" of a clade, whereby organisms are constrained in their ability to change

because they start from a fixed ancestral template molded by even older ancestors and paleoecological prerogatives (Felsenstein, 1978, 1985). Providing the cap on this hierarchical complexity is the ghost of competition past (Connell, 1980) where recent, but non-observable ecological pressure has structured community dynamics.

Difficulties for a research program investigating trait evolution and phenotypic diversity primarily lie in the inability of modern evolutionary models to predict and extrapolate change in complex, interacting anatomical structures (constructional morphology; Barel et al., 1989). In terms of understanding the rules of evolutionary change, our knowledge of the tendencies of molecular evolution far exceeds that of our understanding of morphological change - making it difficult to formulate null hypotheses when testing, predicting, and confirming how evolution gives rise to complex forms. Recent advances in molecular and anatomical investigations of development may in the future allow us to have greater insight into how complex traits arise. Only within the last twenty years, coincident with the maturation of modern comparative phylogenetic methods, has our ability to tie phenotype to performance and these paradigms to fitness, borne fruit. These challenges have been discussed in depth by Wainwright (1994), Schluter (2000) and others (Losos, 2010; Ingram and Mahler, 2013) as critical to our understanding of trait evolution. Finally, our ability to discuss diversification depends heavily on our taxon sampling and understanding of the fossil record (Slater et al., 2010). In order to thoroughly understand the evolution of traits across a group of animals, we must first understand how these animals are related to each other, which requires dedicated research in both systematics and natural history. Habitat transitions have featured prominently in discussions of adaptive radiation and trait evolution. Marine to freshwater transitions are one such example where researchers have documented considerable changes in the ecology of organisms that managed to overcome the hurdles of a new habitat, new competitors and predators, and new

resources (Betancur et al., 2012; Davis et al., 2012, 2014a,b; Bloom and Lovejoy, 2012). Marine to freshwater transitions have been associated with increased rates of speciation and extinction, but perhaps not sheer diversification within some freshwater fish clades (Orti et al., 2012; Vega and Wiens, 2013; Bloom et al., 2013). With the exception of Davis et al. (2012, 2014a,b), no studies have documented changes in morphological diversification rates upon the transition from marine to freshwaters while tying these traits to actual ecological function. If marine-derived lineages (MDLs) like needlefishes, drum, ariid catfishes, and potamotrygonid stingrays were engaged by ecological opportunity after the habitat transition to freshwater, we should observe an early-burst pattern of subclade disparity commiserate with this transition. These transitions have been well-documented from a phylogenetic context (Orti et al., 2012; Bloom and Lovejoy, 2012; Bloom et al., 2013; Bloom et al., 2014) and provide an apt model system for determining whether or not this particular kind of habitat transition has provided the opportunity for these clades to adaptively radiate in freshwaters.

Stingrays (Myliobatiformes) comprise over half of the morphological diversity of the batoid fishes – the most ecologically diverse group of cartilaginous fishes (>600 species; Aschliman et al., 2012). These fishes are found in all major ocean basins as well as in freshwater habitats across the tropics. Numerous stingray clades have undergone substantial habitat transitions, either between marine and freshwaters or from benthic habitats to pelagic ones. The dietary breadth and diversity of stingrays in these habitats is considerable, ranging from molluscivory to planktivory, repeated across several major lineages. The repeated instances of the evolution of novel dietary strategies after habitat transitions make stingrays an ideal system for study of adaptive radiation commiserate with these habitat transitions. In particular, the families Potamotrygonidae and Myliobatidae have at some point in their evolutionary history undergone habitat transitions, followed by drastic shifts in trophic ecology. In Potamotrygonidae, marine

dasyatoid rays invaded the Amazon basin during a period of marine incursions into continental South America (Lovejoy et al., 2006). These rays were subsequently isolated within the interior waterways by the volcanism and continental uplift by the Andes chain, after which potamotrygonids diversified to fill a myriad of specialized trophic roles ranging from molluscivory, insectivory, to piscivory (Charvet-Almeida, 2001; Moro et al., 2012). In the Myliobatidae, there was an ancient shift from a demersal to a pelagic habitat, with subsequent diversification in this clade along two divergent resource gradients, that of shelled prey (here: crustaceans, bivalves, gastropods, and brachiopods) for myliobatids proper, and all manners of plankton and nekton (schooling fish) in related mobulid rays (Aschliman, 2014; Adnet et al., 2014).

## 1.1 Thesis Objectives

My dissertation is composed of three data chapters, each representing a manuscript intended for submission (or already submitted) to an international scientific journal. The objectives of this research are to examine broad trends in functional trait evolution in several groups of stingrays, reconciling disparity in character state (morphology, behavior) evolution with patterns of resource use. Ultimately I ask the question, 'How do characteristics of prey mold the evolution of the predator' from several standpoints: material, structural, and biomechanical. Specifically, I am interested in how animals adapt to novel diets and diets which pose unique challenges, including prey that are tough, stiff, hard, or generally robust. The ultimate goal is to approach these questions at the macroevolutionary level in order to understand how biological complexity is generated and maintained in the light of fundamental physical and engineering principles, in addition to natural selection.

In Chapter 1 (Kolmann et al., 2015b), I examined the role of differing jaw morphologies in hardprey crushing stingrays (Myliobatidae), with regards to their performance feeding on mollusks of

varying material and structural characteristics. 'Hard' prey, generally meant as prey made of stiffened ceramic-like materials, are typically amalgamated into a single category in ecological and evolutionary studies. Biomaterials researchers would predict that predators exploiting these kinds of prey might have drastically disparate morphologies given variation in prey material. Therefore, I expected that jaw morphology (curvature) would have a differing effect on crushing performance either through conveying size-selective advantages (i.e. some curvatures crush smaller or larger shells more easily than others) or prey-material advantages (i.e. some curvatures crush nacreous over composite shell materials more easily than others). This study had four goals: (1) compare jaw cross-sectional curvature among four genera of durophagous stingrays, and evaluate metrics for this comparison; (2) use physical models (jaw replicas) from the four durophagous stingrays to compare crushing performance; (3) quantify and compare differences in performance for the crushing of live prey items, complex physical models, and simple physical models; and (4) quantify the "crushability" of three different species of mollusk (one gastropod and two bivalves). Differences in jaw curvatures may explain differences in resource partitioning (i.e., dietary preferences in hard prey preference) between durophagous stingray taxa, which feature drastically different jaw morphologies. In Chapter 2 (accepted, Proceeding of the Royal Society: Part B), I investigated the feeding behavior and performance of *Potamotrygon motoro* – a generalist feeder on fishes, insects, and crustaceans (Lonardoni et al., 2006; Shibuya et al., 2012). Insect-feeding is an evolutionary anomaly for elasmobranchs (sharks and rays), and our understanding of this behavior will elucidate the manner in which complex prey processing behaviors have evolved across the vertebrates. I expected that *Potamotrygon motoro* uses greater overall kinesis of the jaws while feeding on insects than other prey items. Specifically I expected greater asymmetrical jaw protrusion, higher incidence of symphyseal flexion, and more frequent and variable (duration) of

jaw protrusion. These measures are analogs for the sort of transverse jaw actions seen in mammalian insectivores, which characterize feeding on tough prey through use of shearing occlusal forces. In addition, I expected that prey-handling times will also be greater for insect prey over other (more compliant) prey.

The primary objectives of this study were to (1) test whether P. motoro uses chewing to process prey, as assessed by asymmetric motions of the jaws that shear and compress food between the occluding dentition. I predicted that, across a range of prey types, chewing motions would be more exaggerated for chitinous food items (insects and crustaceans) than other prey items (fish). I also tested the hypothesis that (2) P. motoro dissociates prey capture and processing by using the whole body (disk) to capture prey items, and the mouth and jaws for processing, as observed in two other batoid species (Wilga et al., 2012). Finally, as chewing is typically associated with heterodonty, I determined (3) whether *P. motoro*, a generalist insectivore, and *P. orbignyi*, a specialist, are capable of reorienting their teeth, producing a functionally heterodont dentition. In Chapter 3, I examined patterns of lineage accumulation and ecomorphological diversification in Potamotrygonidae, and the relationship between these phenomena and dietary niche. From a generalist marine ancestor, potamotrygonid freshwater rays diversified to fill a multitude of specialist dietary niches, including piscivory, molluscivory, and insectivory. I expected that both shifts in diversification and the generation of novel adaptive peaks will correlate with some, if not all, these instances of dietary specialization. Finally, I expected that in general, potamotrygonids will show an early-burst of morphological disparity and initial exponential increases in lineage accumulation through time. Whether these findings are confirmed will lend credence to an expansion of functional space relative to the generalist ancestor of potamotrygonids, and lays the groundwork for determining if potamotrygonids have undergone an adaptive radiation.

This study addressed the following objectives: (1) what are the phylogenetic relationships within the potamotrygonid stingrays? (2) What taxa represent the nearest-related marine outgroup to potamotrygonids? The marine sister taxa of this family has been controversial, as is the conclusive monophyly of the potamotrygonid genera, and the geological age of the clade in general. Finally, I used this phylogeny to ask (3) how patterns of ecomorphological diversification have proceeded in potamotrygonids, and (4) whether patterns of lineage accumulation and morphological disparity suggest these rays are adaptively radiating. I predicted that potamotrygonids will show an early-burst pattern of ecomorphological diversification, corresponding to dietary mode, the presumed resource gradient over which these rays have partitioned. I expected that shifts in rates of lineage evolution in this clade correlate with geography or dietary mode Finally, I expected that the generation of novel adaptive peaks in this lineage are commensurate with distinct dietary modes such as insectivory, piscivory, and molluscivory.

## 2 Morphology does not predict performance: jaw curvature and prey crushing in durophagous stingrays

### 2.1 Abstract

All stingrays in the family Myliobatidae are durophagous, consuming bivalves and gastropods, as well as decapod crustaceans. Durophagous rays have rigid jaws, flat teeth that interlock to form pavement-like tooth plates, and large muscles which generate bite forces capable of fracturing stiff biological composites (e.g., mollusk shell). The relative proportion of different prey types in the diet of durophagous rays varies between genera with some stingray species specializing on particular mollusk taxa, while others are generalists. The tooth plate module provides a curved occlusal surface on which prey is crushed, and this curvature differs significantly among myliobatids. I measured the effect of jaw curvature on prey-crushing success in durophagous stingrays. I milled aluminum replica jaws rendered from computed tomography scans, and crushed live mollusks, 3D printed gastropod shells, and ceramic tubes with these fabricated jaws. Our analysis of prey items indicate that gastropods were consistently more difficult to crush than bivalves (i.e. were stiffer), but that mussels require the greatest workto-fracture. I found that replica shells can provide an important proxy for investigations of failure mechanics. I also found little difference in crushing performance between jaw shapes, suggesting that disparate jaws are equally suited for processing different types of shelled prey. Thus, durophagous stingrays exhibit a many-to-one mapping of jaw morphology to mollusk crushing performance.

Key words: Myliobatidae, biomaterials, rapid prototyping, toughness, bite force

## 2.2 Introduction

Batoids (rays, skates, sawfishes and guitarfishes) comprise over half of the cartilaginous fish diversity and include several lineages that independently evolved hard prey crushing. The myliobatid stingrays are a monophyletic group in which the members either eat shelled prey that exhibit high toughness, stiffness, and/or strength (Myliobatinae, Rhinopterinae, Aetobatinae) or have abandoned biting altogether and filter feed (Mobulinae) (Summers, 2000; Aschliman, 2014). Myliobatid stingrays arose approximately 65-70 mya, coincident with the rise of other durophagous fishes as well as a shift in the ecomorphological structure of molluscan

communities (Vermeij, 1977; Aschliman et al., 2012). Compared to non-durophagous stingrays, myliobatids have reduced cranial mobility (e.g. due to jaw symphyseal fusion), several instances of duplicated or reoriented muscles, and increased skeletal reinforcement, all features convergent with other durophagous vertebrates (Summers, 2000; Kolmann et al., 2014; Mulvany and Motta, 2014). Durophagous stingrays also feature robust teeth, interlocking at their bases to form shallow-domed tooth plate arrays (Figure 2.1). Batoids and sharks have continuous dental replacement; in durophagous ray tooth modules, younger teeth mineralize and are conveyed labially to replace older, worn teeth.

Myliobatid rays have considerable inter-taxon variation in the morphology of the jaw complex, with the jaws and teeth varying in overall shape, length, width, and cross-sectional curvature (Figure 2.1). Some species, such as eagle rays (*Aetobatus narinari*), prey almost exclusively on gastropods (Schluessel et al., 2010), while others, such as bat rays (*Myliobatis*), appear to prey preferentially on decapods (Gray et al., 1997; Szczepanski & Bengston, 2014) (Figure 2.1). Finally, cownose rays (*Rhinoptera*) feed on a wide variety of hard and soft prey, depending on geographic distribution (Collins et al., 2007; Ajemian et al., 2012). By examining how performance differs among jaw shapes, I may be able to determine whether or not disparate jaw shapes are optimized for crushing different types of hard prey.

The crushing of hard prey provides a simple, direct, and useful performance metric for investigating the relationship between form and function. There is little ambiguity in deciding whether a prey item has been crushed, so there is a clear relationship between morphology and performance. The main determinant of predator success is the ability to exert high loads (Pfaller et al., 2011). For this reason it is possible to explore the implications of different predator and prey morphologies and to determine their interactions (Bertness and Cunningham, 1981; Whitenack and Herbert, 2015). Not only is there variation in the crushing jaws of the predators, but there are also material and structural differences in the shells of the prey. Mollusk taxa differ in the microstructure of the material that comprises the shell (involving so-called fibrous, prismatic, cross-lamellar, or nacreous mineral-organic composite layers or combinations of these), and the incorporated polymorphs of calcium carbonate mineral (aragonite and/or calcite). The relationship between taxon-specific structural differences and shell mechanics is yet to be clarified, but it is clear that the organic component of the composite layers results in drastic increases in shell toughness, relative to aragonite or calcite alone (Currey, 1980).

The simple metric of crushing allows us to ask whether the predator's morphology is a strong predictor of feeding performance or if crushing success is more contingent on morphological (structural) and/or material composition of the shells of prey. Here, I investigate the effect that variation of jaw shape in durophagous stingray taxa has on crushing success. Our study had four goals: (1) compare jaw cross-sectional curvature among four species of durophagous stingrays, and evaluate metrics for this comparison; (2) use physical models (jaw replicas) from the four durophagous stingrays to compare crushing performance; (3) quantify and compare differences in performance for the crushing of live prey items, complex physical models, and simple physical models; and (4) quantify the "crushability" of three different species of mollusk (one gastropod and two bivalves).

Shelled prey are not all created equal in terms of the mechanical properties of their shells; I investigated three parameters of crushing performance (peak load, yield load, and work-to-fracture/toughness; Figure 2.2, the combination of which characterize the ability of shelled prey to absorb energy before fracture (toughness) and to withstand forces (stiffness) before total failure, illuminating mechanical differences in prey exoskeletons and jaw performance. Yield loading, designated here as the amount of force required to plastically deform the shell (indent) is contrasted with peak loading, the amount of force required to cause the shell to fail outright (Fig 2.2).

## 2.3 Methodology

#### 2.3.1 Jaw replica construction and jaw metrics

Whole specimens of *Aetomylaeus nichofii* (mottled eagle ray), *Aetobatus narinari* (spotted eagle ray), *Myliobatis tobijei* (Japanese bat ray), and *Rhinoptera bonasus* (cownose ray) were obtained from museum collections during a prior study (Dean et al., 2007). These species represent the four extant genera of durophagous myliobatid rays, which cover the range of ecological variability in this clade. These specimens were computed tomography (CT) scanned with a 16-slice medical grade Siemens RS SOMATOM Sensation (MDCT-16, Siemens Medical Solutions, Malvern, PA, USA) with 0.75 mm slice thickness and helical-spiral scans. Specimens were wrapped in alcohol saturated cheesecloth and scanned in large Ziploc© bags. Scans were reconstructed as 8 bit .TIFF stacks and rendered as three-dimensional visualizations using Amira software (v. 5.2.2, Visage Imaging, Inc., Richmond, VIC, AUS).

The upper and lower jaws (palatoquadrate and Meckel's cartilage, respectively) and tooth plates were segmented (digitally dissected) from the rest of the body. A medial sagittal section of each jaw complex (including jaws and teeth) was manually traced in Adobe Illustrator CS (Adobe Systems, Inc., San Jose, CA, USA). These two-dimensional images were then extruded (extended into the z-axis), resulting in four pairs of simplified three-dimensional jaw models scaled to 40mm standard width and cropped to include only the relevant occlusal surface (in an anterior-posterior direction) in 123D Design (v. 1.4.51, Autodesk, Inc., San Rafael, CA, USA). This functional occlusal surface was determined by examining the pattern of wear on specimen tooth plates (e.g. note the wear in the lingual and sagittal images in Figure 2.1). Jaw models were exported as .stl files into SprutCAM7 Pro (v. 7.1.5, Sprut Technologies, Inc., Tormach Inc., Waunakee, WI, USA), to generate tool paths for CNC (computer numerical controlled) milling. Models were fashioned from 6061T aluminum stock using a 4-axis mill (Tormach PCNC1100, Tormach Inc., Waunakee, WI, USA), deburred with a belt sander, and polished (Figure 2.2 inset image).

Radius of curvature (RoC) of the occlusal surface of each jaw complex was measured by fitting a circle to the upper and lower jaw of each species using ImageJ. Larger curvatures correspond to increasingly "flatter" or more broadly-curved jaw sets, while smaller curvatures indicate a more peaked or domed morphology. I used two metrics to characterize the jaws of each species: 1) the average curvature of the upper and lower jaws together, and 2) a measure of the disparity between upper and lower jaw curvatures which I generated by dividing the upper jaw curvature by the lower.

#### 2.3.2 Prey sample collection & 3D-Printing

Several types of "prey" were subjected to materials testing: (1) live common blue mussels (*Mytilus edulis*; shell height size range = 6.0 - 20.5 cm), (2) live varnish clams (*Nuttalia obscurata*; shell height size range = 6.0 - 19.3 cm), (3) live frilled dogwinkles (*Nucella lamellosa*; shell height size range = 6.0 - 23.3 cm), (4) 3D-printed replica shells (ZPrinter 310, ZCorporation, Inc. Rock Hill, SC, USA) of the frilled dogwinkle *Nucella lamellosa* (1.0 - 2.5 cm, four size classes at 0.5 cm intervals), and (5) ceramic tubes (FluVal BioMax filter media rings, Hagen, Inc., Montreal, QC, CAN; size ranges: height = 0.9 - 1.3 cm, length = 1.3 - 2.1 cm, inner dia. = 0.3 - 0.45 cm, outer dia. = 0.9 - 1.3 cm). Replica shells were based on .stl files

generated from micro-CT scans from Crofts and Summers (2014). Replica shells were printed in plaster, hardened using a solution of magnesium chloride and water, and then placed in a vacuum heater for 12 hours to dry and harden. Ceramic tube dimensions were measured using ImageJ (v. 1.40, National Institute of Health, Bethesda, MD, USA) prior to crushing. Live prey were measured with digital calipers. Replica shells and ceramic tubes represent our "complex" and "simple" artificial prey types, respectively.

Although the live prey species used in these experiments have not been reported from the diets of the rays in question, congeneric or confamilial taxa are known to be consumed by myliobatids (Capape, 1977; Gray et al., 1997; Yamaguchi et al., 2005; Jardas et al., 2004; Collins et al., 2007; Schluessel et al., 2010; Ajemian and Powers, 2012). Live shellfish size series were collected from the region around Friday Harbor, San Juan Island, WA from intertidal tide-pool communities. Shell length, height, and depth were recorded for each specimen. Shell length (spire length) was measured from the tip of the spire to the tip of the siphon in dogwinkles and from the umbo to the anterior-most edge of the valves in mussels and clams. Shell width was measured from the valves in mussels and clams. Shell with the operculum lying flat, to the vertical-most extent of the spire in dogwinkles, with height being the maximum distance from the upper and lower valves in mussels and clams. Shell height is presumed to be the shape parameter of greatest relevance to compression resistance, as it is orthogonal to the normal (compressive) loading scenario (Kolmann and Huber, 2009; Crofts and Summers, 2014).

#### 2.3.3 Prey-crushing simulations

Aluminum jaw replicas were threaded and attached to a mechanical loading frame (Synergie 100, MTS Systems Corp.) coupled to a 500 N load cell (Figure 2.2 inset image). To explore the ability of artificial prey types to mimic the failure of natural specimens, I measured the performance (peak load, yield load, and work-to-fracture) required by each set of jaws to crush ceramic media (n = 20 per jaw), and live and printed *Nucella* shells (n = 40 per jaw), all of approximately similar size. Shell spires were positioned facing lingually for gastropods (Figure 2.2 inset image). Bivalves were placed with the hinge facing labially, as seen in videos of preyhandling events of some durophagous rays (Fisher et al., 2011). Shells were crushed using a

compressive loading regime of 1.27 mm/s (Crofts and Summers, 2014). Peak load (N) and yield load (N) were determined from stress/strain curves generated by TestWorks4 software (v. 4.08, MTS Systems Corp.) and recorded after each trial. Work (Nm) was calculated using a custom R script which estimates the area under the load-displacement curve for each trial, with the maxima of the given loading event being the point at which peak load was achieved (Figure .22).

#### 2.3.4 Statistical analyses and experimental design

Wilks-Shapiro and Levene tests were used to test for normality and equal variances as a prerequisite for determining if data should be transformed prior to further analyses. The interaction between shell size, jaw morphology, and prey type (tubes *versus* live or printed snails) were compared using ANCOVA, with prey type as a covariate. Because the size and shape of the prey items varied, especially in the live *Nucella* specimens, I also used an ordinary least-squares (OLS) regression of prey height on crushing performance to determine the size-corrected residuals of loading or work-to-fracture, and tested these data against prey type using a two-way ANOVA. By contrasting the crushing performance across jaw morphologies between printed dogwhinkles (n = 40) and live *Nucella* (n = 30) using ANCOVA with live *versus* printed as a covariate, I were also able to determine how material and structural properties of live shells contribute to differences in overall crushing performance.

To determine the effect of different shell sizes on jaw crushing performance, I used a two-way ANOVA to test four size classes (n = 10 per jaw) of printed *Nucella* shells. Finally, I tested whether different jaw morphologies convey any inherent advantage to crushing live snail (n = 30), mussel (n = 15), and clam (n = 15) shells, which vary considerably in shape and presumably material and structural properties. The interaction between jaw morphology and shell dimensions were investigated using two-way ANOVA. I also used an ordinary least-squares (OLS) regression of prey height on crushing performance to determine the size-corrected residuals of loading or work-to-fracture, and tested these data against jaw morphology using ANOVA.

Post-hoc Tukey Honest Significant Difference (HSD) tests were run on ANOVAs to determine pairwise differences between variables. All analyses were run in R (v. 2.15.0, <u>www.theRproject.org</u>).

## 2.4 Results

Data were found to be non-normally distributed and in some cases to show unequal variances among variables and were subsequently transformed before further analyses (Supplemental Table 2.1). Performance variables generally increased with shell height. The residuals of the regression of shell height on each performance variable were used as our size-corrected dataset (Supplemental Table 2.2).

#### 2.4.1 Differences in performance and morphology among stingray genera

*Myliobatis* had the broadest (flattest) occlusal surfaces when averaging both upper and lower jaws, followed by *Rhinoptera* and *Aetomylaeus*, while *Aetobatus* had the most curved jaw overall. *Rhinoptera* jaws showed the least amount of disparity in curvature between upper and lower and jaws, and *Aetobatus* had the largest disparity in curvature (Tables 2.1-2.5).

Comparing between the myliobatid taxa, *Aetobatus* generally displayed lower performance values (i.e. lower peak and yield loads and work-to-fracture) when compared to *Aetomylaeus*, *Rhinoptera*, and *Myliobatis*, which exhibited similar peak and yield loads in addition to work-to-fracture. There were differences between taxa for peak load (F: 3.211; p = 0.0233), but not yield load (F: 2.04; p = 0.108) for all prey items. Tukey HSD results showed differences in peak loading performance between *Aetobatus* and most other taxa (*Myliobatis*; p = 0.036 and *Rhinoptera*; p = 0.069). According to Tukey HSD comparisons, yield loads were different for all prey types (p < 0.0001). Work-to-fracture did not differ between stingray taxa (F: 2.476; p = 0.0615), and post-hoc analyses show that work-to-fracture differed between *Aetobatus* and *Rhinoptera* only (p = 0.048). However, mussels tended to have higher work-to-fracture than gastropods.

Overall, *Aetomylaeus*, *Rhinoptera* and *Myliobatis* exhibited similar peak and yield loads and work-to-fracture, whereas *Aetobatus* had the lowest values for all performance metrics. There was an effect of predator jaw shape on peak loading across the three live prey categories (F: 3.091; p = 0.0279), and of prey type (F: 177.46; p <  $2.0 \times 10^{-16}$ ) on yield load.

## 2.4.2 Artificial vs Natural Prey Types

Performance metrics (peak load, yield load, and work-to-fracture) varied by live prey type (Tables 2.1-2.3). Yield loads were different for all prey types (p < 0.001), and post-hoc analyses

showed differences between all pairwise comparisons of prey types (p = 0.014) and between live and printed snail shells which behaved more similarly to each other than either did to ceramic tubes (Figure 2.3). Overall, when size was taken into account, ceramic tubes required greater loading forces (peak load and yield load) to initiate fracture than either live or replica *Nucella* snails (Figure 2.3). Work-to-fracture did not differ among prey types (F: 2.399; p = 0.093), and ceramic tubes were shown to have generally higher work-to-fracture values than live *Nucella* snails, albeit not significantly different (p = 0.08).

Using a multiple regression framework to examine how much prey size affected crushing performance, prey type was found to be the most informative variable (35.3% of variance), followed by shell width (22.2%), shell height (21.5%), and shell length (19.2%) when explaining trends in peak loading. Yield load showed a similar trend, with prey type explaining over half (55.2%) of the model variance, followed by shell width (15.0%), shell height (14.4%), and shell length (14.5%). Finally, for work-to-fracture, prey type was again the most explanatory variable, explaining 33.5% of the variance, followed by shell width (22.6%), shell height (22.5%), and shell length (18%).

#### 2.4.3 Crushing live prey

Live *Nucella* snails generally required 1.5 to 3.0 times greater force to crush or indent (peak and yield loading, respectively) than varnish clams or mussels, and mussels failed under noticeably lower loads (1.8 to 3.2 times lesser) than the other prey items (Figure 2.4; Tables 2.3-2.5). After correcting for size, differences between prey species were still significant for all performance metrics. *Nucella* required more force (peak load and yield load) to fail than the bivalves, but mussels required higher peak loadings to fracture than clams. When corrected for size, mussels require the greatest work-to-fracture (generally 1.25 times greater), followed by gastropods, and then clams (Figure 2.5).

There was also a notable effect of shell height on peak load (F: 163.25; p < 0.001) and yield load (F: 234.97; p < 0.001), with yield and peak loads increasing as shell height increased. Correcting for shell size, only prey type was significant for peak load (F: 91.24; p < 0.001), with post-hoc comparisons showing that all prey taxa differed from one another (p < 0.001). Similarly, after correcting for size, only prey type was predictive of yield load (F: 155.9; p < 0.001), with both

bivalve taxa virtually indistinguishable from one another, but conspicuously different from *Nucella* (p < 0.001).

Both shell height and prey type were correlated with work-to-fracture (F: 339.94; p < 0.001; F: 7.256; p < 0.001 - respectively). However, as with the loading variables, once corrected for prey size, only prey type (F: 42.28; p < 0.001) was predictive of work-to-fracture, and post-hoc comparisons showed that all prey taxa differed from one another (p < 0.001) in terms of work-to-fracture.

When examining the effect of prey size on fracture mechanics explicitly, size consistently affected crushing performance across all trials, whereas predator species accounted for less than 2% of all variance. Not unsurprisingly, the larger the shell, the more difficult it was to crush in terms of both loading and work. Multiple regression results show that when all variables were included, shell size parameters, typically shell height, were the most explanatory variables for predicting fracture. For peak loads on natural prey, shell height and prey species were found to explain 33.7% and 31.5% of the variance. Yield load showed a similar trend, but with prey species explaining over half (55.6%) of the model variance, followed by shell height (25.4%), and shell length (11.0%). For work-to-fracture, only shell height (35.6%), shell length (26.9%), and prey type (20.6%) were informative.

#### 2.4.4 Fracture behavior of prey items

Printed and live snails consistently showed crack formation at the base of the spire in almost all trials (Figure 2.6). Crack propagation continued dorsally along the spire suture, paralleling the shell aperture. This pattern was repeated across shells regardless of shell size. Generally, live *Nucella* differed from both simple and complex prey models in having greater variability in the ranges of both loading and work required to fracture the shell, 2.2 to 3.0 times greater than those of artificial prey. Fracture in live clams and mussels typically started along the dorsal surface, beginning at the umbo and continuing along the right valve (dorsal, in this case) anteriorly. There was periodic failure at the conjoining margins of the valves as thinner material buckled outwards.

## 2.5 Discussion

There are many differences in the feeding apparatus of durophagous rays, including the size, shape, insertion, and pennation of muscles, and the arrangement of connective tissue (Kolmann et al., 2014), but I cannot ascribe any performance difference to one of the most obvious differences in morphology - the shape of the jaws. With minor exception, the shape of myliobatid jaws had little effect on the crushing performance of hard prey, regardless of prey type. Aetobatus and Rhinoptera, at opposite ends of a curvature continuum (larger to smaller curvature ratio), had significant but small differences in the peak load required to crush some prey types. Rather than evidence for the superiority of *Rhinoptera*'s morphology I take this to be indicative of the power of our test scheme, which revealed a difference of just 221 N (for Rhinoptera) versus 188 N (for Aetobatus) as statistically significant. The use of metal models isolated the effect of the morphology of the jaws from the any material differences in the jaws and from any effect of the shape and interdigitating pattern of the teeth. In addition to the musculoskeletal differences among these stingrays I might expect that the tooth interdigitation pattern, long recognized as a taxonomic character (Claeson et al., 2010), has some effect on crushing performance. Regardless, the forces necessary to crush any of the examined live prey (from 22 to 486 N, peak loading) were well within the performance bounds (> 500 N) calculated for *Rhinoptera bonasus*, the only myliobatid ray for which bite force has been examined to date (Kolmann et al., 2015a). However, evidence by Fisher et al. (2011) has shown that Rhinoptera can consume some large oysters requiring in excess of 800-1000 N to crush. These crushing behaviors on the largest oysters took *Rhinoptera* in excess of 60 minutes, a duration which seems at odds with the low energy expenditure/high energy gain strategies predicted by optimal foraging theory. Perhaps the curvature of the jaws in these stingrays conveys some performance advantage at prey size extremes which our experimental design could not replicate.

Artificial prey, either simple (tubes) or complex (3D printed shells), had less individual variation in crushability than live prey, as previously proposed (Crofts and Summers, 2014). Although artificial prey and live snails were found to be significantly different from one another in terms of the magnitude of loading required to fracture the shell (2.0-3.0 times greater in printed prey), printed shells approximated the general mechanical behavior of live snails. That is, both live and 3D printed gastropods showed consistent fracture patterns, with stress fractures occurring at the base of shell spire and then continuing dorsally along the spire suture. Work-to-fracture was indistinguishable between artificial prey and live *Nucella*, suggesting that this important characteristic of shell material can be mimicked by a powder-based 3D printer. I confirm that replica shells can provide an important proxy for investigations of failure mechanics, clarifying that features other than shell shape (e.g. shell material and structural properties) could contribute to inter-individual variation in failure properties.

Live prey species differed significantly from one another in their ability to absorb energy before fracture (work) and to withstand high forces (loading) before total failure. *Nucella* and *Nuttalia* were stiffer and required higher forces to crush, whereas *Mytilus* required greater energy investment per unit size. This suggests that inherent species-specific differences in shell properties (e.g. shell materials, gross morphologies, and microarchitectures) provide different strategies for avoiding predation that, in turn, perhaps, demand suites of feeding behaviors from predators with diverse diets (e.g. the species examined in this study). In this way, the predator and prey communities are not only shaping each other's ecologies, but also the material and mechanical properties of their skeletal and dental structures. This is underlined by fossil data: prior to the Jurassic, most mollusks were predominantly thin-shelled, non-ornamented, stationary, and epifaunal (Vermeij, 1977), whereas modern molluscan morphology and ecology are thought to have been precipitated by the rise of durophagous predators during the late Mesozoic (75-65 mya).

Our results show little direct relationship between crushing performance and jaw shape in durophagous stingrays, despite observed variation in diet among these taxa. This may indicate that the jaws of durophagous stingrays are an example of "many-to-one mapping", where multiple and varied morphologies meet the performance requirements for a certain ecological role (e.g. hard prey crushing) (Wainwright et al., 2005). This pattern is common across the vast diversity of vertebrate feeding morphologies in the context of dietary specialization (Wainwright et al., 2005; Young et al., 2007). Although most previous studies of elasmobranch durophagy have focused primarily on musculoskeletal specializations for eating hard prey (e.g., Kolmann and Huber, 2009; Mara et al., 2010), anecdotal evidence suggest that the diversity of strategies for durophagy in elasmobranchs have only begun to be characterized. For example, the bonnethead (*Sphyrna tiburo*) and horn shark (*Heterodontus francisci*) both purportedly use rapid, repeated jaw contractions to crush prey (Wilga and Motta, 2000; Huber et al., 2005; Mara et al., 2010), a method of cyclical loading to fatigue stiff exoskeletal materials that has been

documented in durophagous crabs as well (Kosloski & Allmon 2015). In our study, I only tested the effects of constant rates of compression, but observations of myliobatid prey capture suggest that they may also use cyclical jaw movements in prey crushing (Sasko et al., 2006). Additionally, Mara et al. (2010) suggested that bonnethead sharks may also use stomach acidity to weaken or dissolve the shell, supplementing their comparatively low bite forces to further reduce hard-shelled prey to something more easily digestible. Therefore, although high bite forces are clearly paramount for processing hard prey, durophagous elasmobranch taxa may use a suite of mechanical and non-mechanical methods to reduce prey, suggesting that in elasmobranchs, the concept of "many-to-one mapping" need be expanded to include more than just morphological variation.

Our results underline that durophagous vertebrates are more morphologically variable than previously expected (Crofts and Summers, 2014), even among closely related taxa, highlighting the potential for alternative functional strategies in high-performance systems. The requirement for durophagous taxa to resist high loadings and accumulative fatigue is imperative to the survival of these animals, which tend to have delayed maturity and be generally long-lived (Schluessel et al., 2010; Fisher et al., 2013). As myliobatid jaws appear to represent a "many-toone" system in terms of prey crushing performance, further work is required to determine why the jaws exhibit such disparate curvatures across species. Our study focused on shape parameters; however, other yet-to-be-examined features, including hard anatomy (skeletal and/or dental), soft anatomy (tendons and/or muscles) or physiology (e.g. gut chemistry), may dictate performance differences among these stingrays. Finally, durophagous systems are frequently highlighted for their mechanical performance or structural strength, but infrequently are both paradigms considered simultaneously, especially in relation to prey structural or material properties. Properties of prey are frequently overlooked in the typical reliance on just one aspect of performance (e.g. bite force). The work and energy required to process prey may relate more intimately to optimal foraging strategies than purely biophysical estimates (e.g. maximum bite force), especially when feeding behaviors may be more complex than simply biting with as much force as possible.

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<u>Current</u>	Jaw	r	Avg	Ratio	Peak Load	Yield Load	West (New)
Species	Morphology	Curvature	Curvature	Curvature	(IN)	(IN)	work (INM)
Phinoptara	more similar	595.9	5177	547.7 0.84	$90.0\pm38.4$	$55.8\pm28.9$	$29.9\pm20.3$
Кніпоріета		499.5	547.7	0.04	36.7-169.5	15.4-145.4	9 $29.9 \pm 20.3$ 4 $3.9-83.6$ 2 $27.8 \pm 23.0$ 5 $3.0-96.9$ 1 $27.5 \pm 19.2$
Astonniania		304.9	580.2	2.01	$90.3\pm47.3$	$55.7\pm36.2$	$27.8\pm23.0$
Aelomyldeus		855.4	380.2	2.81	31.4-207.9	21.5-181.5	3.0-96.9
Mulishadia		231.1	E2E E	2.62	$100.9\pm42.4$	$64.3\pm34.1$	$27.5\pm19.2$
Myliobatis		839.9	535.5	3.03	38.8-212.1	25.1-182.8	3.9-92.0
Actobatus		152.4	157.6	5.00	$101.1\pm68.2$	$61.1\pm41.1$	$34.1\pm37.3$
Aetobutus	more disparate	762.8	457.0	5.00	22.6-350.8	15.2-180.2	4.6-173.7

## Table 2.1 Forces and Work to fracture for artificial snails

Values are the mean  $\pm$  s.d.

**Bolded** and *italicized* values are upper and lower jaw radius of curvature (*r* Curvature), respectively (from Kolmann et al., 2015b)

- ·	Jaw	r	Avg	Ratio	Peak Load	Yield Load	
Species	Morphology	Curvature	Curvature	Curvature	(N)	(N)	Work (Nm)
Phinoptara	more similar	595.9	5177	0.84	$183.8\pm29.0$	$165.1 \pm 31.7$	$23.6\pm5.4$
Кппортеги		499.5	547.7	0.84	131.1-235.2	108.5-229.2	Work (Nm)      7 $23.6 \pm 5.4$ 2 $15.5-33.5$ 9 $25.5 \pm 7.9$ 16.9-53.3      9 $23.1 \pm 7.2$ 5 $13.5-46.5$ 1 $21.9 \pm 7.1$ 3 $12.8-46.5$
Astonniania		304.9	580.2	2.01	$170.8\pm30.1$	$137.5\pm29.9$	.9 $25.5 \pm 7.9$
Aelomyldeus		855.4	380.2	2.81	121.0-254.4	88.0-224.5	16.9-53.3
Mulishadia		231.1	525 E	2 (2	$183.8\pm40.6$	$165.5\pm37.9$	$25.5 \pm 7.9$ 16.9-53.3 $23.1 \pm 7.2$ 13.5-46.5
Myllobatis		839.9	555.5	3.03	128.1-273.3	109.3-251.5	
A		152.4	157 6	5.00	$173.4\pm39.7$	$145.2\pm36.1$	Work (Nm) $23.6 \pm 5.4$ $15.5-33.5$ $25.5 \pm 7.9$ $16.9-53.3$ $23.1 \pm 7.2$ $13.5-46.5$ $21.9 \pm 7.1$ $12.8-46.5$
Aetobatus	more disparate	762.8	437.6	5.00	131.6-314.1	110.0-274.3	12.8-46.5

## Table 2.2 Forces and Work to fracture for ceramic tubes

Values are the mean  $\pm$  s.d.

**Bolded** and *italicized* values are upper and lower jaw radius of curvature (*r* Curvature), respectively (from Kolmann et al., 2015b)
## Table 2.3 Forces and Work to fracture for live snails (*Nucella* sp.)

	Jaw	r	Avg	Ratio	Peak Load	Yield Load	
Species	Morphology	Curvature	Curvature	Curvature	(N)	(N)	Work (Nm)
Rhinoptera	more similar	595.9	547.7	0.84	$\begin{array}{c} 276.9 \pm \\ 104.4 \end{array}$	200.4 ± 85.4	57.9 ± 28.8
		499.5			96.6-486.4	65.3-384.0	11.6-126.6
Aetomylaeus		304.9	580.2	2.81	281.8 ± 121.4	$\begin{array}{c} 206.0 \pm \\ 102.9 \end{array}$	$60.9 \pm 44.2$
		855.4			69.6-483.7	46.1-448.6	5.5-171.5
Myliobatis		231.1	535.5	3.63	276.4 ± 133.9	$\begin{array}{c} 207.0 \pm \\ 118.8 \end{array}$	$61.2\pm36.9$
		839.9			66.5-483.0	52.7-444.0	7.3-153.9
Aetobatus		152.4	457.6	5.00	219.4 ± 103.5	$160.9 \pm 81.4$	$45.9\pm36.0$
	more disparate	762.8			53.4-436.3	37.4-336.1	4.1-165.3

Values are the mean  $\pm$  s.d.

**Bolded** and *italicized* values are upper and lower jaw radius of curvature (*r* Curvature), respectively (from Kolmann et al., 2015b)

Table 2.4 Forces and	Work to	fracture	for	live	mussel	s
(Mytilus sp.)						

	Jaw	r	Avg	Ratio	Peak Load	Yield Load	
Species	Morphology	Curvature	Curvature	Curvature	(N)	(N)	Work (Nm)
Rhinoptera	more similar	595.9	547.7	0.84	$\begin{array}{c} 147.6 \pm \\ 65.58 \end{array}$	60.16 ± 33.39	54.61 ± 32.8
		499.5			57.2-314.3	15.6-145.8	11.6-217.2
Aetomylaeus		304.9	580.2	2.81	$\begin{array}{c} 159.5 \pm \\ 60.85 \end{array}$	58.16 ± 33.48	84.65 ± 41.9
		855.4			58.5-285.8	23.8-140.8	5.5-171.5
Myliobatis		231.1	535.5	3.63	151.1 ± 69.11	57.62 ± 22.07	69.36 ± 43.1
		839.9			35.6-292.1	15.3-92.2	2.3-157.6
Aetobatus		152.4	457.6	5.00	$142.3\pm59.9$	$65.9 \pm 32.41$	$\begin{array}{c} 68.36 \pm \\ 43.1 \end{array}$
	more disparate	762.8			56.4-237.5	20.3-121.2	2.9-165.3

Values are the mean  $\pm$  s.d.

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**Bolded** and *italicized* values are upper and lower jaw radius of curvature (*r* Curvature), respectively (from Kolmann et al., 2015b)

# Table 2.5 Forces and Work to fracture for live clams (*Nuttalia* sp.)

	Jaw	r	Avg	Ratio	Peak Load	Yield Load	
Species	Morphology	Curvature	Curvature	Curvature	(N)	(N)	Work (Nm)
Phinoptora	more similar	595.9	5177	0.84	$182.9\pm78.7$	114.7 ± 51.2	$69.9\pm60.0$
кппортега		499.5	547.7	0.04	72.0-373.2	53.1-253.1	13.5-217.2
Aetomylaeus		304.9	580.2	2.81	$195.2\pm95.7$	$116.1\pm69.1$	$57.8\pm41.9$
		855.4	580.2		50.8-335.9	26.1-257.5	7.7-112.4
Myliobatis		231.1	525 5	2.62	$167.6\pm85.4$	$114.7\pm65.3$	$45.5\pm39.5$
		839.9	555.5	3.03	32.3-288.5	18.9-229.5	2.3-157.6
		152.4	157 6	- 00	$166.7\pm84.8$	$105.9\pm65.9$	$53.3\pm40.9$
Aetobatus	more disparate	762.8	437.6	5.00	32.3-380.2	24.9-277.0	2.9-112.5

Values are the mean  $\pm$  s.d.

**Bolded** and *italicized* values are upper and lower jaw radius of curvature (*r* Curvature), respectively (from Kolmann et al., 2015b)



Figure 2.1 External and internal jaw morphology of myliobatid rays. Computed tomography (ct) scans of *Aetobatus narinari, Myliobatis californica, Aetomylaeus bovinus,* and *Rhinoptera bonasus* (top to bottom) in labial, lingual, lateral, and sagittal views (left to right). Prey contribution to dietary proportions based on % frequency or % index of relative importance of decapods, bivalves, and gastropods. Diet data from: Schluessel et al., 2010; Gray et al., 1997; Capape, 1977; Ajemian & Powers, 2012 (in same order as species). (from Kolmann et al., 2015b)



Figure 2.2 Example force-displacement trace during crushing of live shells. Peak load (N) represented the max load (N) reached at shell fracture. Yield load (N) represented by the characteristic slope change suggesting plastic deformation of shell material prior to actual fracture. Work-to-fracture (Nm) calculated as the area beneath the curve leading to peak loading. Figure inset features mounted jaw replicas on mechanical loading frame with a replica shell placed between the occlusal surfaces. (from Kolmann et al., 2015b)



Figure 2.3 Box whisker plots showing size-corrected crushing performance on artificial and natural prey. 3D printed *Nucella* shells in light grey, live *Nucella* in medium grey, and ceramic filter tubes in dark grey. Size-corrected by taking the residuals of the linear regression of shell height on respective performance metric (peak and yield loading, work-to-fracture). Boxes represent 50% quantiles, with bar representing median values, whiskers represent standard errors, and outlying data are represented by black dots. Jaw morphologies of each taxon are represented. (from Kolmann et al., 2015b)



Figure 2.4 Box whisker plots showing actual (not corrected for size) crushing performance on live mollusks. Dogwhinkles (*Nucella lamellosa*) in medium grey, varnish clams (*Nuttalia obscurata*) in dark grey, and common mussels (*Mytilus edulis*) in light grey. Boxes represent 50% quantiles, with bar representing median values, whiskers represent standard errors, and outlying data are represented by black dots. Jaw morphologies of each taxon are represented. (from Kolmann et al., 2015b)



Figure 2.5 Box whisker plots showing size-corrected crushing performance on live mollusks. Dogwhinkles (*Nucella lamellosa*) in medium grey, varnish clams (*Nuttalia obscurata*) in dark grey, and common mussels (*Mytilus edulis*) in light grey. Size-corrected by taking the residuals of the linear regression of shell height on respective performance metric (peak and yield loading, work-to-fracture). Boxes represent 50% quantiles, with bar representing median values, whiskers represent standard errors, and outlying data are represented by black dots. Jaw morphologies of each taxon are represented. (from Kolmann et al., 2015b)



Figure 2.6 Evident fracture patterns in *Nucella* shell models. Printed and live gastropods consistently showed crack formation at the base of the spire in almost all trials. Crack propagation continued dorsally along the spire suture, paralleling the shell aperture. This pattern was repeated across shells regardless of shell size. (from Kolmann et al., 2015b)

# 3 Feeding kinematics of a generalized insectivorous stingray, *Potamotrygon motoro*

#### 3.1 Abstract

Chewing, characterized by shearing jaw motions and high-crowned molar teeth, is considered an evolutionary innovation that spurred dietary diversification and evolutionary radiation of mammals. Complex prey processing behaviors have been thought to be lacking in fishes and other vertebrates, despite the fact that many of these animals feed on tough prey, like insects or even grasses. I investigated prey capture and processing in the insect-feeding freshwater stingray, Potamotrygon motoro, using high-speed videography. I find that Potamotrygon motoro uses asymmetrical motion of the jaws, effectively chewing, to dismantle insect prev. However, CT-scanning suggests that this species has simple teeth. This suggests that in contrast to mammalian chewing, asymmetrical jaw action is sufficient for mastication in other vertebrates. I also determined that prey capture in these rays occurs through rapid uplift of the pectoral fins, sucking prey beneath the ray's body and dissociates the jaws from a prey capture role. We suggest that the decoupling of prey capture and processing facilitated the evolution of a highly kinetic feeding apparatus in batoid fishes, giving these animals an ability to consume a wide variety of prey, including mollusks, fishes, aquatic insect larvae, and crustaceans. We propose *Potamotrygon* as a model system for understanding evolutionary convergence of prey processing and chewing in vertebrates.

Key words: cranial kinesis, chitin, Odonata, toughness, insect cuticle

#### 3.2 Introduction

For predators to effectively digest prey, considerable mechanical or chemical processing is often required (Fänge et al., 1979; Moore and Sanson, 1995). Chewing is used to break down tissue, expose digestible elements, and increase surface area for chemical digestion. Mastication varies across diet, but typically involves multi-axis shearing by dental occlusal surfaces rather than a uniaxial compression-only loading regime. Complex teeth or the possession of multiple types of teeth (heterodonty) are frequently associated with chewing, enabling some teeth to be used for capture (e.g., canines) while others are used for processing (molars) (Evans and Sanson, 2003). In terrestrial taxa, mastication is generally considered to be restricted to mammals (the 'definitive

chewers' of Reilly et al., 2001). Chewing is considered an evolutionary innovation in mammals that spurred dietary diversification, allowing exploitation of food sources ranging from insects to grasses. Dietary flexibility and efficiency made possible by chewing is thought to have contributed to the evolutionary radiation of mammals (Lumsden and Osborn, 1977).

Many mammals capture and process prey with the oral jaws and associated teeth, with chewing and prey processing therefore occurring within the mouth. However, in many other vertebrates, prey capture and processing are accomplished using anatomically distinct modules, meaning that these functions have become dissociated. For example, birds and some other archosaurs use a beak or jaws to seize and rend prey, while a muscular gizzard is used to grind prey further (Reilly et al., 2001). Most fishes use expansion of the oral jaws for prey capture through suction feeding, but in many cases use pharyngeal dentition (posterior jaws derived from gill arches) to crush or grind prey (Gidmark et al., 2014, 2015). In some cartilaginous fishes, notably batoids (skates, stingrays, etc.) prey capture and processing may be handled by two systems—prey capture is achieved with the disk (the morphological structure derived from encircling pectoral fins; Wilga et al., 2012), leaving the jaws and teeth for prey processing. In all these cases, different anatomical modules handle different functions, allowing for independent modular evolution (Collar et al., 2014) and increased evolutionary flexibility.

In fishes, considerable variation in tooth shape, muscle motor activity, and jaw kinematics suggests that prey-processing is more diverse than previously expected (Korff and Wainwright, 2004; Konow et al., 2008). Stingrays (Myliobatiformes) have conspicuously 'loose' jaw joints (Kolmann et al., 2015a), a trait in common with mammals. Loose jaw joints allow for transverse (medio-lateral translation, as in bovids and cervids), as well as propalineal (longitudinal translation, as in rodents) translation of the jaws against one another (Reilly et al., 2001). Unlike mammals, stingrays have homodont dentitions; however, some species can reorient the teeth during feeding to a cusped occlusal surface, making them functionally heterodont (Ramsay and Wilga, 2007; Dean et al., 2008). Batoid fishes also exhibit unilateral, asymmetric muscle activity and asymmetric jaw action during lengthy prey-processing behaviors which strip invertebrate prey of their exoskeleton (Dean and Motta, 2004; Gerry et al., 2010). Loose jaw joints, transverse or longitudinal translation of the lower jaw, asymmetrical (unilateral) jaw activity, and heterodonty are all chewing-associated traits shared with mammals (Herring, 1993). Stingrays

then offer an interesting example of convergence in function with other masticating vertebrates, and are an apt model system for comparisons with other vertebrates.

Here, I analyze both prey capture and processing in the Neotropical freshwater stingray *Potamotrygon motoro* (Müller & Henle, 1841). This species is a member of a lineage of stingrays (20+ species) that likely invaded South American freshwaters during the Miocene (Lovejoy et al., 2006) and diversified across a range of dietary niches. I selected P. *motoro* as a model because it represents one of a few chondrichthyan species whose diet includes insects. Several *Potamotrygon*id stingray species, including P. *motoro*, feed on chironomid, orthopteran, dipteran, and odonate larvae (Lonardoni et al., 2006; Silva and Uieda, 2007; Almeida et al., 2010; Shibuya et al., 2009). Chitin, a main component of insect cuticle, provides considerable toughness (fracture resistance) and strength (elastic energy storage) (Vincent and Wegst, 2004). For these reasons, insect cuticle generally survives digestion through the gut of many insectivores, albeit in pieces. It is possible that the challenges of insect-feeding in the freshwater stingrays prompted the evolution of mastication in this clade, providing an interesting parallel with early mammals, many of which also fed on insects (Mills, 1972).

I used high speed videography to investigate feeding behavior by *Potamotrygon motoro* on different prey types. Our primary objective was to test whether this species uses chewing to process prey, as assessed by asymmetric motions of the jaws that shear and compress food between occlusal surfaces. I predicted that, across a range of prey types, chewing motions would be more exaggerated for more chitinous food items (insects and crustaceans). I also tested the hypothesis that P. *motoro* dissociates prey capture and processing by using the whole body (disk) to capture prey items, and the mouth and jaws for processing, as observed in two other batoid species (Wilga et al., 2012). Finally, as chewing is typically associated with heterodonty, I determined whether P. *motoro* are capable of reorienting their teeth, to produce a functionally heterodont condition.

#### 3.3 Methods

#### 3.3.1 High-Speed Videography

*Potamotrygon motoro* (n = 4) were purchased from aquarium wholesalers and kept in 284 L aquaria with sand bottoms. All animals were fed a diet of processed seafood mixes and

commercial elasmobranch foods. Rays were kept at a 12/12 hr day/night cycle. Food was buried in the sand to promote natural foraging. I chose three experimental prey items: whole silversides (fishes), Palaemonetes shrimps, and Libellula and Aeshna genera odonate larvae. These three prey types span the diversity, in taxonomic and material terms, of P. *motoro* prey in the wild. In general, calcified chitin (crustaceans) is stiffer and less tough than insect chitin, which without mineralization can deform more freely during compression or tension (Vincent and Wegst, 2004). For feeding trials, rays were introduced into the filming aquarium and allowed to acclimate in water from their home aquarium for at minimum two hours, with food being withheld for a prior 24 hr period. Prey items were weighed prior to being placed in the feeding aquarium.

Stingrays were filmed in a custom 290 L clear acrylic aquarium which allowed viewing from all sides and the bottom. Rays were filmed feeding either at 250 frames/sec for most prey-capture behaviors or, if processing behaviors were particularly lengthy, 150 frames/sec in order to not exceed the integrated memory of the camera system. High-speed cameras (model SPR-I, High Speed Imaging, Inc. AOS Technologies AG, Baden Daettwil, Switzerland) were placed at orthogonal angles to the tank in order capture ventral, lateral, and frontal views simultaneously. High intensity lights mounted on a retractable gantry could be raised/lowered to provide lighting. Video data were streamed via Ethernet to AOS Imaging Studio software (AOS Technologies AG, Baden Daettwil, Switzerland), cropped to only the pertinent behaviors, and saved as .RAW files. These files were later converted to .AVI formats and read into ImageJ (ImageJ version 1.40, National Institute of Health, Bethesda, MD) as an iterated .TIFF stack for kinematic analysis. A prey capture event was considered successful if prey was captured, processed, and then ingested.

#### 3.3.2 Feeding kinematics

To quantify asymmetrical jaw action, I examined the angular deviation of the jaws during feeding relative to a resting state (when the rays were not feeding) (Figure 3.1). This metric specifically quantified to what degree jaw protrusion during biting was asymmetrical (i.e. unilateral or restricted to only one side of the jaw). This method was also used to quantify the degree to which the medial symphysis of the lower jaw was flexed and then extended during feeding, which shears prey held in place by the opposing lateral rami of the jaw (Gerry et al.,

2010). In either case, prey is pinched and held in place by one side of the jaw, and either sheared against the opposing upper or lower jaw or pulled in tension when the symphysis is extended. Both cases allow for either propalineal translation of the upper and lower jaws against each other as well as transverse shearing of opposing left and right rami of the jaws.

Prey processing is a complex routine and the distinction between capture and processing is difficult to pinpoint, but processing typically occurs when prey is ingested (moves beyond the gape into the pharynx). I observed that rays rarely swallowed insect prey immediately after ingestion, and instead spat prey back out of the mouth, then re-ingested it before maceration with the jaws. Thus, the second biting event was designated as the beginning of the prey-processing sequence for analyses, the start of prey winnowing. To determine whether feeding on insect prey required longer prey-handling times and more frequent biting, I tallied the number of bites as well as the duration of prey handling for processing events for 15 feeding events per prey type, and calculated the bite rate as the number of bites divided by the duration of prey-handling.

For determining whether asymmetrical kinetic action of the jaws was more frequent when rays fed on chitinous prey, I analyzed the first fifteen bites from a processing cycle. I note that in addition to obvious biting, many more rapid and non-stereotyped motions of the jaws were observed, suggesting that prey-processing also occurs deeper in the pharyngeal cavity. During these quick bites, the magnitude of jaw protrusion and gape size were limited, but considerable asymmetrical jaw motion (symphyseal flexion and asymmetrical jaw protrusion; Figure 3.1) was observed. These motions presumably reflect internal processing events that cannot be recorded with these methods. Beginning with the onset of lower jaw depression and finishing at time of upper jaw retraction (which proceeded after jaw closure, i.e. the actual "bite"), I measured the maximum deviations for symphyseal flexion and angle of jaw protrusion from a resting state. For prey capture, the onset of rostral lifting was chosen as time zero with the rest of the kinematic variables examined occurring relative to this moment. Peak rostral lifting occurred when the anterior edge of the disk, closest to the prey reached peak height.

#### 3.3.3 Statistical analyses

Mixed models were used in order to account for multiple recordings from single individuals in our dataset, essentially a One-Way ANOVA with Fixed and Random Effects. Linear mixed

models (LMM) account for individual variation as a random effect as long as data follow a normal distribution. The R package lme4 was used to generate LMM on our capture dataset with "Individual" as our random effect, and uses maximum likelihood to estimate the parameters of our equation model. Prey type and prey mass were treated as co-varying explanatory variables against separately-analyzed kinematic response variables. To determine which distribution best fit our data, I visualized each variable using the MASS package in R while simulating a normal, log-normal, Poisson, gamma, and negative binomial distribution. In the case of non-normal variables, I used generalized mixed models (GLMM) with the corresponding coefficient modifier to fit the data. Analysis of Deviance (Type II Wald chi-square tests) were used to generate pvalues for LMM and GLMM results, coupled with Tukey tests for finding significant differences between specific variables, represented as the mean and standard error (s.e.m.) in the Results.

Since prey are comminuted over time and predator feeding behavior reflects this change in prey integrity, analysis of how aspects of feeding kinematics change over time are essentially autocorrelated, with measurements closer in time expected to resemble one another more than measurements taken farther apart in time. In order to examine how asymmetrical jaw action varied between prey types generally, as well as how patterns of asymmetrical kinesis change over time when feeding on different prey, we used time-explicit growth models using a linear mixed model framework. I contrasted increasingly complex models accounting for individual variability, auto-correlated error structure, slope and intercept variability and used the Akaike Information Criterion (AIC) to determine the models which best fit our data for measurements of symphyseal flexion and asymmetrical protrusion, separately. As with regular LMMs, Analysis of Deviance (Type II Wald chi-square tests) were used to generate p-values, coupled with Tukey tests for finding significant differences between specific variables (Bliese, 2013). All statistical analyses were performed in R (version 2.15.0, www.theRproject.org).

#### 3.3.4 Computed tomography scanning

In order to examine tooth morphology, and whether teeth reoriented during jaw protrusion, I used one of the experimental animals (P. *motoro*) from filming trials in computed tomography (CT) scanning. For comparison, I also examined a specimen of *Potamotrygon* orbignyi (Castelnau, 1855), a congeneric insectivorous freshwater stingray species. For both specimens, the oral cavity was filled with flexible hobby foam until the jaws attained a protruded state.

Specimens were scanned with a Bruker Skyscan 1173 at the Karel F. Liem Bioimaging Center at Friday Harbor Labs at 60 kV and 100  $\mu$ A and a voxel resolution of 35.5  $\mu$ m. Specimens had their pectoral fins removed in order to fit the specimens within the CT scanner, and wrapped in alcohol saturated cheesecloth in large Ziploc<sup>®</sup> bags. The images were reconstructed and visualized with Amira (v. 5.0, Mercury Computer Systems, Inc., USA).

#### 3.4 Results

#### 3.4.1 Prey processing kinematics

I observed asymmetrical jaw action in all prey processing trials as evidenced by consistent symphyseal flexion and asymmetrical jaw protrusion across all prey types (Tables 3.1 and 3.2). During each bite, some asymmetrical jaw action (symphyseal flexion and/or asymmetrical protrusion) is observed. Prey-handling, including degree of asymmetric protrusion and symphyseal flexion differed between insect prey and less-tough prey like fish and shrimp. Handling durations were, on average, at least twice as long for insects (18.6  $\pm$  1.76; mean  $\pm$ s.e.m.) compared to other prey (shrimp,  $9.19 \pm 0.12$  s.e.; fish,  $5.84 \pm 0.71$  s.e.; Table 3.1). Linear mixed model results show that the duration of prey-processing differed significantly among prey types (p < 0.0001), regardless of prey mass (p = 0.241). LMM results also showed handling duration times for insect prey to be significantly different from other prey types (Insects | Fish, p < 0.0001; Insects | Shrimp, p < 0.0001), while fish and shrimp prey did not differ from one another (p = 0.084) (Table 3.1). Insect prey incurred twice as many bites on average as shrimp prey (insects,  $47.13 \pm 4.21$  s.e.; shrimp,  $22.40 \pm 3.07$  s.e.), and greater than three times the number of bites compared to feeding on fish (fish,  $13.70 \pm 1.32$  s.e.). The number of bites during a prey-handling bout also significantly differed among prey types (p < 0.0001), but did not differ significantly with prey mass (p = 0.241) according to LMM analyses. LMM results confirmed that the number of bites during feeding on insects was significantly different from other prey types (Insects | Fish, p < 0.0001; Insects | Shrimp, p < 0.0001), while fish and shrimp prey did not differ from one another (p = 0.241) (Table 3.1). Finally, bite frequency did not differ significantly among prey types (p = 0.46) or according to prey mass (p = 0.39) (Table 3.1) and averaged about 2.5 Hz ( $\pm 0.08$  s.e.) across prey types.

Time-explicit growth modeling revealed similar trends as general LMM results for the effect of prey type on asymmetrical jaw protrusion during prey processing (Figure 3.2; Table 3.2). For

both asymmetrical jaw protrusion angle and symphyseal flexion, I found that models which incorporated an autoregressive structure with serial correlations incorporating how feeding variables changed over the course of the processing event were necessary to improve model fitting (AIC = 3792 vs. 3847). The random effect of individual variation explained approximately 0.9-6.0% of the variance in symphyseal flexion and asymmetrical jaw protrusion, respectively. Feeding bouts on insect prey were characterized by a greater significant (mean  $4.5^{\circ} \pm 1.06$  s.e.; p < 0.0001) asymmetrical angular deviation from other prey types, decreasing over the extent of the prey processing event (mean  $-0.3^{\circ} \pm 0.08$  s.e.; p < 0.0001). Effect of prey mass on asymmetrical protrusion was not found to vary significantly with time or prey type (p = 0.588). Flexion at the medial jaw symphyses was significantly greater (insects,  $3.3^{\circ} \pm 0.52$  s.e., p < 0.0001; shrimp,  $1.3^{\circ} \pm 0.53$  s.e., p = 0.01) than during prey processing on other prey, and the amount of flexion decreased over the extent of the prey processing event (mean  $-0.2^{\circ} \pm 0.04$  s.e.; p < 0.0001). As with asymmetrical protrusion, prey mass was not found to contribute to symphyseal flexion during prey processing (p = 0.72).

#### 3.4.2 Prey capture kinematics

*Potamotrygon motoro* captures prey with a rapid uplift of the anterior disk region, drawing prey beneath the body. Once prey is "corralled" beneath the disk, subsequent body repositioning maneuvers prey towards the mouth, assisted by pelvic fin "punting" (Macesic et al., 2013). After the disk was sealed against the substrate, prey was either sucked into the mouth by lower jaw depression or gripped by simultaneous lower jaw depression and upper jaw protrusion. Mouth closure occurred quickly (mean =  $0.09s \pm 0.02$  s.e.), before jaw protrusion concludes. Once mouth closure occurs, jaws are retracted and then almost immediately (< 0.01s) re-extended, opened, and closed again in a sequence which marks the beginning of prey-processing. Capture kinematics, such as jaw protrusion, disk-lifting, and jaw closure, were broadly comparable across prey categories; however, feeding events on larger prey items were associated with more failed capture attempts and involved repositioning of the body over the prey item. In all cases, across all prey types, I observed asymmetric, unilateral jaw motions during capture (Table 3.3).

Fundamentally, the kinematic behavior of the jaws differed between chitinous (shrimp, insect larvae) and non-chitinous (fish) prey. Most variables followed a normal distribution except for velocity of jaw protrusion (to peak protrusion), which followed a log-normal distribution. The

results of the LMM of angular deviation during jaw protrusion (angular protrusion) were significantly different among prey types ( $p = 5.96 \times 10^{-12}$ ), with chitinous prey eliciting greater jaw protrusion than fish (Insects | Fish, p = 1x10-04; Shrimp | Fish, p = 1x10-04), but not differing significantly from one another (Insects | Shrimp, p = 0.0587). The effect of prey mass on angular deviation during jaw protrusion was not significant (p = 0.055). The results of the LMM of jaw protrusion distance (protrusion distance) were also significantly different between prey types ( $p = 3.93 \times 10^{-5}$ ), with chitinous prey eliciting greater protrusion distances (Insects | Fish, p = 0.0031; Shrimp | Fish, p < 0.001), but not significantly from one another (Insects | Shrimp, p = 0.760). The effect of prey mass on jaw protrusion distance was not significant (p =(0.4838). The best-fitting LM models suggest that jaw protrusion velocity was also significantly different between prey types (p = 0.02805); however, Tukey results found only weak support for this, with feeding events on shrimp eliciting faster velocities than fish, though differences were not significant (Shrimp | Fish, p = 0.0525; Insects | Fish, p = 0.9968; Insects | Shrimp, p =(0.0659). The effect of prey mass on jaw protrusion velocity was not significant (p = 0.9968). LM model-fitting demonstrated that mouth closure velocity was significantly different between prey types (p = 0.01084), with insect prey eliciting faster closures than fish (Insects | Fish, p =(0.0102), but not significantly different than shrimp (Insects | Shrimp, p = 0.6145; Shrimp | Fish, p = 0.0718). The effect of prey mass on mouth closure velocity was not significant (p =0.11165).

#### 3.4.3 Tooth Reorientation

I did not observe noticeable reorientation in the teeth of *P. motoro* when the jaws were protruded and during symphyseal jaw flexion. However, in the related insectivore *Potamotrygon orbignyi*, I observed noticeable tooth reorientation during jaw protrusion. The average angle of tooth cusp reorientation in *P. orbignyi* varied from 8.5-28.1° (mean = 15.8°) from the resting angle (Figure 3.3).

#### 3.5 Discussion

While several definitions of chewing have been proposed (Moore and Sanson, 1995; Reilly et al., 2001; Evans and Sanson, 2005; Menegaz et al., 2015), I define chewing as an interaction of upper and lower teeth which both compresses and shears food between occlusal surfaces during asymmetric motion of the jaws. Although one of the most frequently cited hallmarks of

mammalian chewing is translational, opposing motion of the upper versus lower jaws, i.e. the 'power stroke,' some of the most successful mammalian chewers, rodents, lack a translational action of the jaw, relying instead on propalineal, longitudinal shearing instead (Reilly et al., 2001). When processing all types of prey, P. *motoro* stingrays show clear evidence of asymmetrical, transverse motion of the jaws, owing to loose mandibular symphyses and jaw joints, which allow the left and right rami of the jaws to move independently. Videos also appear to show longitudinal translation of the upper versus lower jaws, although confirming these kinematic sequences requires detailed imaging of internal anatomy, perhaps through x-ray videography. P. *motoro* uses asymmetric, unilateral movements of the jaws to successfully reduce tough insect cuticle during feeding, suggesting that stingrays and mammals have found a similar kinematic solution for feeding on tough prey. Loose jaw and symphyseal joints coupled with documented unilateral muscle activity (Gerry et al., 2010), allow for independent movement of the upper and lower jaws relative to one another in batoid fishes, a hallmark of mammalian-style chewing kinematics.

Another hallmark of mammal-like chewing is the use of high-crowned tribosphenic molars, which raises the question: does chewing really require complex teeth? Our results indicate that complex, asymmetrical jaw motions enable insect consumption by *Potamotrygon motoro*, despite the homodont dentition of this species. This contrasts with mammals, which rely on similar kinematic behaviors, but also have intricate tooth morphologies (Oron and Crompton, 1985; Freeman, 1972, 1992). However, P. *motoro* is a dietary generalist, and there is some evidence suggesting that larger individuals include insects in their diet, while smaller individuals eat more crustaceans and fishes (Almeida et al., 2010). I note that the congeneric and sympatric *Potamotrygon* orbignyi, includes more insects in its diet than P. *motoro* (Filho, 2006), and can reorient its teeth, making it facultatively heterodont. Thus, it may be that true or obligate insectivores require both complex kinematic jaw function as well as teeth with complex tooth shapes.

Our finding of chewing behaviour in freshwater stingrays adds this taxon to a list of other nonmammalian animals that use complex jaw kinematics to process complex prey (Reilly et al., 2001). Gerry et al. (2010) documented that both sharks and skates use asynchronous muscle motor patterns during prey processing. Skates in particular use unilateral jaw muscle activation and simple, piercing teeth to effectively 'pinch' prey on one side of the jaw, and use hydrodynamic jetting to dissemble these prey. In fact, there are many examples of vertebrates which feed on tough, stiff, or other manners of complex prey, which do so with relatively simple teeth and complex jaw kinematics, including tuataras and *Uromastix* lizards. Some herbivorous pleurodiran turtles and tortoises process food with an absence of teeth entirely (Gorniak et al., 1982; Reilly et al., 2001; Schumacher, 1973; Bramble, 1974). Translational motion alone, I suggest, is the key to shearing complex prey apart – perhaps mammalian jaws are simply too integrated a system to allow for the complex jaw motions seen in other vertebrate 'chewers,' hence the necessity for tribosphenic molars.

The decoupled nature of the cranial skeleton (euhyostyly) in skates and rays allows these fishes to independently position the jaws relative to one another, as well as the cranial skeleton (Gerry et al., 2010). A similar effect of decoupled feeding structures is evident in some teleost fishes, notably black carp and grass carp (Cyprinidae), which use the independent rami of the lower pharyngeal jaws to shear prey against upper pharyngeal dentition, and show complex asymmetrical muscle activation patterns (Gidmark et al., 2014, 2015). The anatomical traits and behaviors characterizing 'chewing' as a prey processing behavior are found in a broader context than just mammal, or even amniote systems. Stingrays have many of the hallmarks of chewing: loose jaw joints, medio-lateral movement of occluding tooth surfaces, asymmetrical jaw and muscle action, and at least in the insect-feeding specialist P. orbignyi, heterodont dentitions. But in the case of large generalist insectivores like P. *motoro*, as well as many reptiles and archosaurs, complex heterodont tooth morphologies are not required to process tough prey.

Batoid fishes represent much of the trophic ecological diversity within the elasmobranch fishes, perhaps facilitated by behavioral plasticity of the feeding apparatus (Dean et al., 2007; Aschliman et al., 2012; Jacobsen and Bennet, 2013). Stingrays spend a puzzling amount of time processing prey, during which they are conspicuous to predators and competitors in the wild. Gerry et al. (2010) documented that both sharks and skates behaviorally change prey processing behavior to suit complex or tough prey, as documented here for *Potamotrygon*. Greater overall jaw kinesis and longer prey-handling times occurred when *Potamotrygon* feed on chitinous prey, markedly greater for insects over shrimp, and for both kinds of chitinous, arthropod prey over fish. The rate of biting during processing remained constant regardless of prey, while asymmetrical action of the jaws and frequency of biting decreased over the duration of the feeding event. This suggests that P. *motoro* uses some stereotypy for feeding on complex prey,

biting at comparable rates regardless of prey, but changing the manner in which jaw action occludes against prey, as well as how long processing cycles continue. The decreasing extent to which these rays use abrupt, asymmetrical action of the jaws suggests that *Potamotrygon* modifies its behavior to reflect the progressive dismantling of prey during processing. This mechanical feedback allows predators to respond to nuances of prey material and structural toughness during feeding. This ability to modulate prey processing has allowed stingrays to access myriad trophic niches across numerous habitats, faced with novel prey like aquatic insect larvae. This plasticity of feeding behavior may explain why, despite entrenched competitors in novel habitats, freshwater rays were successful in making the transition from marine to aquatic environments (Thorson and Watson, 1975).

I determined that *Potamotrygon motoro* uses its appendicular skeleton (its disk) for prey capture and its jaws for prey processing, effectively dissociating the functional anatomy of these two processes. In other fishes, decoupling of prey capture from prey processing is a function of having two sets of jaws, oral and pharyngeal, of which the latter performs the majority of processing (Liem and Sanderson, 1986). In some fish lineages, decoupling of anatomical modules during feeding behavior has led to radical changes in the morphology of formerly integrated cranial modules, correlated with increased diversification in these lineages (Collar et al., 2014). Using the body as a method of prey restraint or to outright capture prey is prominent in other vertebrates outside fishes, e.g. predatory birds use talons to hold and suffocate prey, bats capture fish from rivers with their hindlimbs, and small and large mammals alike often grasp and rend prey using their forelimbs (Csermely et al., 1998; Siemers et al., 2001; Rasa, 1973). Although rare in bony fishes, prey capture using the pectoral fins occurs in other batoids, such as guitarfishes and skates (Wilga et al., 1998, 2012; Mulvany and Motta, 2014). We suggest that using the appendicular skeleton to trap prev is an innovation that was made possible by the evolution of the pectoral fins to encircle the front of the head, forming a flexible, flattened disk, and has evolved at least twice, independently in modern stingrays (Myliobatiformes) and skates (Rajiformes) (Aschliman et al., 2012). In turn, this innovation may have facilitated the evolution of extreme jaw kinesis and chewing behaviour in these stingrays. I suggest that asymmetrical jaw kinematics are a frequent motif of stingray, and more generally, batoid feeding – a function of their decoupled, loose jaws and cranial skeletal architecture. These properties establish

*Potamotrygon* as a model system for understanding evolutionary convergence of prey-processing and chewing in vertebrates.

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Model	Covariate	Tukey Results	Estimate s	Standard Error	t	р
Number of Bites	Prey Type	-	17.48	4.41	3.96	$1.53 \times 10^{-15}$
n = 15 / prey item		Fish   Insect	34.96	4.45	7.86	< 1x10 <sup>-4</sup>
		Shrimp   Fish	10.55	4.60	2.29	0.057
		Shrimp   Insect	-24.41	4.25	-5.74	< 1x10 <sup>-4</sup>
	Prey Mass	-	-16.75	14.30	-1.17	0.241
Duration of Prey-Handling	Prey Type	-	7.08	1.79	3.95	9.56x10 <sup>-</sup> 14
n = 15 / prey item		Fish   Insect	13.31	1.81	7.36	< 1x10 <sup>-4</sup>
		Shrimp   Fish	3.98	1.87	2.13	0.084
		Shrimp   Insect	-9.32	1.73	-5.40	< 1x10 <sup>-4</sup>
	Prey Mass	_	-5.55	5.81	-0.96	0.339
Bite Rate	Prey Type	-	2.57	0.19	13.2 6	0.457
n = 15 / prey item		Fish   Insect	0.23	0.20	1.16	0.480
		Shrimp   Fish	0.05	0.20	0.24	0.968
		Shrimp   Insect	-0.18	0.19	-0.94	0.612
	Prey Mass	-	-0.55	0.63	-0.87	0.386
Significance level ( $\alpha = 0.05$ ).						

## Table 3.1 Linear Mixed Model results of Prey-Processing Kinematics for Prey Type and Mass

(Kolmann et al. 2016)

Model	Covariate	Tukey Results	Value	Standard Error	t	р
Angular Jaw Protrusion	Prey Type	-	8.48	1.37	6.17	< 0.001
n = 15 / prey item		Fish   Insect	4.52	1.06	4.27	< 6.0x10 <sup>-5</sup>
		Shrimp   Fish	-0.09	1.08	-0.08	0.996
		Shrimp   Insect	-4.61	1.02	-4.53	< 1.6x10 <sup>-5</sup>
	Prey Mass	-	1.97	3.64	0.54	0.588
	Time	-	-0.39	0.08	-5.17	< 0.001
Symphyseal Flexion	Prey Type	-	4.33	0.49	8.77	< 0.001
n = 15 / prey item		Fish   Insect	3.34	0.52	6.46	< 0.001
		Shrimp   Fish	1.35	0.53	2.54	0.030
		Shrimp   Insect	-1.99	0.50	-4.02	< 0.001
	Prey Mass	-	-0.61	0.53	2.54	0.721
	Time	-	-0.21	0.04	-5.33	< 0.001
Significance level ( $\alpha = 0.05$ ).						

Table 3.2 Growth Model results for angular deviations of the jaws during prey-processing (Kolmann et al., 2016)



Figure 3.1 Functional morphology of asymmetrical jaw protrusion in *Potamotrygon motoro*. (A) Resting jaw and hyomandibular articulations; (B) asymmetrical protrusion of jaws relative to kinetics of angular cartilage and hyomandibular articulations, inset and pointer: photo of asymmetrical protrusion of live *Potamotrygon* while feeding on insect larvae; (C) medial flexion of mandibular (Meckelian) symphyses; (D) computed tomography scan of *Potamotrygon motoro* crania, inset: articulation of jaws to hyomandibular cartilage via dual angular cartilages. (from Kolmann et al., 2016)



Figure 3.2 Growth model showing declines in asymmetrical jaw activity during prey-processing across three prey types. Top panel is jaw protrusion angle, bottom panel is symphyseal flexion angle. (from Kolmann et al., 2016)



Figure 3.3 Computed tomography scans of: (A) *P. motoro* teeth, the animal analyzed in this study, an insect-feeding generalist predator and (B) *P. orbignyi*, an insect-feeding specialist. (from Kolmann et al., 2016)

## 4 Evolution of feeding specialization in Neotropical freshwater stingrays

#### 4.1 Abstract

The interplay between ecological and evolutionary forces drives morphological differentiation by mitigating competition, promoting niche specialization, and ultimately structuring macroecological communities. Although several studies have examined marine-freshwater transitions in fishes, as well as the ecomorphological novelty present in many of these taxa, little focus has been spent investigating whether marine-derived lineages are representative of adaptive radiations, despite the fact that habitat transitions promoting ecological diversification are a common theme in radiations. We investigated the macroevolutionary history of marinederived, South American river rays (Potamotrygonidae). The ancestral potamotrygonid lineage invaded freshwater some 40 mya and subsequently diversified taxonomically and ecologically, with several instances of piscivory, molluscivory, and insectivory (the only example within elasmobranchs) evolving within this clade. I present a time-calibrated, multi-gene phylogeny for Potamotrygonidae and examine evolutionary trends in diet specialization and ecomorphology. Potamotrygonid morphological disparity exhibited two patterns, showing an early burst around 40 mya with the majority of morphological disparity partitioned among subclades. There is a gradual shift to greater within-subclade disparity starting at 20.4 mya, with an abrupt spike in morphological disparity peaking around 7.6 mya. These findings correspond with a rate shift in lineage evolution for lower Amazon stingrays, and the occupation of novel adaptive peaks corresponding to dietary specialization. The first of these peaks corresponds with the evolution of fish-eating *Paratrygon* and *Heliotrygon*. More recent adaptive peaks correspond to the evolution of mollusk-feeding, insect-feeding, and crustacean-feeding specialist stingrays within Potamotrygon. These findings are not broadly consistent with an early-burst mode of morphological disparity common to other adaptive radiations. Although potamotrygonids exhibit an initial burst in morphological disparity, recent lineage and morphological diversification suggests that new niche exploration has occurred relatively recently in potamotrygonids.

Key words: adaptive radiation, ecomorphology, insectivory, disparity, Pebas Mega-Wetland
# 4.2 Introduction

Some degree of habitat transition is considered common or even necessary for the onset of an adaptive radiation (Schluter, 2000; Gavrilets & Losos, 2009). In Darwin's finches, for example, a mainland lineage arrived in the Galapagos and subsequently colonized and re-colonized various isles (Grant & Grant, 2008). These finches diversified to feed on a variety of seeds, with different species evolving more or less robust beaks according to the hardness of seeds they preferentially feed on. A similar situation explains the repeated evolution of multiple *Anolis* lizard "ecomorphs" on various Caribbean islands. Lizards living on different islands repeatedly adopted similar limb morphologies according to their shared substrate preferences (plant trunks, limbs, and branches) (Losos et al., 1997).

However, few studies have explicitly examined fishes that have diversified across the marinefreshwater threshold in the context of adaptive radiation, despite well-documented ecomorphological novelty in these clades (Weitzmann & Vari, 1988). Davis et al. (2012) tested differences in lineage diversification rates in terapontid fishes across a marine-freshwater transition, finding that freshwater clades had increased dietary breadth and faster rates of diversification. However, Bloom et al. (2013) found that although silverside fishes have higher speciation and extinction rates in freshwater, net lineage diversification was not significantly different between marine and freshwater clades. These studies did not closely examine morphological and ecological diversification in marine-derived lineages (MDLs).

Although habitat transitions can provide the ecological opportunity for diversification, the ordering of species across some resource gradient (e.g., microhabitat and seed hardness for *Anolis* and *Geospiza*, respectively) is mandatory for such a radiation to be termed "adaptive" (Gavrilets & Losos, 2009), with phenotypically-variable species partitioning resources according to their performance capacities. Also, adaptively radiating clades are expected to show both an early-burst pattern of morphological disparity concomitant with similar patterns in lineage accumulation (Simpson, 1953; Schluter, 2000; Pybus and Harvey, 2000). As niche space is filled, both lineage and morphological diversification should slow, as competition limits diversification (Simpson, 1953). This pattern has been seen recently in a number of radiations of new world cichlids, several clades of which show tight relationships between foraging mode, feeding morphology, and body shape changing commensurate with the generation of novel

adaptive peaks (Arbour and López-Fernández, 2013, 2014). In marine systems, patterns of putative adaptive radiations are less obvious, with some clades seemingly to show an early-burst pattern in their initial history, but with subsequent bursts in morphological diversification and lineage accumulation occurring more in line with subsequent changes in habitat (Near et al., 2012; Frédérich et al., 2013).

Evolutionary transitions of lineages from marine to freshwater habitats represent an interesting opportunity for investigating adaptive radiation and evolution. Davis et al. (2013, 2014) illustrated how terapontid fishes diversified across dietary niches upon the transition to freshwater, these fishes diversifying into herbivorous, detritivorous, and benthic invertebrate specialist niches from a generally carnivorous ancestor. Freshwater environments may offer novel resources (e.g. aquatic insect larvae) that are unavailable to marine species or may offer resources which are relatively free from competition. Indeed, Davis et al. (2013, 2014) found signals of adaptive radiation: an early-burst in both lineage accumulation and morphological diversity, followed by a gradual decline in lineage and ecological diversification. Similarly, potamotrygonid stingrays (Potamotrygonidae, Myliobatiformes), a South American MDL, appear to have diversified across an array of feeding niches, including piscivory, molluscivory, insectivory, and generalist invertebrate feeding (Shibuya et al., 2009, 2012; Moro et al., 2012). Potamotrygonids originated at least 12-38 mya (million years ago) when a marine ancestral lineage invaded an ancient lowland brackish lagoon in central South America (Lovejoy et al., 1998, 2006). The diversity of potamotrygonid feeding ecology is in stark contrast to the hypothesized marine sister group of potamotrygonids, the two species of amphi-American *Himantura* (Lovejoy 1996, Lovejoy et al., 1998), which are large, estuarine, generalist predators with a limited geographic range spanning either side of the Panamanian Isthmus.

In general, elasmobranchs (sharks and rays) are conspicuously absent from most freshwater ecosystems, which account for only 0.01% of the water on earth, yet harbor over 41% of all fish species (Horn, 1972). Although the fossil record shows several incidences of elasmobranchs diversifying in freshwater environments, only the potamotrygonid stingrays have diversified entirely in freshwater, while other freshwater rays represent independent invasions of freshwater by single species. Potamotrygonids currently number approximately 30 species in four genera and although they are not the only instance of freshwater elasmobranchs, they display the highest species richness of any extant, monophyletic lineage of freshwater elasmobranchs (Thorson &

Watson, 1975; Carvalho & Lovejoy, 2011). Stingrays account for over half the diversity of elasmobranch fishes, but potamotrygonids may be unique in the amount of dietary, morphological, and coloration diversity they exhibit relative to their species diversity. These rays, when compared to their closest marine relatives, represent an abrupt ecological departure from phylogenetic conservatism. For example, outside potamotrygonids there are very few if any exclusively molluscivorous dasyatoid rays. There are also insectivorous potamotrygonids, but this strategy does not appear in other elasmobranchs. These patterns are impressive, considering that the species richness of the dasyatoid rays excluding Potamotrygonidae (Urotrygonidae, Dasyatidae, Urolophidae, Plesiobatidae) is four times greater than that of potamotrygonids. Potamotrygonids, given their dietary novelty, evidence of habitat transition, and high species richness relative to their marine counterparts, offer a compelling system for the study of adaptive evolution in elasmobranch fishes.

Here, I propose that transitions from marine to freshwater habitats may have spurred both lineage and ecological diversification in potamotrygonids, commensurate with exposure of these lineages to new prey options. Since foraging is time-consuming as well as costing resources in terms of energy expenditure, study of feeding behavior in an adaptive context is particularly relevant to ecological models of diversification (Wainwright et al., 2004). Most studies of adaptive radiation deal with some aspect of feeding morphology matching a gradient in prey resources, but do potamotrygonids follow a similar pattern? Critical to investigations of adaptive radiation is establishing an ecomorphological, functional connection between traits and dietary ecology. While there has been much research regarding the novelty and dynamism of chondrichthyan feeding functional morphology, no studies have attempted to place this research within a larger, explicit evolutionary framework.

To reconstruct the directionality and order of dietary mode and corresponding morphological transitions in potamotrygonids, a robust phylogeny is required. Such a phylogeny would also allow a test of whether or not potamotrygonids show an early-burst pattern of morphological and lineage diversification, as predicted for adaptive radiations. To date, phylogenies of potamotrygonids have been limited by reliance on single genes (Lovejoy et al., 2006; Carvalho and Lovejoy, 2011) or limited sets of genes (mitochondria; Toffoli et al., 2008; Garcia et al. 2015), limited taxon sampling, or issues with alpha taxonomy. Also, the timescale of

potamotrygonid diversification has not been well-established using molecular phylogenetic approaches.

Here, I use potamotrygonid stingrays as a model system for exploring evolutionary diversification of morphology and diet, and attempt to determine whether or not this clade exhibits some of the characteristics of an adaptive radiation. I address the following main objectives: (1) I generate a robust, time-calibrated species-level molecular phylogeny for Potamotrygonidae. (2) I use this tree to determine patterns of ecomorphological diversification in potamotrygonids, and test whether potamotrygonids show characteristics of adaptive radiation (early-burst pattern of ecomorphological diversification, high early lineage accumulation, followed by a slowdown as niches are filled). (3) I determine whether shifts in rates of lineage evolution correlate with shifts in diet, and whether adaptive peaks correspond to distinct dietary types, such as insectivory, piscivory, and molluscivory.

# 4.3 Methods

### 4.3.1 Taxon Sampling, DNA extraction, amplification, and sequencing

Specimens were collected using gillnets, seines, dip-nets, and cast-nets, by various collectors from multiple museums. Specimens obtained personally by the authors are done so under the auspices of UACC protocol (# 2000). Muscle or fin tissue was stored in 95% ethanol. My dataset is the most complete taxonomic sampling of potamotrygonid stingrays to date, including 21 of 28 described species from all four of the currently described genera, including multiple representatives of some polymorphic and widely-distributed species (*Paratrygon aiereba* and *Potamotrygon motoro*), as well as two undescribed species, *Potamotrygon* sp. 'reticulata' from Colombia and *Potamotrygon* sp. 'demerarae' from Guyana. For outgroups, I included seven species representing the immediate relatives of Potamotrygon kuhlii, *Himantura schmardae and H. pacifica, Urotrygon simulatrix,* and *Urobatis halleri*), including previously published sequence data for more distantly-related taxa (see Table 4.1).

Whole genomic DNA was extracted using the DNeasy spin column tissue kit (Qiagen Inc., Valencia, CA, USA). We collected DNA sequence fragments from three mitochondrial loci (co1, cytochrome oxidase I; cytb, cytochrome b; ATP6, ATPase subunit 6), using novel and published

primer sequences (cytb; Aschliman et al., 2012) primers. In addition, I sequenced four nuclear loci (RAG1, Recombination activating gene 1; SCFD2, Sec1 Family Domain Containing 2; ENC1, Ectodermal-Neural Cortex 1; ITS1, internal-transcribed space 1 and 2) using published primers for RAG1 and SCFD2 (Aschliman et al., 2011) as well as novel primers for ENC1 and for ribosomal ITS1. The PCR products for all genes were purified using USB ExoSAP-IT PCR purification kit (Affymetrix Ltd., Santa Clara, CA). Primers for ENC1, ITS 1 and 2 and sequencing primers for SCFD2, developed for this study were as follows: ENC1-FN1 (5' GGAATGTCTGC TTTTGGAA 3'), ENC1-FN1 (5' CAACCATCAGCAAGAGTGAAGA 3'), ENC1-seqN1 (5' CCCAAGGAGGAGCAAGGCATGTCCAG 3'), ITS1-F/ CAS18SF1 (5' TACACACCGCCCGTCGCTACTA 3'), ITS1-R/ POT5.8SR1 (5' CTAGCTGCGTTCTTAATCGACG 3'), ITS1-R/ POT5.8SR2 (5'

GTCGATGATCAATGTGTCCTGC 3') and SCFD2-Seq1 (5'

CTGAGAGCTTACACTGGGTCG 3'). PCR for all genes were performed in 25 µL volumes, which included a 2.5 µL of KCl/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> mixture PCR buffer, 2.5 µL MgCl<sub>2</sub>, 2.0 µL dNTPs (10mM), 1.25 µL of each primer (10mM), 0.5 µL of Tag polymerase, 1-4 µL genomic DNA, with the remaining volume of H<sub>2</sub>O. Touchdown PCR thermocycler conditions for ATP6 were 94 °C for 5 min, followed by 15 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min to a final sustained annealing temperature of 55 °C for 30 s, for another 15 cycles, followed with a final extension of 72 °C for 7 min. PCR thermocycler conditions for cytb & CO1 were 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 48 °C for 30 s, 72 °C for 1 min and a final extension of 72 °C for 7 min. Touchdown PCR thermocycler conditions for RAG1 were 95 °C for 5 min, followed by 20 cycles of 95 °C for 30 s, 54 °C for 1 min, 72 °C for 1.5 min to a final sustained annealing temperature of 50 °C for 1 min, for another 20 cycles, followed with a final extension of 72 °C for 7 min. PCR thermocycler conditions for ITS1 2 were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1.5 min and a final extension of 72 °C for 7 min. PCR thermocycler conditions for ENC1 were 95 °C for 4 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 1 min, 72 °C for 1.5 min and a final extension of 72 °C for 5 min. PCR thermocycler conditions for SCFD2 were 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 1 min, 72 °C for 1.5 min and a final extension of 72 °C for 5 min. Samples were sequenced at the SickKids Centre for Applied Genomics, Toronto, Canada.

#### 4.3.2 Alignment and Analysis

Forward and reverse sequences were used to construct de novo consensus sequences, which were then edited by trimming the distal ends of ambiguous base-pair (bp) calls in GENEIOUS v6 (Kearse et al. 2012). The resulting sequences were aligned in GENEIOUS using the MUSCLE plugin and protein-coding genes were translated to amino acids to confirm an open reading frame. Aligned sequences were then used to generate the following datasets: (1) the three mitochondrial genes combined, (2) RAG1, (3) ENC1, (4) SCFD2, and (5) ITS1, concatenated to form a single matrix of 8270 bp. This final matrix includes data for more than 75% of all described potamotrygonid taxa and comprises data for 37 taxa in total. I used PartitionFinder to determine the best-fit model of evolution and partition schema simultaneously for each gene and possible reading frame. Models were selected using a Bayesian Information Criterion (BIC), using a 'greedy' search scheme (Lanfear et al., 2012). Partitioned maximum likelihood (ML) tree searches were performed with GTR+G models for each partition using the program RAXML (Stamatakis, 2006) to create our gene trees. ML bootstrap estimates were based on 1000 replicates using the rapid boot-strapping algorithm in RAXML. For our consensus Bayesian analysis, I used the gene partition recommendations from PartitionFinder: for ATP6, RAG1, and cytb, the GTR+ gamma; for CO1, the GTR+ I+ gamma; for ENC1, the TrNef + I; for ITS1, the HKY+ I+ gamma; and for SCFD2, the K80+ gamma.

For the consensus phylogeny used in our final analyses, I used BEAST (v. 1.8.3; Drummond and Rambaut, 2007) to simultaneously estimate the phylogeny and diversification times of potamotrygonids using a Bayesian framework. I partitioned my data according to gene, with unlinked parameters and default priors. I used an uncorrelated lognormal tree prior and a pure birth-death prior for our expectation of cladogenesis. I ran two separate BEAST analyses for 100 million generations, sampling every 5000 generations, and automatically discarding the first 10% of trees as burn-in. I used Tracer 1.6 (Drummond and Rambaut, 2007) to assess convergence and mixing of runs and to verify that effective sample sizes (ESS) were >200 for all parameters. An additional first 20 million generations from each run were discarded as burn-in. To determine calibrated, dated divergence times I used two fossil and two geological time calibrations (see Appendix 1 for details), corresponding to: (1) the earliest dasyatoid fossil (~51.0 mya), (2) the earliest potamotrygonid fossil (~41.0 mya), (3) the separation of modern potamotrygonid taxa spanning the Rio Magdalena/Rio Atrato and Orinoco-Maracaibo basins (~12.0 mya), and (4) the

separation of amphi-American *Himantura* by the closure of the Isthmus of Panama (mean ~7.0 mya). Specifically:

*†Dasyatis muricata* (Carvalho & Maisey, 2012), from the Eocene of Italy – as the most recent common ancestor (MRCA) for Dasyatoidea; with an exponential prior set to a mean of 1.9, st dev = 1.0; offset = 50.0 mya, median of 71.7 mya; 95% quantile interval of (65.9, 112.5) mya.

*†Potamotrygon ucayaliensis* (Adnet et al., 2014), from the Miocene of Uruguay – as the most recent common ancestor (MRCA) for the amphi-American *Himantura* + Potamotrygonidae; with an exponential prior set to a mean of 1.9, st dev = 1.5; offset = 41.0 mya, median of 39.5 mya; 95% quantile interval of (38.0, 54.0) mya.

Separation between *Potamotrygon yepezi* from *P. magdalenae* and *P.* 'reticulata' by the Cordillera Oriental Mountains of Colombia, Andean satellite ranges to the north. This node was calibrated with an exponential prior set to a mean of 2.0, st dev = 1.0; offset = 10 mya, median of 13.0 mya; 95% quantile interval of (10.1, 15.0) mya.

Separation of the amphi-American *Himantura* species (*H. pacifica* and *H. schmardae*) by the coalescence of the Isthmus of Panama and greater Central America. This node used a normal prior to represent the ambiguity regarding the particular date for final separation of the Caribbean from the Pacific, with a set mean of 2.0, st dev = 1.0; offset = 10.0, median of 5.0 mya; 95% quantile interval of (3.3, 10.1) mya.

#### 4.3.3 Phylogenetic analysis of diet and feeding biomechanics

Diet data was obtained from the literature and other sources (Table 4.2). Species were considered specialists if >70% of the diet was reported as being a particular prey item, or if no other prey items were listed as part of the diet. If diet data was not available, or if the grey literature or personal communications were used to assess diet (17% of diet data), species were conservatively labeled as dietary generalists. Diet categories were coded as a discrete multistate character, and I used the Phytools package (Revell, 2012) to perform maximum likelihood reconstructions of ancestral states in Potamotrygonidae + amphi-American *Himantura*. Transitions between states were considered as being equally-likely.

To identify the major axes of morphological variation in biomechanical attributes across taxa and to characterize species along ecomorphological axes (diet), I used a phylogenetically-informed Principle Components Analysis (phyPCA; phyl.pca function in the phytools package; Revell, 2011). Morphological and functional traits were measured from computed tomography, or x-ray scanning of museum specimens (ANSP, ROM, MCZ, USP, and CUMV; Table 4.1) spanning the Potamotrygonidae as well as several dasyatoid outgroups (Dasyatidae, Urolophidae, and Urotygonidae). Functional characters were chosen based on demonstrable covariation with feeding performance or behavior (Huber et al., 2005; Dean et al., 2007; Anderson et al., 2009; Arbour and Lopez-Fernandez, 2013; Balaban et al., 2015). The following morphological variables were measured: (a) mechanical advantage (MA - leverage), (b) maximum jaw depth (upper and lower), (c) jaw aspect ratio (upper and lower), (d) symphyseal height (lower jaw), (d) occlusional offset (cm – measure of jaw closing tooth occlusion), (e) lower dental row length, (f) hyomandibular offset (cm – linear measure of jaw protrusion), (e) jaw adductor fossa length (cm), (f) gape width (cm), (g) jaw-hyomandibulae disarticulation (a function of joint range of motion), (h) hyomandibular cartilage aspect ratio, and (i) propterygia aspect ratio (rigidity of the skeletal elements which anchor the pectoral fins). Morphological measurements were scaled relative to head length to standardize for different unit comparisons and thus adjusting for body size.

Description of morphological measures as follows: mechanical advantage is the ratio of the inlever (muscle insertion to jaw joint) to out-lever (bite point to jaw joint) distances and measures proportional force transmittance (leverage) across a structure. High MA represent highefficiency force propagation, low MA suggests higher effective velocities during rotation (mouth closing). Maximum jaw depth, jaw aspect ratios, and symphyseal heights approximate the second moment of area (I) for a beam-like structure; this measure represents the ability of a structure to resist bending (Anderson et al., 2009). Taller jaws and symphyses represent those which are less apt to deform during biting (Anderson et al., 2009). Occlusional offset and hyomandibular offset are linear representations of angular rotations. Occlusional offset is measured by measuring a line drawn tangent from the jaw joint to the plane in which the tooth surface (occlusal plane) resides (for both jaws) (Anderson et al., 2009). Jaw occlusion either has teeth occluding directly, or with a lateral, scissoring action – the former for applying compressive forces, the latter for applying shear to prey. Hyomandibular offset ratio is a linear representation of the angle between the occlusal plane and the hyomandibular at rest and at full extension. The jaw-hyoid gap is a measure of the distance between the proximal ends of the hyomandibular cartilage to the jaw joint. Both hyomandibular offset and jaw-hyoid gap indicate a jaw suspensory apparatus kinesis potential, larger gaps and greater offsets allow the jaws to have higher range of motion (Dean et al., 2007). This measure accounts for ease of comparison between those species with and without an angular cartilage (Dean et al., 2007). Hyomandibular dimensions and aspect ratio portray the relative gracility or robustness of the hyomandibular cartilages; their resistance to bending. Thicker, stouter hyomandibulae are useful for generating oral suction (Balaban et al., 2015). Longer, more gracile hyomandibulae allow for greater jaw kinesis. Gape width is the proportion of the occlusal surface covered by teeth. Adductor fossa length measures the length of muscle attachment area, of the two primary jaw adductors, the adductor mandibulae lateralis and major. Max gape is the lateral distance between the jaw joints, and is predictive of the size of prey consumed (particularly for piscivores). Finally, many batoid fishes capture prey by rapidly raising the pectoral disk, which generates negative pressure under the body and sucks prey beneath (Wilga et al., 2012). The pectoral propterygia support the anterior pectoral fin region, their aspect ratio again gives an idea of the rigidity of the structure and its resistance to bending. Stouter, more robust propterygia will allow for greater muscle attachment area and therefore, greater suction generation.

#### 4.3.4 Species accumulation and disparity-through-time

I expect that if potamotrygonids represent an adaptive radiation, their history should show rapid initial lineage diversification, followed by a slowdown as lineage accumulation reaches equilibrium, the result of niche space filling towards capacity. I used two methods to examine lineage accumulation and diversification rates. To identify whether or not diversification rates have shifted across the potamotrygonid tree, I used MEDUSA, which combines dated tips with relative taxon richness values to account for missing data (i.e. incomplete taxon sampling) across a diversity tree (Alfaro et al., 2009). This method fits various 'breaks' across which evolutionary rates can vary across the diversity tree, comparing among increasingly complex, fitted models of evolution. In order to determine whether lineage accumulation follows different evolutionary patterns (niche-filling, exponential, or linear), I fit constant-rate pure-birth models, birth-death models with fixed and variable rates, exponential (DDX) and logistic decline (DDL) models of lineage accumulation and analyzed their fit to the data using corrected Akaike Information Criterion (AICc) values in the LASER package (Rabosky, 2006). The gamma statistic was also used to determine how cladogenesis might deviate from a constant-rate process (Pybus and Harvey, 2000).

Adaptively radiating clades are also expected to show an initial burst of morphological disparity, rather than a constant increase in overall disparity (Slater et al., 2010). I examined ecomorphological disparity-through-time (DTT) plots generated from our cranial morphology data. I compared cranial anatomical disparity across our time-calibrated tree to a simulated null disparity dataset, using a Brownian motion model of morphological disparity iterated over 10,000 generations. I calculated the morphological disparity index (MDI) as the difference between the simulated Brownian curve and our observed clade disparity and plotted this relative to node age using the 'dtt' function in Geiger (Harmon and Slater). MDI captures the overall difference in relative clade disparity (Harmon et al. 2003), with negative MDI values suggesting lower subclade disparity, a frequently-cited component of early adaptive radiations. If lineage accumulation and morphological disparity increase and then plateau, this would suggest that ecological diversity within Potamotrygonidae has resulting from adaptive radiation.

Finally, I measured the extent of adaptive peak shifts and convergence following Ingram & Mahler (2013), using the SURFACE package in R. SURFACE fits Hansen models with alternative selective regimes in a stepwise fashion, comparing the fit of these models using AICc. The procedure fits adaptive peaks iteratively to the phylogeny, i.e. the 'forward' run followed by a 'backyard' phase which collapses weakly-supported peaks, according to whether the fit of Hansen OU (Ornstein-Uhlenbeck) models was improved or not. Since computations in SURFACE can stall on initially-favorable model conditions, the search function was extended to randomly sample all peaks with delta AICc values < 2, with the 'sample\_shifts' option set to TRUE so the best-fitting peaks were compared at every step of both the forward and backward iterative processes (Ingram and Mahler, 2013; Arbour and López-Fernández, 2014).

## 4.4 Results

# 4.4.1 Molecular data, phylogenetic relationships, and ancestral reconstruction of diet

The total molecular dataset resulted in a concatenated matrix of 8270 bp. This final matrix includes data for more than 75% of all possible tip taxa, and comprises data from 36 species for

ATP6 (955 bp), 37 species for cytb (756 bp), 36 species for co1 (707 bp), 35 species for RAG1 (953 bp), 32 species for ENC1 (548 bp), 22 species for SCFD2 (663 bp), and 28 species for ITS (1154 bp). BEAST was run twice with identical results recovered from each run; the resulting tree is shown in Figure 4.1. Gene trees showed considerable incongruence between gene histories, but generally reconstructed Potamotrygonidae as monophyletic and sister to the amphi-American Himantura, as illustrated in the Appendix as Supplemental Figures 1a-1f. The Paratrygon + Heliotrygon clade was typically recovered intact, while Potamotrygon + Plesiotrygon relationships changed appreciably with each gene. Coalescent analyses supported the monophyly of the family Potamotrygonidae with the amphi-American Himantura, H. pacifica and H. schmardae, recovered as the family's nearest sister taxon. I also recovered separation of the family Potamotrygonidae into two major clades: *Plesiotrygon + Potamotrygon* and *Heliotrygon* + *Paratrygon*, Potamotrygon is paraphyletic because of the nested position of Plesiotrygon, with Potamotrygon brachyura diverging before the split of the remaining Potamotrygon from Plesiotrygon. Potamotrygon from the Guiana Shield generally formed a clade with Orinoco and Magdalena taxa. According to our ancestral state reconstruction, clade inclusion was not predictive of dietary guild. Rather, novel dietary strategies have arisen throughout the tree, typically from generalist (mostly crustacean-feeding) ancestors (Figure 4.2). Insectivorous taxa entirely comprised this clade of northern potamotrygonids. A number of endemic taxa restricted to the lower Amazon, as well as widespread taxa (P. motoro, P. orbignyi) from the Xingu, Tocantins, and headwater drainages of Peru, form another clade.

According to our ancestral state reconstruction, novel dietary strategies have arisen independently throughout the tree, typically from generalist (omnivorous) ancestors (Figure 4.2). Insectivorous taxa have arisen at least four times, once in *Plesiotrygon nana*, although this is supported by anecdotal evidence (Lucanus, pers comm; Table 4.2), again in the entire lineage composed of *P. magdalenae*, *P. yepezi*, and *P.* 'reticulata,' again in *Potamotrygon signata*, and a fourth time in *Potamotrygon orbignyi*. Other feeding modes, like piscivory, appear to have only arisen once (the base of the *Paratrygon* + *Heliotrygon* clade), in molluscivores (*Potamotrygon leopoldi* and *P. henlei*) as well as in crustacean-specialists (*Potamotrygon scobina*, *Potamotrygon motoro*). Interestingly though, these last two transitions are notable in that while other potamotrygonid dietary novelties arise from generalist ancestors, molluscivores arise exclusively from crustacean-feeding specialists, as does the latest iteration of insectivory in *P*. *orbignyi*.

#### 4.4.2 Feeding morphospace

The first three axes of the phyPCA represent 37.6%, 18.0%, 16.5% of the variance in the data. Species which loaded on the positive end of PC1 had larger gapes, robust jaws (thicker symphyses and lower aspect ratios), high jaw mechanical advantages, and greater ability to lift the pectoral disk (stouter pectoral propterygia) (Figure 4.3a). Species on the negative end of PC1 had increased jaw protrusion ability (long hyomandibulae) and greater jaw kinesis (longer angular cartilages). Rays which loaded positively on PC2 had higher anterior jaw leverages, larger gapes, scissoring jaw action, greater disk lifting ability, and increased jaw protrusion. Species on the negative end of PC2 had higher posterior mechanical advantages, larger jaw muscle attachment areas (adductor fossa length), more robust jaws, more robust hyomandibulae, and more rigid jaw symphyses (Figure 4.3a). Rays which loaded positively on PC3 had higher mechanical advantages and thus greater force efficiency, robust jaws, scissoring jaw action, and greater jaw protrusion, while those species which loaded negatively on PC3 had larger gapes, larger jaw muscles, and stouter pectoral propterygia (Figure 4.3b).

Figures 4.3a & b also highlight ecological trends in feeding morphology relative to diet. In general most dietary guilds overlapped in the medial region of the morphospace. Overlap in large part is driven by insectivores occupying the largest area of morphospace of all the dietary modes, perhaps surprisingly. The two most outlying insectivores are *Potamotrygon signata* and *P. orbignyi*, the former is a relatively older taxon, the latter relatively recent (but see discussion of adaptive peaks, below) (Figure 4.3a). *P. signata* is characterized by generally higher mechanical advantage jaws and wide gape. *P. orbignyi* has greater overall cranial kinesis, with increased jaw protrusion (longer hyomandibulae and angular cartilages), flexible, gracile jaws with much lower force-efficiency. Insectivores encompass crustacean specialist convex hulls almost entirely, prey items which are presumably similar to insects although presumably stiffer given mineralization of the chitinous cuticle in crustaceans. Molluscivores like *P. leopoldi* and *P. henlei* ordinated low and negative on PC2 and positively on PC3, corresponding with more rigid jaw skeletons, owing to a fusion of the medial symphyses of the jaw in these species (Figure 4.3a, b). Molluscivores were also characterized by high mechanical advantages and large jaw

adductor muscle attachment areas. The diet of *Potamotrygon brachyura* is largely uncertain, and interestingly it falls out within the morphospace adjacent to both large crustacean specialists and molluscivores, suggesting this species may in fact be specializing on stiff or tough prey. The piscivorous taxa *Paratrygon* and *Heliotrygon* were characterized by large gapes, increased ability to life the disk, as well as rounder, more gracile jaw cross-sectional shape (Figure 4.3a, b). The middle of both phylomorphospaces is densely-packed, showing frequent shifts between omnivorous, crustacean, and insect-feeding dietary modes (corroborated by the ancestral state reconstructions), with PC2 and PC3 showing a greater divergence between piscivorous taxa and other dietary guilds (Figure 4.3b).

#### 4.4.3 Patterns of lineage and morphological diversification

The  $\gamma$  statistic provided no evidence for declining rates of lineage accumulation after an early burst of diversification ( $\gamma = 0.196$ , p = 0.577), which is also evident in the lineage-through-time plots (Figure 4.4). The fit of the constant-rate models versus the two density-dependent models (DDX and DDL) did not clearly support one model over another, although a pure-birth Yule model with multiple rate shifts (three) was slightly favored over other models according to  $\Delta$ AIC (Table 4.3). Although  $\gamma$  was recovered as positive, I cannot entirely rule out a density-dependent, ecologically-driven process given almost equivocal support for one model over another. Even the fitted Yule model showed a decline in rates of speciation through time (r1 = 0.061, r2 = 0.172, r3 = 0.035). MEDUSA, however, revealed strong support for a single rate shift at a node uniting lower Amazon Potamotrygon ( $\Delta$ AIC = 10.12) (Figure 4.5a). The net diversification rate for this clade (r = 0.254 lineages Myr–1) was almost four times higher than the background rate of diversification for most potamotrygonids (0.066 lineages Myr–1).

Adaptively radiating clades are expected to show an initial burst of morphological disparity, rather than a constant increase in overall disparity (Harmon et al., 2003). This trend, counterintuitively, is represented by high initial morpho-disparity followed by negative subclade disparity relative to simulated, random processes, i.e. accumulation of disparity-through-time (Harmon et al., 2003; Slater et al., 2010). This means that subclades tend to overlap in their overall disparity, with multiple convergent incursions into similar morphospace regions (Harmon et al., 2003; Slater et al., 2010). The subclade disparity for potamotrygonids starts as lower than expected given the null, i.e. was less than the simulated median for a

Brownian motion model of morphological evolution (Figure 4.5b). However, at approximately 20.4 mya, subclade disparity starts to exceed simulated levels with a sharp upturn occurring approximately 9-10.0 mya – culminating in a major spike of disparity around 7-7.6 mya (approximately 0.825-0.9 relative time). This spike is roughly coincident with the diversification of potamotrygonids in the Lower Amazon, including species such as *Potamotrygon leopoldi* and *P. henlei*, *P. motoro*, *P. falkneri*, and *P. histrix* (Figure 4.5). The resultant, recent trend in MDI for the clade as a whole is therefore overwhelmingly positive (0.0554, p = 0.93), starting at approximately 20 mya, due to recent increases in subclade disparity starting at 20 mya, with a peak at approximately 8 mya.

SURFACE analyses revealed five adaptive peaks, with four local, as well as one ancestral peak reconstructed at the base of Potamotrygonidae, with no convergent peaks indicated. These novel adaptive peaks correspond to (1) the common ancestor of the piscivorous *Heliotrygon* + *Paratrygon* clade, (2) the common ancestor of durophagous *P. leopoldi* + *P. henlei*, (3) the base of the *P. motoro* lineage (from the Rio Ucayali) and finally, (4) the origin of insectivorous *P. orbignyi* (Figure 4.5a). Model parameters for both forward and backward simulation runs resulted in a single best-fit model, with evolutionary rates of  $\sigma 2$  for PC1 = 3.287,  $\sigma 2$  for PC2 = 130.26, and  $\sigma 2$  for PC3 = 2.77, and with selective constraint values of  $\alpha$  for PC1 = 0.221,  $\alpha$  for PC2 = 9.221, and  $\alpha$  for PC3 = 0.281.

#### 4.5 Discussion

The combined mitochondrial and nuclear gene dataset resulted in a well-resolved phylogeny for freshwater rays. Ancestral reconstruction of dietary traits on this tree indicates that generalist lineages have given rise to several dietary specialist lineages, such as piscivores, insectivores, and crustacean-feeding specialists. However, molluscivores appear to have arisen from crustacean-feeding specialist ancestors. These different dietary strategies largely overlap in their trait values according to phylomorphospace plots, with insectivores occupying a surprisingly large and disparate region of potamotrygonid morphospace. This pattern may be a result of the several independent derivations of insectivory across potamotrygonids, including *P. orbignyi*, which appears to be a morphological outlier even within insect feeding specialists. SURFACE analyses found that this species, as well as both species of molluscivorous rays, and all fisheating rays (*Paratrygon* and *Heliotrygon*) represent novel adaptive peaks relative to the ancestral

adaptive peak for the majority of potamotrygonids. Finally, patterns of lineage accumulation and morphological diversification throughout the history of potamotrygonids are seemingly decoupled; lineage accumulation appears mostly constant throughout time while morphological diversification in potamotrygonids showed an early-burst in disparity, with recent shifts towards positive subclade disparity. The case for a potamotrygonid radiation is more complex than expected, since lineages do not show greater accumulation rates over log-linear expectations, nor is there evidence of overall morphological diversity or lineage accumulation tapering off in more recent time series. However, morpho-disparity does show an early-burst pattern, with positive subclade trends the product of relatively recent phenomena.

#### 4.5.1 Phylogenetic systematics of Potamotrygonidae

Our analyses show that Potamotrygonidae is monophyletic, and that marine, amphi-American Himantura (H. schmardae and H. pacifica) are its sister lineage, relationships supported by previous morphological and molecular studies (Lovejoy, 1996; Lovejoy et al. (1998); Aschliman et al., 2012; Garcia et al., 2015). Within Potamotrygonidae, I find that *Plesiotrygon* is nested within Potamotrygon, consistent with findings of previous studies (Toffoli et al., 2008; Carvalho and Lovejoy, 2011; Figure 4.1), and making *Potamotrygon*, as currently composed, paraphyletic. The sister taxon to *Plesiotrygon* and most species of *Potamotrygon* is *Potamotrygon brachyura*, the largest species of *Potamotrygon* (greater than 90 cm disk width; Achenbach and Achenbach, 1976). P. brachyura is endemic to the Paraguay and Paraná basins, and has a poorly understood life history and ecological role. I found that *Paratrygon* and *Heliotrygon* form a monophyletic group, and together represent the sister group to all other potamotrygonids. These two genera are specialized piscivores, and their close relationship indicates that their shared ancestor was piscivorous. *Paratrygon*, currently considered a mono-specific genus, exhibits considerable genetic diversity, perhaps unsurprising given its large range. Our results support the findings of Frederico et al. (2012), who identified a lower Amazon clade (Xingu and Araguaia; in our findings, Xingu and Iriri) and an upper Amazon clade (in our findings: Negro + Nanay), with the latter having Guiana Shield (Rupununi River) *Paratrygon* as its nearest relative, a potential signal of the Rupununi Portal, a seasonal ephermeral connection between the Branco/Negro rivers basins and the rivers of the Guianas (de Souza et al., 2012).

Our dated tree suggests that the bulk of potamotrygonid morphological disparity was in place by the early Miocene (see Lovejoy et al., 2006), starting with the piscivorous Paratrygon + Heliotrygon clade diverging from other potamotrygonids at approximately 28.7 mya. By 20.1 mya all major lineages of potamotrygonids were present, including deepwater crustacean and insect-feeding Plesiotrygon, fish-eating Paratrygon and Heliotrygon, and more omnivorous but diverse *Potamotrygon*. Ancestral state reconstructions show that the ancestor of the amphi-American Himantura and potamotrygonids was a generalist predator, with generalists being strongly represented across the entire history of the freshwater clade (Figure 4.2). Piscivory was presumably the first instance of dietary specialization in the potamotrygonids, with the *Heliotrygon* + *Paratrygon* clade splitting from the rest of the family and being uniformly piscivorous. Generalist lineages consistently gave rise to different kinds of dietary specialists, with insectivory evolving in the northern Colombian-Venezuelan Cordillera clade and again in both Potamotrygon signata and Potamotrygon orbignyi. Crustacea-feeding specialists also evolved from lineages reconstructed as generalists, with the *Potamotrygon motoro*, as well as Potamotrygon scobina, species complex feeding predominantly on crabs and prawns. Finally, stemming from this crustacean-feeding specialist lineage, durophagous potamotrygonids like P. henlei and P. leopoldi evolved. Interestingly, dietary specialists (excluding piscivorous *Paratrygon*) are largely restricted to the lower Amazon, particularly the clear-water rivers of the Xingu, Tocantins, and Tapajos.

The phylogeny reveals interesting biogeographical patterns within *Potamotrygon*. The topology suggests a vicariant split between a northern cordillera clade, including Orinoco/Magdalena taxa and rays from the Guiana Shield, from an Amazon clade dating to 17.8 mya. This latter clade has several early-diverging taxa which are endemic to rivers in the Guianas (*P. marinae* – Maroni River, *P. boesemani* – Corantyne River), and in the Negro River (*P. wallacei*), suggesting a connection between the Guianas and the lower Amazon basin, with the Rupununi savannah serving as a potential portal. I recovered a northern Colombian/Venezuelan clade including [*Potamotrygon yepezi* + (*P. magdalenae* + *P.* 'reticulata'), with *P. yepezi* an endemic to the Maracaibo basin, and *P. magdalenae* and *P.* 'reticulata' found in both the Rio Atrato and Rio Magdalena basins. The high incidence of insectivory in this Colombian/Venezuelan clade is fascinating, and perhaps a function high-gradient river systems in these regions having high aquatic insect larvae diversity, with relatively few consumers. The separation of northern

Orinoco-Magdalena rays at around 17 mya is coincident with the contraction and ultimate fragmentation of the Pebas mega wetland, which during its zenith, united upper Amazon, proto-Orinoco, and Guiana Shield drainages (references). Amazon basin potamotrygonids appear to have experienced a noticeable radiation around 7-8 mya ago, giving rise to much of the extant diversity of the genus *Potamotrygon* in particular. Geologically, these times correspond with the Amazon River achieving its modern flow pattern, with Andean sediments being documented in Atlantic facies at this time (Hoorn et al., 2010). During this global period of cooling and declining sea-levels, the Acre-wetlands of middle South America would have disappeared, suggesting that modern *Potamotrygon* diversity is associated less with wetlands and more with increasing channelization of the Amazon basin.

# 4.5.2 Morphological and functional diversity in the feeding mechanism of freshwater stingrays

Piscivorous potamotrygonids (*Paratrygon* and *Heliotrygon*) generally have wide jaws with dentition restricted to only a narrow margin at the medial symphysis of the jaws. This is in many ways similar to the overall jaw morphology of other piscivorous rays, including marine butterfly rays (*Gymnura*, Kolmann et al., 2014) and the pelagic stingray, *Pteroplatytrygon* (Kolmann, pers obs.). Freshwater piscivorous stingrays also have reduced eyes, and tails, as well as a greatly expanded pectoral disk, traits they again share in common with *Gymnura*. The thickness of the pectoral propterygia in these species presumably serves as be a robust anchor for pectoral fin musculature, which rapidly raise the anterior disk to capture elusive prey (Wilga et al., 2012; Mulvany and Motta, 2013; Kolmann et al., 2016). *Paratrygon* and *Heliotrygon* go so far as to hypertrophy and fuse the anterior-most radials of the disk, which may serve to facilitate faster elevation of the disk during suction-capture. In other piscivorous fish lineages, strong sustained selection for suction feeding performance has been posited as enforcing commensurately strong morphological integration on the jaws, hyoid, and opercular apparatuses (Collar et al., 2009, 2014). In turn, piscivory for predators has generally been thought to be a highly lucrative niche role, while simultaneously being an ultimate impediment for further morphological diversification (Collar et al., 2009). The early divergence of these piscivorous rays from other potamotrygonids, their drastically divergent morphology optimized for disk-driven suction feeding, and their apparent lack of dietary diversification may indicate that a similar scenario might be limiting further diversification in *Paratrygon* and *Heliotrygon*.

Insectivores occupy a large region of the feeding morphospace, with both omnivores and crustacean-feeders subsumed within this variation. The presence of several independent instances of insectivory in this clade can support the presence of such variation, especially since some insectivores appear relatively unchanged from early stem-potamotrygonid adaptive peaks, while more recent insect-feeders (P. orbignyi) exhibit truly novel phenotypes. P. orbignyi, as stated previously, has more gracile jaws with much lower force-efficiency than other potamotrygonids but has greater overall cranial kinesis conferred by flexible jaw symphyses (Kolmann et al., 2016). This species feeds on a diet entirely composed of aquatic insect larvae, which it presumably processes using considerable jaw kinesis, facilitated by both long hyomandibulae and long angular cartilages (Kolmann et al., 2016). Angular cartilages, a synapomorphy for potamotrygonids (Lovejoy, 1996) are an accessory skeletal element connecting the paired hypomandibulae to the jaws, and may act as a novel joint within the feeding apparatus of these rays. Similar novel joints in the jaw mechanisms of fishes have been identified in many teleost lineages, including pomacanthids, acanthurids, and pomacentrids (Konow et al., 2008; Gibb et al., 2008; Ferry-Graham and Konow, 2010). If specialization for insectivory depends on increasing kinetic ability of the feeding apparatus, the angular cartilages may facilitate such mobility, necessary for the masticatory behaviors of some Potamotrygon species (Kolmann et al., 2016). This disambiguation between the jaws and hyomandibular apparatus coupled with a dietary focus on complex prey seems to be common for both insectfeeders and marine invertebrate-feeding batoids, although the presence of angular cartilages is unique to potamotrygonids (Lovejoy, 1996; Dean et al., 2007).

Molluscivores like *P. leopoldi* and *P. henlei* and crustacean-feeding specialists like *P. motoro* and *P. scobina* have rigid jaw skeletons and high mechanical advantages, owing to compact, robust jaws, tall symphyses, and broad muscle attachment areas (Figure 4.3a). The jaws of molluscivores like *P. leopoldi* and *P. henlei*, as well as the recently-described *Potamotrygon rex*, exhibit some degree of fusion at the medial jaw symphyses as well as tightly-interlocking, flattened teeth (Carvalho, 2016). The overall morphology of the jaws of these species are remarkably similar to the feeding mechanism of marine durophagous rays such as eagle, bat, and cownose rays (Summers, 2000). Both marine and freshwater molluscivorous rays have hypertrophied jaw adductor muscles, fused jaw skeletons, and closely-interdigitating teeth which effectively transfer force to shelled prey while maintaining structural resiliency (Summers,

2000; Kolmann et al., 2014, 2015a,b). The disparity between the jaws of an insect-feeder like *P. orbignyi* relative to the jaws of *P. leopoldi* is shocking, with the former highly kinetic, and the latter fused and highly mineralized. The jaws of *Plesiotrygon iwamae* are seemingly found somewhere between these morphologies, with tall jaws forming a narrow gape, closely-interlocking teeth, and large angular cartilages (Charvet-Almeida, 2001). This species seemingly preys in large part on freshwater prawns, which are structurally intermediate between tough and stiff prey (insects and mollusks, respectively), having exoskeletons made of lightly-mineralized chitin. The diet of many potamotrygonid species is largely unknown, pending greater study into the ecology of these animals. We suggest that the ecomorphological gradient observed above may largely be a function of a dietary resource gradient: a prey biomaterials spectrum spanning from more compliant, elusive prey (fishes) to tough prey like insect larvae, to progressively stiffer, more mineralized prey such as decapod crustaceans, bivalves, and gastropods. This scenario very broadly resembles the ecomorphological variation of one of the 'poster-child' clades for adaptive radiation, *Geospiza* spp., or Darwin's finches (Van der Meij and Bout, 2004, Van der Meij et al., 2004).

Similar patterns of morphological diversification reflecting evolution along a dietary gradient have been documented in only one other lineage of marine-derived fishes (Davis et al., 2012, 2013, 2014). Terapontid grunters invaded freshwaters of Australasia only once, and subsequently diversified from a carnivorous ancestor to novel instances of carnivory, detritivory, herbivory, and omnivory (Davis et al., 2012). Terapontids similarly experienced increased rates of morphological evolution and the advent of novel adaptive peaks which correlated with new dietary modes (Davis et al., 2013, 2014). However, these fishes invaded a freshwater system with a comparably depauperate ecological community structure; almost all of Australasia's freshwater fishes are 'secondary' freshwater taxa, having originally evolved in the oceans. Potamotrygonids in contrast invaded one of the most complex and presumably competitivelyintensive aquatic ecosystems on earth, the Amazon and adjacent river systems (Orti et al., 2012). However, the presence of several lineages of marine-derived fishes in the Amazon would imply that this competition is infrequently, maybe even occasionally, overcome. Although niche conservatism has been suggested for some other MDLs (anchovies; Bloom and Lovejoy, 2012), ecomorphological and dietary diversification is clearly evident in freshwater stingrays. Perhaps the success of terapontids and potamotrygonids is due to the plastic ecological role of their

pioneering ancestors, with the invasion of novel ecosystems led by dietary generalist species, whereas engraulids are overwhelmingly specialized for suspension-feeding and only diversified into other niches (piscivory in *Lycengraulis*) after colonization of freshwater (Bloom and Lovejoy, 2012).

#### 4.5.3 The case for an adaptive radiation of potamotrygonids

Potamotrygonid morphological evolution shows patterns consistent with other examples of adaptive radiation, including early, high overall disparity partitioned among subclades (i.e. negative subclade diversity). Establishment of several disparate clades with non-overlapping trait morphospaces early in the history of these rays suggests that partitioning of ecological niches was rapid, consistent with other adaptively radiating lineages. However, this pattern shifts midway through the history of potamotrygonids, with a gradual shift in subclade disparity towards a positive trend starting around 18 mya. This pattern suggests that one or several clades, notably within *Potamotrygon*, started exploring larger regions of morphospace; modern potamotrygonid clade disparity is therefore accounted for by later diversification. This positive upturn in disparity at 16.4 mya drastically changes from Brownian estimates at around 12 mya and peaks at 5-6 mya, coincident with the drainage of the Pebas Mega Wetland and increased channelization of the Amazon, respectively. MEDUSA analyses also resolved a shift in rates of lineage accumulation within this interval (~ 8 mya), with a secondary radiation of Potamotrygon species in the Amazon River proper as well as its lower tributaries. This secondary radiation includes the majority of the novel adaptive peaks identified by SURFACE, corresponding to the rise of stingrays specialized for insect-feeding, crustacean-feeding, and molluscivory.

As I predicted, potamotrygonids exhibit high subclade disparity, at least early during their diversification throughout the Amazon basin (Figure 4.5). However, lineage accumulation is essentially constant, rather than exponential and shows no indication of decreasing rates (Figure 4.4), contrary to our expectations (Harmon et al., 2003). The presence of at least one rate shift in lineage diversification within this clade, constant accumulation of extant species, and initially high subclade disparity also suggests that high background extinction, either sustained or intermittent, could be masking signals of adaptive radiation (Pybus and Harvey, 2000; Rabosky and Lovette, 2008). Slater et al. (2010) reported a similar phenomenon for modern cetaceans, i.e. relatively constant, log-linear rates of lineage accumulation coupled with high levels of early

clade disparity, and ascribed this pattern to considerable background extinction in whales and their allies. This suggestion is strengthened by an exemplar fossil record for stem and crown cetaceans, which documents quite clearly the rise and fall of many lineages, now lost to molecular methods of phylogenetic reconstruction (Uhen & Pyenson, 2007). Unfortunately batoid fossils are typically restricted to isolated hyper-mineralized teeth, spines, or dermal bucklers – which make confident taxonomic identification difficult. I have no way of knowing how or if early extinct potamotrygonids were particularly morphologically diverse, given that teeth in this clade are generally similar (Adnet et al., 2014).

I suggest that adaptive radiation in Potamotrygonidae is probable. However, the situation is complicated by recent shifts in diversification; modern *Potamotrygon* lineages seem to have undergone recent radiations in the channels of the Amazon, potentially overlapping in their morphospace with confamilials and complicating the overall tempo of the potamotrygonid radiation. More recent or secondary radiations into novel niche space, presumably with novel disparity present in more recent taxa, like *Potamotrygon leopoldi*, *P. henlei*, and *P. orbignyi* may have obscured the overall pattern of feeding diversification, which although originally partitioned evenly among subclades, is now dominated by *Potamotrygon*, which is both ecologically diverse as well as producing some novel ecological modes not explored by other freshwater ray genera. Interestingly, Ingram et al. (2012) found that radiations which have high proportions of omnivores, particularly if the clade is ancestrally omnivorous, are susceptible to having the early divergence within phenotypic space masked by frequent transitions and reversions back to omnivory. Early in the diversification of potamotrygonids, generalist omnivore taxa gave rise to early specialists like *Plesiotrygon* and *Paratrygon*.

The pattern whereby generalists give rise repeatedly to specialists has been documented in other lineages with later bursts of morpho-disparity, such as damselfishes and notothenioids (Frédérich et al., 2012; Near et al., 2012). Concomitant with the end of the Pebas formation and the advent of positive clade disparity in potamotrygonids a shift occurs whereby several lineages of specialist taxa (molluscivores and insectivorous *P. orbignyi*) arise from crustacean-specializing stingrays instead of generalists. This trend explains why I see the generation of novel adaptive peaks in these clades, relative to the ancestral, global adaptive peak at the base of Potamotrygonidae + *Himantura*. Potamotrygonid rays therefore offer a complicated history of feeding adaptation, starting with ancestral rays diversifying within the Pebas wetland, with

distinct clades having distinct ecological roles as piscivores, insectivores, and generalists. Later diversification within *Potamotrygon* involved a shift away from omnivory, with the advent of crustacean-feeding specialists giving rise to dietary specialists like mollusk-feeders, and novel experiments with insect feeding in the case of *P. orbignyi*. I maintain that signals of adaptive radiation are retained in the recovered trends in morphological diversification, with recent colonization of the lower Amazon potentially obscuring any pattern of gradual decrease in lineage accumulation. I agree with Slater et al. (2010) that testing for patterns regarding both lineage richness (accumulation) and diversity (morphological disparity) is imperative for understanding adaptive evolutionary patterns.

# 4.6 Conclusion

The time frame of adaptive radiations is controversial and centers mostly on the observation that diversification and lineage accumulation are in some way accelerated, particularly early on within the diversifying clade (Gavrilets and Losos, 2009). Early negative subclade disparity, as seen in early potamotrygonids, suggests rays partitioned the majority of their ecomorphological diversity quickly. Later trends towards positive morphological disparity, starting around 16.4 mya, suggests that the ecological playing field for potamotrygonids shifted drastically. This more recent accumulation of diversity suggests that modern potamotrygonid ecomorphology has been shaped by more recent events, perhaps access to novel habitats and resources of the lower Amazon basin. The magnitude of morphological disparity in clades undergoing radiation is expected to become less disparate as lineages partition ecological niches, and potamotrygonids show negative subclade disparity early in their history up until 20.5–16.4 mya. This era would have been characterized by rays initially diversifying in the sub-Andean river systems which drained northward into the Caribbean (Lovejoy, 1996; Lovejoy et al., 2006). The advent of the Pebas system, which dominated western-central Amazonia 23-10 mya (Hoorn et al., 2010), seems to have perturbed the slowdown of diversification in early potamotrygonids. Increases in positive subclade disparity and the generation of new adaptive peaks occurs more recently, coincident with increasing channelization of the Amazon basin and the dissolution of the Acre system (Hoorn et al., 2010), which presumably exposed potamotrygonids to new habitats and new resources.

Potamotrygonid rays diversified in the Eocene solely in freshwater, the only extant monophyletic clade of elasmobranchs to do so. Modern freshwater ray diversity was established early on, coincident with shallow interior wetlands of the Pebas and Acre phenomena, and exhibits a secondary increase in ecomorphological disparity coincident with later channelization of the Amazon basin. I propose that exposure to novel, clear-water habitats in the lower Amazon promoted the advent of durophagy in this clade of fishes. Recent changes in the evolutionary dynamics of freshwater rays, which I ascribe to geological processes extending ecological opportunity through exposure to novel habitats, have complicated the tempo of diversification in this clade.

# 4.7 Acknowledgements

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	Speci	Voucher								SCF
Genus	es	#	Locality	ATP6	CO1	cytb	ENC1	ITS	RAG1	D2
Heliotry	gomes	CUMV	Rio Nanay,	NL-	NL-	NL-	NL-	NL-	NL-	83
gon	i	78485	Peru	849	849	849	849	849	849	-
Paratryg	aiereb	ANSP1	Iriri River,	ROM-	ROM-	ROM-	ROM-	ROM-	ROM-	
on	а	98641	Brazil	15254	15254	15254	15254	15254	15254	-
Paratryg	aiereb	CUMV	Rio Nanay,	PU092	PU092	PU092	PU092	PU092	PU092	PU0
on	а	78489	Peru	7	7	7	7	7	7	927
Paratryg	aiereb	CUMV	Rio Negro.	RN04	RN04	RN04	RN04	RN04	RN04	RN0
on	a	78490	Brazil	59	59	59	59	59	59	459
Paratryg	aiereb	ROM87	Rupununi	ROM-	ROM-	ROM-	ROM-	ROM-	ROM-	
on	a	233	River. Guvana	0966	0966	0966	0966	0966	0966	-
Paratryg	aiereb	ANSP1	Xingu River.	TO05	TO05	TO05	TO05	TO05	TO05	TO0
on	a	98642	Brazil	79	79	79	79	79	79	579
Plesiotry	iwam	CUMV	Rio Nanav	PA031	PA031	PA031		PA031	PA031	
gon	ae	78494	Peru	04	04	04	-	04	04	-
Plesiotry	ue	CUMV	Rio Nanav	PU094	PU094	PU094	PU094	PU094	PU094	PU0
ann	nana	78813	Peru	5	5	5	5	5	5	945
8011		70015	Corantyne	5	5	5		5		715
Potamot	boese	ROM91	River	ROM-	ROM-	ROM-	ROM-	ROM-	ROM-	
rvaon	mani	269	Suriname	10800	10800	10800	10800	10800	10800	_
Potamot	brach	MZUSP	Parana River	AC06	AC06	AC06	AC06	AC06	AC06	AC0
rvaon	wura	104224	I arana Kiver, Brazil	004	004	004	004	004	004	AC0 6004
Potamot	falkna	ANSD1	Diazii Dorono Divor	MS04	MS04	MS04	004	MS04	MS04	0004
T Olumol	juikne ri	ANSE 1 72783	Falalla Kivel, Brazil	32	32	32		32	32	
Potamot	11	CUMV	Toconting	32 TO05	52 TO05	J2 TO05	- TO05	32 TO05	J2 TO05	- TO0
Folamol	honloi	79401	Divor Drozil	70	70	70	70	70	70	576
Potamot	neniei	70491 ANSD1	River, Diazii	70 MS04	70 MS04	70 MS04	70 MS04	70 MS04	70 MS04	570
Polamol	histrix	79009	Rio Nallay,	MS04	MS04	MS04	MS04	MS04	MS04	
Tygon Dotant of	loomol	/0090	Vincu Divor	14 TO05	14 TO05	14 TO05	14 TO05	14 TO05	14 TO05	- TO0
Polamoi	ieopoi 1:	ANSP1 08642	Aingu Kiver,	1005	1003	1003	1003	1003	1005	505
rygon	ui	98043	DIazii	09	09	09	09	09	09	393
Determent	maga	ANCD7	Rio Atrato,	NI	NI	NI	NI	NI	NI	
Polamol	aiena	ANSP/	Colombia	NL- 2170	NL- 2170	NL- 2170	NL- 2170	INL- 2170	NL- 2170	
rygon	е	91/4 DOM07	Marin	5170 DOM	5170 DOM	5170 DOM	5170 DOM	5170 DOM	3170 DOM	-
Potamot	marin	KOM9/	Maroni River,	KOM-	KOM-	KOM-	KOM-	KOM-	KOM-	
rygon	ae	978	Suriname	18145	11827	11827	11827	11827	11827	-
	Orino	ROM88	Orinoco	V712	V712	V712	V712	V712	V712	V71
Potamot	со	355	Kiver,	VZ13	VZ13	VZ13	VZ13	VZ13	VZ13	VZ1 200
rygon	* *	NH 1011	Venezuela	08	08	08	08	08	08	308
Potamot	Ucaya	NLIUII	Rio Ucayali,	NL-	NL-	NL-	NL-	NL-	NL-	
rygon	11	ð ANGD1	Peru	10118	10118	10118	10118	10118	10118	-
Potamot	Xingu	ANSPI	Xingu River,	ROM-	ROM-	ROM-	ROM-	ROM-	ROM-	
rygon	1.	99/58	Brazil	0/146	0/146	0/146	0/146	0/146	0/146	-
Potamot	orbig	NLIOII	Rio Ucayali,	TA06	TA06	TA06	TA06	TA06	TA06	TA0
rygon	nyı	4	Peru	18	18	18	18	18	18	618
י י ת			K10	<b>NTT</b>	NT	NT	ът	ът	NT	
Potamot	reticul	NH 0000	Magdalena,	NL-	NL-	NL-	NL-	NL-	NL-	
rygon	ata	NL9800	Colombia	9800	9800	9800	9800	9800	9800	-
Potamot	schro	ANSPI	R10 Negro,	RN04	RN04	RN04	RN04	RN04	RN04	
rygon	ederi	61193	Brazil	94	94	94	94	94	94	-
<b>D</b> .	scobin	ANSP1	Rio	DAGG	DAGG	DAGG		DAGG	DAGGG	
Potamot	a	98639	Amazonas,	PA038	PA038	PA038		PA038	PA038	
rygon	<u> </u>		Brazil	0	0	0	-	0	0	-
Potamot	signat	ANSP6	Rio Paranaiba,	PA079	PA079	PA079	PA079	PA079	PA079	
rygon	a	9344	Brazil	1	1	1	1	1	1	-
Potamot	tigrin	ANSP1	Rio Nanay,	NL-	NL-	NL-	NL-		NL-	
rygon	a	95978	Peru	1230	1230	1230	1230	-	1235	-
Potamot	walla	ROM25	Rio Negro,	RN04	RN04	RN04		RN04		
rygon	cei	798	Brazil	19	19	19	-	19	-	-
Potamot		USNM1	Maracaibo,	VZ11	VZ11	VZ11		VZ11	VZ11	VZ1
rygon	yepezi	21659	Venezuela	02	02	02	-	02	02	102

Potamot	demer	ROM10	Demerara	NL-		NL-		NL-	NL-	
rygon	arae	0073	River, Guyana	10113	-	10113	-	10113	10113	-
	guttat	MCZ40	Rio de	ROM-	ROM-	ROM-	ROM-		ROM-	
Dasyatis	a	418	Janeiro, Brazil	20323	20323	20323	20323	-	20323	-
	geijsk	MCZ40	Rio de	ROM-	ROM-	ROM-	ROM-		ROM-	
Dasyatis	esi	425	Janeiro, Brazil	20697	20697	20697	20697	-	20697	-
Himantu	schma	ROM10	Demerara	JN184	JN184	JN184	ROM-	ROM-	JN184	JN18
ra	rdae	0989	River, Guyana	062	063	064	20690	20691	126	4169
Himantu	pacifi	ROM66	Boca del Toro,			NL-				
ra	ca	839	Panama	-	-	1761	-	-	-	-
				NC-	NC-	NC-				NL-
Pteropla	violac	ROM25	Georges Bank,	02457	02457	02457	NL-		KT18	1183
tytrygon	ea	636	Canada	0	1	2	11838	-	7542	8
	lymm	ROM40	Queensland,	JN184	JN184	JN184	NL-		JN184	JN18
Taeniura	а	480	Australia	079	080	081	1162	-	117	4160
			Gulf of							
Neotryg		ANSP1	Thailand,	JN184	JN184	JN184	NL-		JN184	JN18
on	kuhlii	71541	Thailand	065	066	067	1159	-	115	4158
			Gulf of							
	haller	MCZ-	California,	JN184	JN184	JN184	NL-		JN184	JN18
Urobatis	i	S1419	USA	083	084	085	11835	-	128	4171
Urotryg	simul	ROM66	Boca del Toro,	NL-	NL-	NL-	NL-		NL-	
on	atrix	841	Panama	1758	1758	1758	1758	-	1758	-

Table 4.1 Summary of specimens used in this study, the matched molecular sequences data, and collection locality.

Genus Species		Diet	Study		
Heliotrygon	gomesi	Piscivore	Lucanus, pers comm		
Paratrygon aiereba		Piscivore	Shibuya et al., 2009		
Plesiotrygon	iwamae	Crustaceans	Charvet-Almeida, 2001		
Plesiotrygon	nana	Insects	Charvet-Almeida, 2001		
Potamotrygon	boesemani	Unk/Omnivore	Lucanus, pers comm		
Potamotrygon	brachyura	Omnivore	Achenbach and Achenbach, 1976		
Potamotrygon	falkneri	Omnivore	Lonardoni et al., 2006; Silva & Uieda, 2006		
Potamotrygon	henlei	Molluscivore	Pantano-Neto & Souza, 2002; Charvet-Almeida, 2006		
Potamotrygon	histrix	Omnivore	Achenbach and Achenbach, 1976; Lasso et al., 2013		
Potamotrygon	leopoldi	Molluscivore	Charvet-Almeida, 2006		
Potamotrygon	amotrygon magdalenae Insectivore Ramos-Socha & Grijalba-Bendeck, 24		Ramos-Socha & Grijalba-Bendeck, 2011		
Potamotrygon	Potamotrygon marinae		Lucanus, pers comm		
Potamotrygon	motoro Ucayali	Crustaceans	Shibuya et al., 2007; Almeida et al., 2010; Vasconcelos & Oliveira, 2011		
Potamotrygon motoro Orinoco Omnivore		Omnivore	Almeida et al., 2010; Vasconcelos & Oliveira, 2012		
Potamotrygon	motoro Xingu	XinguOmnivoreMelo et al., 2007; Almeida et al., 2010; Vasconcelos & 2011			
Potamotrygon	<i>Potamotrygon orbignyi</i> Insectivore Shibuya et al., 2009; Moro et al., 2011;		Shibuya et al., 2009; Moro et al., 2011; Gama & Rosa, 2015		
Potamotrygon reticulata		Insectivore	Lasso et al., 2013		
Potamotrygon schroederi Unk/Omnivore		Unk/Omnivore	Góes de Araújo, 2009; Lasso et al., 2013		
Potamotrygon scobina Crustaceans Braganca, 2004; Gama		Braganca, 2004; Gama & Rosa, 2015			
Potamotrygon	signata	Insectivore Moro et al., 2011			
Potamotrygon	tigrina	Unk/Omnivore Lasso et al., 2013			
Potamotrygon	wallacei	Omnivore	Shibuya et al., 2009		
Potamotrygon	yepezi	Insectivore	Góes de Araújo, 2009; Lasso et al., 2013		
Potamotrygon	demerarae	Omnivore	Kolmann, pers obsv		

Table 4.2 Summary of references for potamotrygonid dietary information. 'Unk/' designates those sources which are ambiguous or personal communications.

Model	Lk	AIC	dAIC	
	-			
Yule	26.1303	54.260	2.311	
birth-	-			
death	26.0724	56.144	4.195	
	-			
DDL	26.1217	56.243	4.294	
	-			
DDX	26.0396	56.079	4.129	
	-			
yule2rate	23.7171	53.434	1.484	
	-			
yule3rate	20.9746	51.949	0.000	

Table 4.3 Model fitting results (log likelihood,  $\Delta AIC$ ) for the comparison of constant-rate versus variable rate models for rates of evolution.



Figure 4.1 Time-calibrated Bayesian phylogeny estimated from a partitioned mixed-model analysis of all nuclear and mitochondrial genes. Numbers above nodes represent posterior probabilities (PP). Marine outgroups in dark blue, marine in-group (amphi-American *Himantura*) in light blue, and Potamotrygonidae *sensu stricto* in green. Scale of x-axis is in millions of years from the present.



Falamatrygon mainro Orinoco tamotrygon motoro Ucayal amolygon motors form amaliygon magdalena iamotrygon orbiging tamotrygow miculata Polamotrygon schroeder temotrygon horseman Polamotrygon wallacet tamotrygon fallment sentron nugricum Pleniotrygon nama Pleniotrygon iwawae ninetry wight a partie innotrygon leopoid tamoiny you acolding tamotrygon tigring otamotrygow demerus Potamotrygon fraction tamotrygon hunles otanotygon jepezi Parabygoe Ruparant simultygon histric anarygon Aingu Partitrygore Manay Heliotrygon goment Partitrygon Negro anutrygon livit

Figure 4.2 Maximum likelihood ancestral state reconstruction of diet in Potamotrygonidae. Pink are generalists, blue are piscivores, orange are molluscivores, red are insectivores, and green are crustacean-specialist taxa.



Figure 4.3a Scores of the first two phylogenetically-informed principal component (PC1 and PC2) axes for feeding morphology of potamotrygonid stingrays. Points represent actual species scores, convex hulls represent the area over which trophic modes dominate. Inset shows the connecting branches of the phylomorphospace along the same PC axes. Text adjacent to the axes generalizes the functional gradient indicated by the loadings of the phyPCA.



Figure 4.3b Scores of the second two phylogenetically-informed principal component (PC2 and PC3) axes for feeding morphology of potamotrygonid stingrays. Points represent actual species scores, convex hulls represent the area over which trophic modes dominate. Inset shows the connecting branches of the phylomorphospace along the same PC axes. Text adjacent to the axes generalizes the functional gradient indicated by the loadings of the phyPCA.



Figure 4.4 Lineage-through-time plots for Potamotrygonidae. First plot denotes the actual relationship between lineage accumulation and time since the present. Second plot denotes log-lineage accumulation through time relative to the split of potamotrygonids from their nearest outgroup. The final plot denotes the frequency of branching times, which are biased towards recent cladogenic events.



Figure 4.5 Disparity-through-time (DTT) analyses for Potamotrygonidae. The clade in red has undergone a significant shift in the rate of evolution, starting at the node denoted by the red circle. The numbered grey boxes denote novel adaptive peaks. For the DTT plot, the dashed lines represent the median simulated (Brownian motion) subclade disparity across 10 000 simulations. The solid line represents the observed subclade disparity for potamotrygonids. The grey shade region represents the 95% range of simulated Brownian subclade disparity.
## 5 Concluding Discussion and Synthesis

#### 5.1 Conclusions

In this thesis I examined how predator morphology and behavior vary with regard to aspects of prey material and structural properties, both within individuals and across multiple species. In Chapter 2 I analyzed how morphology does or does not predict performance across the myliobatid stingrays. In Chapter 4 I considered how ecomorphological diversity in New World freshwater stingrays has changed the tempo and general evolutionary dynamics of the clade, by generating a molecular phylogenetic tree for these stingrays. I also tested assumptions regarding how predator feeding behavior might change in relation to the material challenges of prey, with a focus on insectivory in freshwater stingrays (Chapter 3). Below, I summarize the findings of the each chapter and finish with a synthesis of what this research has contributed to our understanding of biomaterials and evolution from the perspective of predator-prey interactions.

In Chapter 2, I examined the performance of differing jaw morphologies in hard-prey crushing stingrays, with regards to their ability to crush mollusks of varying material and structural properties (Kolmann et al., 2015b). I found that these morphologies are functionally equivalent despite prey varying in their response to compressive loading (along a toughness-stiffness gradient) indicating that these durophagous rays represent an example of equifinality: morphological variability does not predict functionally redundant outcomes. Many-to-one mapping describes a property of organismal complexity that allows diversity; the optimizing tendencies of evolution do not necessarily result in overtly-similar bauplans, as seen in studies of convergence or parallelism (Stayton, 2008). Equifinality of form has been documented elsewhere and is thought to at least partially override (or circumvent) phylogenetic conservatism in morphological evolution, as many different morphological configurations can lead to mostly equivalent functional consequences (Young et al., 2007; Wainwright et al., 2005). This study is the first example of morpho-functional equifinality documented in elasmobranch fishes, and underscores how durophagous vertebrates, despite often being used as case studies for morphological convergence, are more morphologically diverse than expected.

In Chapter 3, I investigated the feeding behavior of *Potamotrygon motoro*, particularly with respect to insect-feeding (Kolmann et al., 2016). Chewing, characterized by shearing jaw motions and high-crowned molar teeth, is considered an evolutionary innovation that spurred

dietary diversification and evolutionary radiation of mammals. Complex prey processing behaviors have been thought absent in fishes and other 'lower' vertebrates, despite many of these animals consuming tough prey, like insects or even grasses (Reilly et al., 2001; Gidmark et al., 2014, 2015). I find that *Potamotrygon motoro* uses asymmetrical motion of the jaws, effectively chewing, to dismantle insect prey. Cranial kinesis, with respect to asymmetrical jaw action and symphyseal flexion were evident when *Potamotrygon* feed on any kind of prey, but are particularly exaggerated for tougher prey items like insect larvae or shrimp. Incidences of asymmetric jaw action were elevated and maintained for longer when feeding on insects over other prey, and these feeding bouts lasted twice as long as when feeding on other prey. These findings were consistent regardless of prey size, meaning that 'complex' prey, i.e. prey that are either mechanically difficult or particularly large, are approached in two different manners by rays. Most interestingly, across a survey of the requisite anatomical and behavioral components of chewing across vertebrates, stingrays share many of traits in common with other complex prey-processors, including mammals. These traits include loose jaw joints, shearing (transverse) jaw motions, cuspidate teeth (at least in insectivore specialists), ability to reposition the prev bolus (e.g. hydrodynamic tongue), amongst others.

In Chapter 4, I determined the phylogenetic relationships within the potamotrygonid stingrays and used this molecular tree to establish (A) the marine sister taxa for the family, (2) whether potamotrygonid genera are monophyletic, and (3) what the geological age of the clade is in general. I used this phylogeny to ask how patterns of ecomorphological diversification has proceeded in potamotrygonids, and whether patterns of lineage accumulation and morphological disparity suggest these rays are adaptively radiating. I predicted that potamotrygonids would show an early-burst pattern of ecomorphological diversification, corresponding to dietary mode. I also determined whether or not shifts in rates of lineage evolution in this clade correlate with geography or dietary mode, and whether the generation of novel adaptive peaks correlate with the evolution of insectivory, piscivory, and molluscivory. Piscivory, molluscivory, and a single instance each of insectivory and crustacean-feeding indeed represent novel evolutionary adaptive optima which evolved relatively recently, seemingly in parallel with the channelization of the Amazon and the linking of the upper and lower regions of the basin. Potamotrygonids do show an early-burst pattern of morphological disparity; however, this trend shifted toward positive subclade disparity around the time of Pebas wetland formation and peaked during the final channelization of the upper and lower Amazon. Conversely to expectations, lineage accumulation was mostly linear throughout time. This early burst pattern of diversification coupled with constant lineage accumulation suggests on-going, recent diversification. This may suggest either substantial extinction for potamotrygonids midway through their occupation of South American basins or recent expansion into regions which offered new resources and novel opportunity. Modern potamotrygonids, particularly *Potamotrygon*, may be representative of a relatively new trend in the evolution of these animals in riverine habitats, with earlier forms adapted to Pantanal-style wetland habitats of the Pebas and Acre floodplains. With these more lacustrine or swamp-adapted rays having gone extinct, modern potamotrygonids seem to have adapted to the increasingly channelized and isolated basins of the Amazon, particularly in the lower Amazon tributaries (Tocantins, Xingu, and Tapajos, in particular).

#### 5.2 Synthesis

Considerable research effort has been spent investigating habitat transitions because they offer replicated evolutionary experiments in which ecological opportunity for diversification is presumably present. Transitions like these stand at the interface of drastic changes in clade diversity and richness, suggesting that some radical change in the evolutionary dynamic of these lineages occurred. For biomechanists, these systems are potent models for understanding how physical and biomechanical constraints were overcome or have shaped modern biological form. Some examples of classic transition studies include the transition from water to land by early tetrapods and the advent of flight (at least, the second instance of powered flight in the vertebrates) stemming from the transition between non-avian theropods to birds. Each of these transitions is ultimately a physics discussion before it can be an evolutionary one – feathers alone do not make a bird fly; lighter bones, powerful muscles, and an efficient respiratory apparatus clearly contribute as well - in addition to myriad changes in brain morphology and physiochemistry. Transitioning terrestrial sarcopterygians faced musculoskeletal challenges in supporting their mass given the higher gravity found on land, compared to water. All of these transitions required animals to drastically change their behavioral and anatomical interface with the primary forces of nature: gravity, momentum, and inertia, to name a few.

Combining experimental research in combination with historical, phylogenetic reconstructions allows researchers to examine trait evolution within the malleable setting of the laboratory and

compare these observations to evolutionary pathways in deep-time, the relationships among species and their communities. Within my dissertation I approach questions regarding the evolution of form and function from the perspective of both biomechanics and evolution. Recently three critical considerations on the evolution of form have arisen and my research bears directly on some aspects of the questions raised by these discussions. Firstly, what is the role of equifinality (or the trendier 'many-to-one mapping'; Wainwright et al., 2005) in generating morphological diversity (Young et al., 2007)? In my first chapter I found that despite considerable differences in the shape and size of the jaws of myliobatid stingrays, their function was equivalent despite clear differences in the material properties of their prey. Since these species show demonstrable differences in diet, i.e. diet studies have shown these rays consume more of one particular kind of mollusk over another, one might expect predator jaw morphologies to reflect such a difference in ecology – this is the essence of functional morphology. More and more studies have shown however, that quite variable morphologies can result in equal or near-equal functional outcomes. For this reason, despite typically conserved patterns of trait evolution due to phylogenetic constraints, animals can evolve similar functional and ecological roles despite vastly different morphologies.

Our data demonstrate that while myliobatid jaw shape may not be predictive of feeding performance, the jaws of these rays as well as other durophagous vertebrates, show remarkable convergence in their form and function (Kolmann et al., 2015b). Many vertebrates which consume particularly rigid or stiff prey like mollusks, bones, or nuts have reinforced skeletal structures – robust teeth, thickened skeletons, and hypertrophied muscles (Summers, 2000). Myliobatid rays share all these traits in common with animals as distinct as hyenas, Galapagos finches, and modern carp (Binder and van Valkenburg, 2000, Soons et al., 2010; Kolmann et al., 2014; Gidmark et al., 2015). Myliobatid rays as well as durophagous potamotrygonids (Chapter 3) have reinforced their jaws externally by thickening the mineralized perichondrium, as well as reinforcing the inner matrix with beam-like struts of mineralized cartilage called trabeculae (Summers, 2000; Dean et al., 2006). So although many-to-one mapping may be influential at more immediate, local phylogenetic levels – the challenges of mechanically robust prey seem to curve the evolution of durophagous animals towards robust, akinetic skeletons which can both resist internal deformation while transmitting muscles forces to prey.

For mollusk-crushing predators, efficient force-transfer through the skeleton is essential in order to fracture shelled prey, although the jaws must also be able to resist internal forces or stresses incurred during biting. Both these performance prerogatives would be subsumed within a greater sense of 'constructional morphology' (Barel et al., 1989), which sought to determine the competing effects of interacting anatomical modules regarding overall performance. At the time of Barel et al. (1989) however, the necessary sorts of computational modeling approaches (finite element analysis, multibody dynamics, etc.) were not available and constructional morphology as a subdiscipline of comparative anatomy stalled. The majority of the energy within the discipline of functional morphology was redirected to reflect an emerging statistical renaissance in ecology, the result being studies in ecomorphology. The natural next step in investigating notions of equifinality is to examine phenotypic questions from a more holistic perspective. In the case of my Chapter 2, the obvious extension of why these disparate morphologies exist despite equivalent function would be: how do these jaw morphologies mitigate internal stresses incurred during biting? These sorts of questions, involving how traits evolve at the behest of multiple, oft-competing selective forces is still in its infancy – with only some recent papers regarding the trade-offs between feeding performance and cranial strength across artificial or simulated adaptive landscapes being some of the best examples of the field moving forward (Stayton, 2008; Dumont et al., 2014).

Many authors have expounded upon the necessity of using functional or ecomorphological characters to examine the malleable nature of trait evolution within an adaptive radiation context. If equifinality of form and function is as pervasive as some authors are suggesting, this begs the question as to whether much of the patterns of trait diversity which researchers report is grossly underestimated. If functional traits relating to ecological resource use ('ecological traits') are the only relevant characters for understanding adaptive evolution, what relevance are other traits? Do these traits 'count' to understanding the overall disparity of a group of organisms? This paradigm might lend insight in how to account for traits which are under strong immediate selection (functional characters, or 'ecological traits') in a current ecological context, versus those morphological traits which exist under more ancient, conservative, yet stochastic evolutionary flux – if not Connell's 'Ghost' then it's skeleton (the vast majority of morphological traits, or 'historical traits'). In fact, studies of adaptive radiation do not concern themselves with the vast majority of the total morphological diversity of a clade, these 'historical

traits.' Rather, in a comparison to studies of molecular evolution, where differences in selection are now based on frequencies of synonymous versus non-synonymous base changes – could the difference between hypothetical curves documenting overall clade disparity versus functional disparity give us a morpho-disparity deficit or sorts? Meaning, does the difference between functionally-relevant trait disparity and overall trait disparity tell us something regarding a model for morphological evolution? How many studies of adaptive radiation fail to reveal patterns of adaptation simply because these studies do not choose adequate traits within a relevant functional framework? How often are traits misinterpreted without first examining the functional outcomes of a given set of morphological characters?

A perfect example of this is the presumed mechanical equivalency of many kinds of 'hard prey' in the literature. These mechanical and material considerations influence the evolution of dietary transitions. For example, the success of early hominids is frequently predicated upon the evolution of robust molars to masticate less-nutritious but abundant grasses, legumes, and nuts (Lucas et al., 2013). Durophagy as a catch-all term in the ecological and evolutionary literature has often amalgamated prey resources like insects, nuts, fruits, bone, mollusks, and even softshell crab molts; essentially treating these prey as being of comparable structural and material behavior (Gosler, 1986; Herrel et al., 2001; Mara et al., 2009). As a point of comparison, the morphological structures comprising the feeding apparatus of insectivorous (Chapter 3) and molluscivorous (Chapter 2) stingrays are found on opposing ends of an anatomical spectrum which essentially equates to a gradient from kinetic to akinetic jaw skeletons, respectively. Fusion of the upper and lower jaw rami (palatoquadrate and Meckel's cartilages, respectively) and increased imbrication of the teeth towards a singular, plate-like tooth surface is characteristic of increasing rigidity and strength in molluscivorous durophagous stingray jaws (Summers, 2000; Kolmann et al., 2014). The opposing trend seen in insectivorous stingrays is towards incredible jaw kinesis - unfused jaw symphyses, reoriented teeth on flexible dental ligaments, and the evolution of an additional joint via the Meckelian-Angular articulation (Chapter 2). Tough prey like insects requires shearing forces to effectively tear or shred the cuticle, while composite ceramics like mollusk shell require considerable compressive force, either outright or through successive winnowing. One can imagine that the morphological disparity between these two 'durophagous' feeding strategies, insect- and mollusk-feeding, would be grossly

misrepresented in studies of adaptive evolution if insectivores and molluscivores were combined into similar ecological categories.

Perhaps what is so remarkable regarding adaptive evolution is that such disparate morphologies as those underlying dietary modes such as molluscivory and insectivory, can arise in such closely-related animals over admittedly brief time periods. The divergence between insect specialist Potamotrygon orbignyi and molluscivores such as P. leopoldi and P. henlei are on the magnitude of 6 million years (Chapter 3). Potamotrygonids appear to be incredibly plastic in their ability to shift feeding modes and drastically change their morphology to fit this shift in ecology. Whereas other marine-derived fish lineages kept mostly to ancestral feeding strategies, with some exceptions being large piscivorous freshwater anchovies like Lycengraulis, other MDLs like needlefishes, drum, and toadfishes largely remained settled in ancestral niches (Bloom and Lovejoy, 2012). Potamotrygonids represent an abrupt ecological departure from their marine relatives, at least immediately in comparison to amphi-American *Himantura*, but also in general – there are very few if any exclusively molluscivorous dasyatoid rays. As mentioned earlier, there are no examples of insectivorous rays outside potamotrygonids, at least that I am aware of. This pattern is impressive, considering that the species richness of the dasyatoid rays excluding Potamotrygonidae (Urotrygonidae, Dasyatidae, Urolophidae, Plesiobatidae) is four times greater than that of potamotrygonids. Potamotrygonids, given their impressive and rapidly-evolved trophic and morphological diversity offer a potent study system for understanding adaptive evolution.

The identification of model systems such as potamotrygonids and myliobatids, animals which are morphological and ecological outliers within their immediate phylogenetic schema, offer potent model systems for studying adaptive evolution. Not to mention that for evolutionary theory to be tested at a truly holistic scale, as many different organisms as possible must be brought into the fold, not simply model systems (of which elasmobranchs are definitely not). With robust molecular phylogenies being present now for most vertebrate lineages, the opportunity is there to test for repeated patterns of trait evolution across diverse clades.

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Supplemental Figure 1a: Gene tree for ATPase Subunit 6 from PhyML maximum likelihood reconstruction using the GTR + gamma model.

# Appendices



Supplemental Figure 1b: Gene tree for COI from PhyML maximum likelihood reconstruction using the GTR + gamma model.



Supplemental Figure 1c: Gene tree for cytb from PhyML maximum likelihood reconstruction using the GTR + gamma model.



Supplemental Figure 1d: Gene tree for RAG1 from PhyML maximum likelihood reconstruction using the GTR + gamma model.



Supplemental Figure 1e: Gene tree for ENC1 from PhyML maximum likelihood reconstruction using the GTR + gamma model.



Supplemental Figure 1f: Gene tree for ITS1 from PhyML maximum likelihood reconstruction using the GTR + gamma model.



Supplemental Figure 1g Gene tree for SCFD2 from PhyML maximum likelihood reconstruction using the GTR + gamma model.