# MODEL BEHAVIOR OF NON-MODEL ORGANISMS: ADAPTIVE BEHAVIOR IN DESERT RODENTS AND OSTEOGLOSSIFORMES FISHES

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# ABSTRACT

MODEL BEHAVIOR OF NON-MODEL ORGANISMS: ADAPTIVE BEHAVIOR IN DESERT RODENTS AND OSTEOGLOSSIFORMES FISHES

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Model organisms are useful tools for investigating generalities in biology and have greatly advanced current understanding of adaptive behavior. Yet Krogh's principle—that there exists specialized species most convenient for answering specific biological questions—suggests that non-model organisms may be best suited for identifying specific genetic and physiological underpinnings of behavior. In fact, non-model organisms with unique traits have been instrumental for examining the potential selective pressures and underlying genetic mechanisms of adaptive behavior. I present two groups of vertebrates, desert rodents and Osteoglossiformes fishes, as exemplary non-model organisms that support Krogh's principle and are particularly appropriate for the integrative study of predatory and reproductive behaviors, respectively. I first compare the history, benefits, and limitations of standard laboratory and nontraditional model species before describing the particular biological traits that make desert rodents and osteoglossiforms poised to be useful nontraditional model organisms. In Chapter 1, I analyze the predatory behavior of desert rodents and find that carnivorous grasshopper mice (*Onychomys torridus*) are the most persistent and efficient rodent predators of chemically defended beetles and undefended crickets. Their success is due to their attack behavior, even though they are exposed to the beetles' benzoquinone spray. These results suggest that grasshopper mice's predatory behavior enables them to potentially outcompete related rodents and occupy an obligately carnivorous dietary niche in the desert. In Chapter 2, I demonstrate that grasshopper mice are equally gustatorily sensitive and averse to benzoquinone as laboratory house mice (*Mus*

*musculus*), but they also contain mutations in a sensory channel known to be activated by benzoquinone. Grasshopper mice and their relatives may therefore be informative models for exploring chemosensory mechanisms and optimal foraging theory. In Chapter 3, I review the diverse reproductive anatomy, morphology, and behavior of Osteoglossiformes fishes, and the role of sperm competition and sexual selection in shaping their reproductive adaptations. This review highlights the ways in which osteoglossiforms can be used as models for understanding reproductive diversity among teleost fish, as well as the evolution of body plan development, fertilization strategies, and parental care. I conclude with a discussion about how the results from this work provide new species-specific information and exemplify the ways in which these systems can be used within a comparative framework to contribute to biomedicine, conservation, and other broader biological topics. Advancements in the ability to link genes to behavior suggest that focusing on more diverse nontraditional model systems is an increasingly feasible way to expand comprehensive knowledge of adaptive behavior. This dissertation proposes that embracing exemplary species, such as grasshopper mice and Osteoglossiformes, is the next step for developing new investigations inspired from work using model species, and for capturing the complexity of natural variation within experimental research.

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# INTRODUCTION

Humans have observed and evaluated animal behavior as long as there have been interactions with other species. Cave paintings depict horses, bison, and pigs, and record an ancient interest in megafauna dating back tens of thousands of years. Using rough tools like crushed minerals or charcoal, these naturalistic and detailed paintings would have required careful observation to capture an animal's behavior.

In modern times, animal behavior and its underlying complexity has become an interdisciplinary puzzle, as variation in many natural behaviors is now known to have a genetic component (Croston *et al.*, 2015; Polderman *et al.*, 2015). However, the ability to piece together the genetic underpinnings of behavior with its effects on morphology and physiology is still an emerging field, in spite of advances in technological methods for linking genes to behavior. This is partly due to most knowledge of behavioral genetics having been generated from a limited number of model organisms (Juntti, 2019; Niepoth and Bendesky, 2020). Just like the cave painting artists, contemporary researchers often focus on a few specific species; most of these model species are widely studied in the laboratory to understand particular biological phenomena. Yet, as more technological advances in science allow for a growing number of non-model species to become tractable research tools, the term "model organism" has come under controversy for being too loosely defined, having incompatible definitions, or being overused (Katz, 2016). Evaluating non-models for their versatility across biological disciplines may open the door for redefining exemplary species as more specialized, non-traditional models better suited for advancing biological research.

In this section, I will briefly review the history, benefits, and limitations of using traditional laboratory model species for researching adaptive behavior. I will then discuss the concept of Krogh's principle—that there exists specialized species best suited for answering particular research questions (Krogh, 1929)—and the feasibility and advantages of conducting research on specialized non-model species. Subsequently, I will outline my dissertation, which comprises two distinct examples in support of Krogh's principle. This work will examine behaviors of different vertebrate non-model organisms, predatory behavior in grasshopper mice (*Onychomys torridus*) and reproductive behavior in Osteoglossiformes "bony-tongue" fishes, to demonstrate that as nontraditional models, these animals can contribute species-specific and broader biological knowledge on the sensory, physiological, morphological, and genetic bases of animal behavior.

## 1. An overview of model organisms

According to the National Science Foundation, traditional model organisms are small, simple, produce many offspring, and develop quickly (Arriens, 2016); these traits allow them to serve as tractable systems for studying larger themes of biology such as the principles of heredity (Morgan, 1910), the genetic code (Nirenberg and Matthaei, 1961), transcription (Reece-Hoyes *et al.*, 2005; Shaulian and Karin, 2002), translation (Bolton *et al.*, 1984; Gan and Jewett, 2014), DNA replication (Friedman *et al.*, 1997; Pourkarimi *et al.*, 2016), development (Gritsman *et al.*, 1999; Malassine *et al.*, 2003; Nüsslein-Volhard and Wieschaus, 1980), and disease (Dawson *et al.*, 2018; Faillaci *et al.*, 2018; Washington *et al.*, 2009). Classic models include the bacterium *Escherichia coli,* the unicellular green alga *Chlamydomonas reinhardtii*, the nematode *Caenorhabditis elegans,* the plant maize (*Zea mays*), and the fruit fly (*Drosophila melanogaster*). Model vertebrate representatives include house mice (*Mus musculus*), rats (*Rattus norvegicus*), African clawed frogs (*Xenopus laevis*), and zebrafish (*Danio rerio*). Each of these model organisms can be used to provide information about other species or processes that may be more difficult to study directly. For instance, rat tumors were historically chosen as a proxy for studying human cancer because unlike human tumors, rat tissue could be controlled to contain only actively growing, quantifiable cells (Krebs, 1975). Giant squid axons considered to be larger, more malleable representatives of the nervous system compared to smaller, less workable vertebrate axons (Hodgkin and Huxley, 1952). Thus, these organisms also fulfill one of the main criteria of model organisms by being useful for identifying similarities across species (Milani and Ghiselli, 2020). While no model system is able to stand up to all assumptions or be applicable to all situations, some model organisms are more adaptable than others to a greater variety of contexts.

The second half of the 20th century saw major advances in linking genes to behavior as a result of work with model organisms and the rise of inbred lines. Mutations in genes related to flagella motility in *E. coli* were found to affect coordinated swarming behavior (Harshey and Matsuyama, 1994), while mutated olfactory receptor proteins in *C. elegans* were found to disrupt chemotaxis in response to specific odorant ligands (Bono and Villu Maricq, 2005; Sengupta *et al.*, 1996). A large proportion of progress in understanding gene function was due to forward genetics work in *Drosophila*, in which randomly introduced mutations were matched with resultant phenotypes. For example, a single gene, *period*, was found to affect *Drosophila* circadian rhythms, in which three different mutations led to variations in the normal 24-hour rhythm (Konopka and Benzer, 1971). Moreover, male flies with mutations that lead to aberrant splicing in the *fruitless* gene incur a variety of phenotypes related to sex-specific behavior, including a failure to attempt copulation with females and other abnormal patterns in gene-controlled courtship rituals (for a review see Hall, 1994). Studies that found both *fruitless*

(Wheeler *et al.*, 1989) and *period* mutations generate abnormalities in the male courtship song provide striking examples of the role of multiple genes in regulating complex behaviors like reproduction and circadian rhythms (Kyriacou and Hall, 1980).

The convenience of model organisms generated a positive feedback cycle within the scientific community: more infrastructure, inbred lines, community databases, and resources were created to support their use as research tools, which only reinforced their convenience (Matthews and Vosshall, 2020; Russell *et al.*, 2017). Yet, there is still a wealth of information to be gained from studying model organisms. For example, there have been recent calls to integrate genomics with field studies to explore topics such as the role of reproduction in biocontrol and the relationship between microbiology and health (Alfred and Baldwin, 2015; Markow, 2015). Many model systems also remain the easiest tools in several experimental contexts. Since early research in neurobiology, the simple central nervous system of the mollusc (*Lymnaea stagnalis*) has consistently been viewed as highly advantageous for investigating general ways in which single neurons operate as part of behavioral circuitry, such as in feeding behavior (Benjamin and Crossley, 2020), even though advances in technology now allow for those experiments to be performed on vertebrate species as well (Fodor *et al.*, 2020). The low background activity of *Xenopus laevis* oocytes makes them popular in neurobiology and physiology work for characterizing membrane transporters and channels of other systems, including for humans, which are less easy to study *in vivo* (Bröer, 2010). More advanced technical tools, combined with genomic databases, have allowed researchers to revisit previous genotype-phenotype questions with new results. For example, quantitative trait locus (QTL) mapping revealed new genes affecting natural variation in aggressive behavior in two *Drosophila* lines with epistatic effects; these genes were not identified by an older study using genome expression profiling (Edwards and Mackay, 2009). A later study then combined QTL mapping with a Genome Wide Association Study (GWAS) to examine 200 *Drosophila* lines; both new and previously identified genes were confirmed to have epistatic interactions and effects on aggressive behavior (Shorter *et al.*, 2015).

Model organisms have helped clarify many aspects of biology, but they are unlikely to be the best systems for all possible questions or valid models for all biological processes (Goldstein and King, 2016; Russell *et al.*, 2017). Long-term domestication and adaptation to the laboratory can prevent valid comparisons between lab animals and those in nature. The Mouse Genome Informatics database contains over 10,000 artificial mutations that affect behavior in the lab, but would be detrimental to survival if they spontaneously occurred in nature (Niepoth and Bendesky, 2020). Wild populations of zebrafish have more extensive genetic diversity than seen in lab strains (Alfred and Baldwin, 2015). Organisms best suited for laboratory work may be mismatched models for evolutionary studies of adaptation, since the very traits that make model species easy to raise in captivity also allowed them to evade selective pressures that longer-lived, slower-growing species must cope with in the wild (Alfred and Baldwin, 2015). Many models are also not suitable for drawing comparisons with humans, which is a major challenge in biomedical research. For this reason, there has been a call to expand research into the spotted gar (*Lepisosteus oculatus*) as a potential model species that can serve as a bridge between humans and the more distantly related standard zebrafish model (Braasch *et al.*, 2016).

## 2. Models vs. nontraditional models

To overcome the limitations of available models, a growing number of non-model species have been labeled as emerging models. This has led to increased confusion and debate over the meaning of the terminology. The general definition of the word “model” typically refers to a system used as an example of something else. But an animal disease model, a widespread term in biomedical research, does not ascribe to this definition, as the disease itself is being studied directly (Katz, 2016). Alternatively, the National Institutes of Health suggests that a model species is an organism which is genetically modified or most widely studied in the laboratory (National Institutes of Health, 2018). Many traits of these species, however, conflict with the National Science Foundation’s definition of models as species characterized by being small, simple, and fast-growing (Arriens, 2016). Even traditional house mice, widely accepted as models across the scientific community, are relatively long-lived compared to other models such as *e. coli* and *c. elegans,* and they are not particularly simple organisms.

An alternative, more general definition has been offered by Russell *et al.*, which aims to simplify the difference between traditional models and non-model species: all model species serve as useful examples of larger processes, but nontraditional models lack the convenience and infrastructure currently available for traditional models (2017). Nonetheless, it can be challenging to decide whether a non-model species can become a nontraditional model species, even if this infrastructure is developed. More specific phrasing referring to which aspects of particular species are exemplary may help resolve this conflict, especially as more laboratories are built around research on specific organisms. For the purpose of this dissertation, I will use the definition of the word “model” to refer to a species or system that is exemplary of a biological process and consider the difference between models and nontraditional model species as those which are widely and historically studied in the lab compared to those which are not, respecitvely.

Within the next decade, this definition will likely need to be reassessed for the very reason the distinction between model and non-model species became blurred in the first place: The development of genomics tools opens the door for studying nontraditional models that have previously lacked the infrastructure and resources to enable their widespread use as study species. High-throughput sequencing, QTL mapping, GWAS, and clustered regularly interspaced short palindromic repeats (CRISPR-CAS9) genome editing technology have all lately revealed new functional genetics information from non-model organisms (Bendesky *et al.*, 2017; Buniello *et al.*, 2019; Doudna and Charpentier, 2014; Goldstein and King, 2016; Harel *et al.*, 2016; Reese *et al.*, 2020). The rise of these cost-effective techniques could facilitate research with non-model species and ultimately enable more species to be investigated as comprehensively as traditional models.

## 3. Krogh's Principle and nontraditional model species

August Krogh articulated the value of selecting a study organism in 1929, stating "For such a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied" (Krogh, 1929). Krogh argued that comparative and species-specific studies have greater value beyond knowledge of biodiversity for its own sake; building knowledge of biological mechanisms and their modifications will also help solve general problems related to form and function (Krogh, 1929). Krogh's principle refers to specialized organisms serving as models for studying a specific problem, and thus is not limited to popular model species used generally across disciplines due to their convenience. Nearly a century later, accumulated knowledge of diverse species and the ability to apply efficient tools to study them has led to revived interest in studying non-model organisms as research models (Russell *et al.*, 2017).

These nontraditional models typically share some of the traits of traditional models such as genomic resources or high fecundity, as well as unique biological features that can be leveraged to address questions of general importance (Russell *et al.*, 2017). Recently, there has been a focus on organisms with unusual and informative adaptations related to extreme stress survival. Microscopic animals known as tardigrades have species-specific proteins that promote desiccation tolerance (Boothby *et al.*, 2017) and protect them from DNA damage (Hashimoto *et al.*, 2016), which may explain how they can survive intense cold and unfiltered solar radiation in the vacuum of space (Jönsson *et al.*, 2008). These traits do not exist in other models, rendering tardigrades the only known organism for testing mechanisms of tolerating such extremes (Goldstein and King, 2016). The giant ciliate, *Stentor coeruleus*, has long been known to have a profound capacity to heal its own wounds (Morgan, 1901), and *Stentor's* recently sequenced genome makes it a prime candidate for testing the genes involved in regeneration (Russell *et al.*, 2017). Molecular studies of regeneration and repatterning in *Stentor* may also provide new information about the processes by which cells generate, establish internal organization, and regulate morphology (Russell *et al.*, 2017). Versatile non-model organisms can become models for a diversity of research questions and broad biological functions, especially those that can capitalize off of previous foundational work built from traditional models.

In order to use a nontraditional model system to comprehensively explore evolutionary and ecological questions, it is helpful to understand its position within the context of genetic and phenotypic variation (Duffy *et al.*, 2021). A single species may be informative when studied as part of a model system (Duffy *et al.*, 2021), in which researchers compare one question at the same level of biological organization across multiple species (Travis, 2006). While any one species will be representative of some processes in biology and distinctive in others, “there can be as much to learn from a system that is an exception to a rule as from one that adheres to it” (Duffy *et al.*, 2021). Species that have these exceptions can help test the limitations or plasticity of a biological process (Milani and Ghiselli, 2020) and, at a minimum, serve as distinctive experimental systems for modeling unexplained phenomena (Rheinberger, 2007). In line with this concept, my work examines two different groups of species, focusing on organisms with unique traits and behaviors and the way these may be shaped by evolutionary pressures and genetics. Promising nontraditional model species or multispecies systems may therefore comprise attributes of models from both biomolecular and ecology, evolutionary, and behavior standpoints.

## 4. Desert rodents and Osteoglossiformes as nontraditional models

My dissertation will demonstrate the use of two non-model systems, desert rodents and Osteoglossiformes fishes, to improve knowledge in two key areas of biological research: chemosensation and reproduction. Sensory systems and reproductive traits are both incredibly diverse across the animal kingdom, but detailed studies of every species are prohibitively expensive and unrealistic. Conclusions based on traditional biomolecular model species alone, however, can lead to overgeneralized explanations of this diversity (Milani and Ghiselli, 2020). More refined understanding can be accomplished through multispecies comparisons involving carefully selected exemplary species that are feasible and better suited biologically and phylogenetically for addressing these questions. Comparing these species—which possess specialized sensory or reproductive traits—with those that lack them, enables improved identification of conserved features (Niepoth and Bendesky, 2020) and enhances understanding of the rules of selection (Montgomerie and Fitzpatrick, 2009). If desert rodents and osteoglossiforms continue to be incorporated in future research on these topics, they may be classified as new nontraditional model systems that supplement the work of traditional laboratory models.

Chemosensation is the detection of chemicals, such as by olfaction and gustation, and is a key feature of all organisms (Spielman *et al.*, 2019). In particular, the ability to detect noxious, painful substances is highly conserved because it helps organisms detect harmful stimuli and respond accordingly to prevent detrimental effects. The grasshopper mouse is one organism that displays unique chemosensory abilities as an obligate carnivore that predates on numerous chemically defended prey. Grasshopper mouse predatory behavior is much speedier and more effective than that of its sympatric desert rodent relatives; this positions the grasshopper mouse as a model organism well-suited for studying the plasticity of foraging behavior and learning mechanisms (e.g., Langley, 1981). Considering they consume many of the same prey as related insectivorous species, comparing grasshopper mice with other desert rodents may reveal the selective forces and constraints that facilitate carnivory and exceptional hunting speeds within the clade. Grasshopper mice also have a unique mechanism of pain resistance to scorpions (Rowe *et al.*, 2013), one of their venomous prey types, which is unique compared to other traditional rodent models used to study pain (Munro *et al.*, 2017). As a result, grasshopper mice are well suited for studies of natural variation in chemosensation.

As with sensory systems, comprehensive knowledge of reproductive adaptation is impossible if research is limited to a small number of traditional models that express only one or a few variations of each reproductive trait. Between species, genes involved in sexual reproduction evolve rapidly and are often the most divergent; these dynamic genetic changes may be responsible for diverse morphology, fertilization strategies, and subsequent reproductive behaviors (Arnqvist, 1998; Hosken and Stockley, 2004; Singh and Kulathinal, 2000; Swanson and Vacquier, 2002; Torgerson *et al.*, 2002). One example of a related group of organisms that encompasses this diversity is the Osteoglossiformes, an order of fish considered the most ancient living order of teleost fishes. Most species have unpaired gonads and two osteoglossiform families contain the only known vertebrate species with aflagellate sperm. Several families have reproductive anatomy associated with internal fertilization but perform external fertilization, which may be representative of the evolutionary transition from external to internal fertilization. Osteoglossiforms also employ every type of parental care seen in vertebrates, including the ancestral state of absent parental care and cooperative breeding. Taken together, osteoglossiforms' unusual reproductive anatomy and gamete structure emphasize their capacity to be used as models for clarifying the genetic underpinnings of body plan development and sperm cell morphology, while their range of reproductive behaviors suggests they may be an effective system for understanding the evolution of paternal care.

Detailed investigation of the evolution and functional aspects of osteoglossiform reproduction has the potential to yield not only taxon-specific discoveries, but new insight into the genetic basis of reproductive morphology and its relationship to behavior. Many of their unusual traits and behaviors indicate low levels of sperm competition and possible tradeoffs between sperm competition and the environment. Subsequently, their position as a sister clade to other ancestral teleost fishes could potentially be used to help highlight the role of reproduction and sexual selection in speciation (Ritchie, 2007; West-Eberhard, 1983) and radiation (Rabosky *et al.*, 2013), and allow Osteoglossiformes to serve as a model for the diversification of reproductive strategies and teleost evolution.

The following chapters evaluate the underlying factors shaping adaptive behavior in grasshopper mice and related desert rodents and Osteoglossiformes fishes, in which I demonstrate how behavioral data from these species within a comparative framework positions them as nontraditional model organisms. In Chapter 1, I show that grasshopper mice are more efficient and persistent predators of both chemically defended stink beetles and undefended crickets than related desert rodents. These results indicate that grasshopper mice consume these beetles in spite of exposure to the insect's highly concentrated, toxic benzoquinone spray, suggesting that the mice may have an underlying physiological or genetic mechanism that protects them from the spray's noxious effects.

In Chapter 2, I examine the gustatory sensitivity of grasshopper mice and house mice to benzoquinone. In a two-bottle choice test, both species are equally averse to the chemical, in spite of grasshopper mice's consumption of beetles containing far higher concentrations of benzoquinone in nature. I show that the genetic sequence of a highly conserved benzoquinone agonist and sensory receptor, TRPA1 (transient receptor potential ankyrin 1), has single amino acid variants that may affect grasshopper mice TRPA1 sensitivity. I discuss the role of TRPA1 in facilitating grasshopper mice predation of stink beetles within the context of other physiological factors that may influence behavior.

In Chapter 3, I review the remarkable diversity of adaptive reproductive morphology and behavior within Osteoglossiformes fishes, and the role of sperm competition and sexual selection as a whole in shaping their reproductive adaptations. Two notable adaptations include unpaired gonads and the only known occurrence of aflagellate sperm in vertebrates. By addressing opportunities for improving knowledge on reproductive form and function in osteoglossiforms, this review also identifies new pathways for benefitting current understanding of fertilization strategies and parental care.

The vertebrate species presented here as examples of nontraditional model organisms have the capacity to provide novel yet fundamental scientific insights into adaptive behavior. Grasshopper mice have genetic and phenotypic adaptations that appear to be absent in sympatric, related species incapable of handling toxic prey. Osteoglossiform reproduction represents a microcosm of teleost reproductive diversity, potentially signifying the genetic flexibility that contributed to the adaptive radiation of teleost fishes. These nontraditional model organisms' phylogenetic relationships to current model species may also facilitate new work in comparative biology. Studies that use multispecies comparisons, such as work seeking to identify genetic orthologs underlying an adaptive trait, can benefit from information derived from traditional model species.

Resources developed using traditional models may also be applied to related nontraditional models. For example, as demonstrated in this work, research on grasshopper mice can take advantage of knowledge and infrastructure developed from prior studies conducted on house mice and deer mice, another emerging nontraditional model species (Bedford and Hoekstra, 2015). Similarly, research using osteoglossiforms to study teleost radiation can incorporate tools and techniques practiced in zebrafish, the representative teleost fish model species. The use of these animals in laboratory studies has revealed that many of them can be kept in captivity and genomic information is available for grasshopper mice, multiple Osteoglossiformes species, and numerous related taxa on community-wide databases. In short, non-model organisms like grasshopper mice and Osteoglossiforms are essential pieces of the animal behavior puzzle. Only by including more non-model organisms can we come close to a comprehensive understanding of the genetic underpinnings of behavior and associated effects on morphology and physiology.

# REFERENCES

# REFERENCES

Alfred, J. & Baldwin, I. T. (2015). The natural history of model organisms: new opportunities at the wild frontier. *Elife* **4**, e06956.

Arnqvist, G. (1998). Comparative evidence for the evolution of genitalia by sexual selection. *Nature* **393**, 784-786.

Arriens, J. (2016). *Earth's weird and wonderful animal models*. National Science Foundation. https://www.nsf.gov/discoveries/disc_summ.jsp?cntn_id=138386

Bedford, N. L. & Hoekstra, H. E. (2015). The natural history of model organisms: Peromyscus mice as a model for studying natural variation. *Elife* **4**, e06813.

Bendesky, A., Kwon, Y.-M., Lassance, J.-M., Lewarch, C. L., Yao, S., Peterson, B. K., He, M. X., Dulac, C. & Hoekstra, H. E. (2017). The genetic basis of parental care evolution in monogamous mice. *Nature* **544**, 434-439.

Benjamin, P. & Crossley, M. (2020). Gastropod Feeding Systems: Evolution of Neural Circuits That Generate Diverse Behaviors. In *Oxford Research Encyclopedia of Neuroscience*.

Bolton, V., Oades, P. & Johnson, M. (1984). The relationship between cleavage, DNA replication, and gene expression in the mouse 2-cell embryo. *Development* **79**, 139-163.

Bono, M. d. & Villu Maricq, A. (2005). Neuronal substrates of complex behaviors in C. elegans. *Annu. Rev. Neurosci.* **28**, 451-501.

Boothby, T. C., Tapia, H., Brozena, A. H., Piszkiewicz, S., Smith, A. E., Giovannini, I., Rebecchi, L., Pielak, G. J., Koshland, D. & Goldstein, B. (2017). Tardigrades use intrinsically disordered proteins to survive desiccation. *Molecular cell* **65**, 975-984. e975.

Braasch, I., Gehrke, A. R., Smith, J. J., Kawasaki, K., Manousaki, T., Pasquier, J., Amores, A., Desvignes, T., Batzel, P. & Catchen, J. (2016). The spotted gar genome illuminates vertebrate evolution and facilitates human-teleost comparisons. *Nature genetics* **48**, 427-437.

Bröer, S. (2010). Xenopus laevis oocytes. In *Membrane Transporters in Drug Discovery and Development*, pp. 295-310: Springer.

Buniello, A., MacArthur, J. A. L., Cerezo, M., Harris, L. W., Hayhurst, J., Malangone, C., McMahon, A., Morales, J., Mountjoy, E. & Sollis, E. (2019). The NHGRI-EBI GWAS Catalog of published genome-wide association studies, targeted arrays and summary statistics 2019. *Nucleic acids research* **47**, D1005-D1012.

Croston, R., Branch, C., Kozlovsky, D., Dukas, R. & Pravosudov, V. (2015). Heritability and the evolution of cognitive traits. *Behavioral Ecology* **26**, 1447-1459.

Dawson, T. M., Golde, T. E. & Lagier-Tourenne, C. (2018). Animal models of neurodegenerative diseases. *Nature neuroscience* **21**, 1370-1379.

Doudna, J. A. & Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *science* **346**.

Duffy, M., García-Robledo, C., Gordon, S. P., Grant, N. A., II, D. A. G., Kamath, A., Penczykowski, R. M., Gómez, M. R., Wale, N. & Zaman, L. (2021). Model systems in ecology, evolution, and behavior: A call for diversity in our model systems and discipline.

Edwards, A. C. & Mackay, T. F. (2009). Quantitative trait loci for aggressive behavior in Drosophila melanogaster. *Genetics* **182**, 889-897.

Faillaci, F., Milosa, F., Critelli, R. M., Turola, E., Schepis, F. & Villa, E. (2018). Obese zebrafish: a small fish for a major human health condition. *Animal models and experimental medicine* **1**, 255-265.

Fodor, I., Hussein, A. A., Benjamin, P. R., Koene, J. M. & Pirger, Z. (2020). The natural history of model organisms: the unlimited potential of the great pond snail, Lymnaea stagnalis. *Elife* **9**, e56962.

Friedman, K. L., Brewer, B. J. & Fangman, W. L. (1997). Replication profile of Saccharomyces cerevisiae chromosome VI. *Genes to Cells* **2**, 667-678.

Gan, R. & Jewett, M. C. (2014). A combined cell-free transcription-translation system from Saccharomyces cerevisiae for rapid and robust protein synthe. *Biotechnology journal* **9**, 641-651.

Goldstein, B. & King, N. (2016). The future of cell biology: emerging model organisms. *Trends in cell biology* **26**, 818-824.

Gritsman, K., Zhang, J., Cheng, S., Heckscher, E., Talbot, W. S. & Schier, A. F. (1999). The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. *Cell* **97**, 121-132.
Hall, J. C. (1994). The mating of a fly. *science* **264**, 1702-1714.

Harel, I., Valenzano, D. R. & Brunet, A. (2016). Efficient genome engineering approaches for the short-lived African turquoise killifish. *Nature protocols* **11**, 2010-2028.

Harshey, R. M. & Matsuyama, T. (1994). Dimorphic transition in Escherichia coli and Salmonella typhimurium: surface-induced differentiation into hyperflagellate swarmer cells. *Proceedings of the National Academy of Sciences* **91**, 8631-8635.

Hashimoto, T., Horikawa, D. D., Saito, Y., Kuwahara, H., Kozuka-Hata, H., Shin, T., Minakuchi, Y., Ohishi, K., Motoyama, A. & Aizu, T. (2016). Extremotolerant tardigrade genome and improved radiotolerance of human cultured cells by tardigrade-unique protein. *Nature communications* **7**, 1-14.

Hodgkin, A. L. & Huxley, A. F. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *The Journal of physiology* **117**, 500-544.

Hosken, D. J. & Stockley, P. (2004). Sexual selection and genital evolution. *Trends in Ecology & Evolution* **19**, 87-93.

Jönsson, K. I., Rabbow, E., Schill, R. O., Harms-Ringdahl, M. & Rettberg, P. (2008). Tardigrades survive exposure to space in low Earth orbit. *Current Biology* **18**, R729-R731.

Juntti, S. (2019). The future of gene-guided neuroscience research in non-traditional model organisms. *Brain, behavior and evolution* **93**, 108-121.

Katz, P. S. (2016). 'Model organisms' in the light of evolution. Current Biology 26, R649-R650.

Konopka, R. J. & Benzer, S. (1971). Clock mutants of Drosophila melanogaster. *Proceedings of the National Academy of Sciences* **68**, 2112-2116.

Krebs, H. A. (1975). The August Krogh principle:"For many problems there is an animal on which it can be most conveniently studied". *Journal of Experimental Zoology* **194**, 221-226.

Krogh, A. (1929). The progress of physiology. *American Journal of Physiology-Legacy Content* **90**, 243-251.

Kyriacou, C. P. & Hall, J. C. (1980). Circadian rhythm mutations in Drosophila melanogaster affect short-term fluctuations in the male's courtship song. *Proceedings of the National Academy of Sciences* **77**, 6729-6733.

Langley, W. (1981). Failure of food-aversion conditioning to suppress predatory attack of the grasshopper mouse, Onychomys leucogaster. *Behavioral and Neural Biology* **33**, 317-333.

Malassine, A., Frendo, J. L. & Evain-Brion, D. (2003). A comparison of placental development and endocrine functions between the human and mouse model. *Human reproduction update* **9**, 531-539.

Markow, T. A. (2015). The natural history of model organisms: the secret lives of Drosophila flies. *Elife* **4**, e06793.

Matthews, B. J. & Vosshall, L. B. (2020). How to turn an organism into a model organism in 10 'easy'steps. *Journal of Experimental Biology* **223**.

Milani, L. & Ghiselli, F. (2020). Faraway, so close. The comparative method and the potential of non-model animals in mitochondrial research. *Philosophical Transactions of the Royal Society B* **375**, 20190186.

Montgomerie, R. & Fitzpatrick, J. L. (2009). Testes, Sperm, and Sperm Competition. In *Reproductive Biology and Phylogeny of Fishes (Agnathans and Bony Fishes): Phylogeny, Reproductive System, Viviparity, Spermatozoa* (Jamieson, B. G., ed.), pp. 1-53: CRC Press.

Morgan, T. H. (1901). Regeneration of proportionate structures in Stentor. *The Biological Bulletin* **2**, 311-328.

Morgan, T. H. (1910). Sex limited inheritance in Drosophila. *science* **32**, 120-122.
Munro, G., Jansen-Olesen, I. & Olesen, J. (2017). Animal models of pain and migraine in drug discovery. *Drug discovery today* **22**, 1103-1111.

National Institutes of Health. (2018). *Model Organisms*. National Institutes of Health. https://public.csr.nih.gov/FAQs/ReviewersFAQs/ModelOrganisms

Niepoth, N. & Bendesky, A. (2020). How natural genetic variation shapes behavior. *Annual review of genomics and human genetics* **21**, 437-463.

Nirenberg, M. W. & Matthaei, J. H. (1961). The dependence of cell-free protein synthesis in E. coli upon naturally occurring or synthetic polyribonucleotides. *Proceedings of the National Academy of Sciences* **47**, 1588-1602.

Nüsslein-Volhard, C. & Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. *Nature* **287**, 795-801.

Polderman, T. J., Benyamin, B., De Leeuw, C. A., Sullivan, P. F., Van Bochoven, A., Visscher, P. M. & Posthuma, D. (2015). Meta-analysis of the heritability of human traits based on fifty years of twin studies. *Nature genetics* **47**, 702-709.

Pourkarimi, E., Bellush, J. M. & Whitehouse, I. (2016). Spatiotemporal coupling and decoupling of gene transcription with DNA replication origins during embryogenesis in C. elegans. *Elife* **5**, e21728.

Rabosky, D. L., Santini, F., Eastman, J., Smith, S. A., Sidlauskas, B., Chang, J. & Alfaro, M. E. (2013). Rates of speciation and morphological evolution are correlated across the largest vertebrate radiation. *Nature communications* **4**, 1-8.

Reece-Hoyes, J. S., Deplancke, B., Shingles, J., Grove, C. A., Hope, I. A. & Walhout, A. J. (2005). A compendium of Caenorhabditis elegans regulatory transcription factors: a resource for mapping transcription regulatory networks. *Genome biology* **6**, 1-12.

Reese, R. M., Dourado, M., Anderson, K., Warming, S., Stark, K. L., Balestrini, A., Suto, E., Lee, W., Riol-Blanco, L. & Shields, S. D. (2020). Behavioral characterization of a CRISPR-generated TRPA1 knockout rat in models of pain, itch, and asthma. *Scientific reports* **10**, 1-11.

Rheinberger, H.-J. (2007). Experimental model systems: An epistemological apercu from the perspective of molecular biology. *Modeling biology: Structure, behaviors, evolution* **37**, 46.

Ritchie, M. G. (2007). Sexual selection and speciation. *Annu. Rev. Ecol. Evol. Syst.* **38**, 79-102.

Rowe, A. H., Xiao, Y., Rowe, M. P., Cummins, T. R. & Zakon, H. H. (2013). Voltage-gated sodium channel in grasshopper mice defends against bark scorpion toxin. *science* **342**, 441-446.

Russell, J. J., Theriot, J. A., Sood, P., Marshall, W. F., Landweber, L. F., Fritz-Laylin, L., Polka, J. K., Oliferenko, S., Gerbich, T. & Gladfelter, A. (2017). Non-model model organisms. *BMC biology* **15**, 1-31.

Sengupta, P., Chou, J. H. & Bargmann, C. I. (1996). odr-10 encodes a seven transmembrane domain olfactory receptor required for responses to the odorant diacetyl. *Cell* **84**, 899-909.

Shaulian, E. & Karin, M. (2002). AP-1 as a regulator of cell life and death. *Nature cell biology* **4**, E131-E136.

Shorter, J., Couch, C., Huang, W., Carbone, M. A., Peiffer, J., Anholt, R. R. & Mackay, T. F. (2015). Genetic architecture of natural variation in Drosophila melanogaster aggressive behavior. *Proceedings of the National Academy of Sciences* **112**, E3555-E3563.

Singh, R. S. & Kulathinal, R. J. (2000). Sex gene pool evolution and speciation: a new paradigm. *Genes & genetic systems* **75**, 119-130.

Spielman, A. I., Yan, W. & Brand, J. G. (2019). Chemosensory systems. *eLS*.

Swanson, W. J. & Vacquier, V. D. (2002). The rapid evolution of reproductive proteins. *Nature Reviews Genetics* **3**, 137-144.

Torgerson, D. G., Kulathinal, R. J. & Singh, R. S. (2002). Mammalian sperm proteins are rapidly evolving: evidence of positive selection in functionally diverse genes. *Molecular biology and evolution* **19**, 1973-1980.

Travis, J. (2006). Is It What We Know or Who We Know? Choice of Organism and Robustness of Inference in Ecology and Evolutionary Biology: (American Society of Naturalists Presidential Address). *The American Naturalist* **167**, 303-314.

Washington, N. L., Haendel, M. A., Mungall, C. J., Ashburner, M., Westerfield, M. & Lewis, S. E. (2009). Linking human diseases to animal models using ontology-based phenotype annotation. *PLoS Biol* **7**, e1000247.

West-Eberhard, M. J. (1983). Sexual selection, social competition, and speciation. *The Quarterly Review of Biology* **58**, 155-183.

Wheeler, D. A., Kulkarni, S. J., Gailey, D. A. & Hall, J. C. (1989). Spectral analysis of courtship songs in behavioral mutants of Drosophila melanogaster. *Behavior genetics* **19**, 503-528.

# CHAPTER 1: DESERT RODENT PREDATION ON BENZOQUINONE-DEFENDED BEETLES

## Abstract

Predators that consume defended prey may outcompete other species by using adaptive behavior, like specialized hunting strategies. Grasshopper mice (*Onychomys torridus*) are obligate carnivores that consume a variety of noxious prey. However, the mechanism by which they consume chemically defended stink beetles (*Eleodes longicollis*) is unknown. This study used a comparative approach to investigate the predatory behavior of grasshopper mice and two related rodent species, insectivorous deer mice (*Peromyscus* spp.) and granivorous pocket mice (*Chaetodipus* spp.). Behavioral video trials showed that in contrast to related desert rodents who avoided stink beetles, grasshopper mice employ behaviors that enable them to be more efficient predators and consume stink beetles. Grasshopper mice attacked beetles more than undefended crickets, used as a control, and oriented their attacks towards the beetles' heads more than their abdomens. Grasshopper mice were the only rodents to perform defensive face shoveling behavior, in response to encountering the beetles' benzoquinone spray. In addition to the spray, beetle size was correlated with grasshopper mice's total handling time of prey, and it took more time for the mice to incapacitate beetles than crickets. In spite of the spray's high concentrations of noxious benzoquinone, the beetles contain high caloric value in comparison to other insect prey. These results suggest that grasshopper mice's predatory traits may enable them to occupy a uniquely carnivorous dietary niche in a resource-poor desert environment. Behavioral variation among predators with different prey types also suggests that desert rodents are a well-suited model for testing the predictions of optimal foraging theory. Further study is needed to resolve

whether the grasshopper mice may be protected from the effects of the beetle’s spray through an underlying protective physiological or genetic mechanism.

## 1. Introduction

Predators use mechanisms that approximate optimal hunting strategies to maximize their success at catching prey (Curio, 1976). Many generalist predators rely on a repertoire of adaptive behaviors to target specific prey and sustain a varied diet. Generalists that choose to consume dangerous and well-defended prey benefit from both specialized attack behaviors and risk assessment (Eisner and Meinwald, 1966; Jackson and Pollard, 1996; Rowe and Rowe, 2006). The ability to eat noxious food, especially in a resource-poor environment, can provide a significant advantage to predators competing with species otherwise incapable of handling these prey items (Lunceford, 2015). However, studies are lacking quantitative descriptions of the strategic behaviors employed by successful predators compared to their relatives when competing for the same food.

Grasshopper mice, *Onychomys spp*., (Rodentia: Cricetidae) are useful for studying adaptive predatory behavior because they are aggressive, carnivorous rodents that frequently encounter and consume dangerous prey. Three species are distributed throughout Western North America and their diet mainly consists of arthropods, including large spiny-legged grasshoppers, stink bugs that exude or spray irritating benzoquinone compounds, whip scorpions that squirt acetic acid, and venomous centipedes, tarantulas, and scorpions (Bailey and Sperry, 1929; Egoscue, 1960; Eisner and Meinwald, 1966; Horner *et al.*, 1965; Slobodchikoff, 1987; Slobodchikoff *et al.*, 1987). They occasionally eat small vertebrates, like lizards and other mice, and have been reported to engage in cannibalism (Bailey and Sperry, 1929). For many of these prey items, however, it remains unclear which conditions, including availability, factor into their dietary choices.

There are several reports that suggest grasshopper mice employ specialized behavior to subdue defended prey. First, grasshopper mice have been described as biting off the legs of lubber grasshoppers, in order to prevent being kicked and harmed by the insects, before incapacitating the grasshoppers by biting their heads (Whitman *et al.*, 1986b). Second, the mice were reported to avoid scorpion stings, which are painful upon first injection, by biting the scorpion's tail first before biting its head (Eisner and Meinwald, 1966; Langley, 1981a; Rowe and Rowe, 2006). Third, they close their eyes when biting stink beetles, potentially to avoid contracting the spray in their eyes (Cyr, 1972). In contrast, grasshopper mice do not appear to show specialized predatory behavior towards less dangerous prey like crickets and mealworms (Langley, 1994; Whitman *et al.*, 1986b). This indicates that behaviors aimed at disabling the defenses of the prey may be intentional for incapacitating those prey (Whitman *et al.*, 1986b). Moreover, grasshopper mice have been shown to attack prey using their forepaws, in contrast to other omnivorous rodents which employ an ancestral strategy of attacking prey with only their mouth (Curio, 1976; Eisenberg and Leyhausen, 1972; Langley, 1987; 1994). The increased complexity of the grasshopper mouse's predation behaviors may be correlated with its specialized, obligately carnivorous lifestyle (Langley, 1994), which is unusual among rodents.

In contrast, other studies suggest that grasshopper mice predatory behavior may not be specialized. Any attack orientation other than biting the prey's head may be circumstantial (Langley, 1981a; Langley, 1987). The mice may bite the first part of the body they encounter, like a raised scorpion tail or grasshopper leg, because it is easier to grasp than the prey's head, which is usually lower to the ground (Cyr, 1972; Rowe and Rowe, 2006; Whitman *et al.*, 1986b). Grasshopper mice have been described as indiscriminate predators primarily motivated to attack based on visual cues and prey movement (Kemble and Lewis, 1982; Langley, 1981b; Langley, 1983a; b; 1989; 1991; Langley and Knapp, 1982). There is no need for grasshopper mice to develop a specific attack behavior if they never take time to assess prey until after it is killed. This lack of specialization may underlie the adaptive value of their predation strategy; succinctly summarized as “kill first, ask nutritional questions later” (Whitman *et al.*, 1986a), a rapid attack, rather than one targeted to subdue specific prey, may be more beneficial for grasshopper mice that rarely encounter prey (Langley, 1991; Whitman *et al.*, 1986a; b).

One of the grasshopper mice’s main prey—the black, aposematic beetle species *Eleodes longicollis* (Coleoptera: Tenebrionidae)—performs a defensive headstand that warns a predator of its noxious spray and allows the beetle to aim the spray at an oncoming predator (Doyen and Somerby, 1974; Eisner *et al.*, 2005; Eisner and Meinwald, 1966; Tschinkel, 1975). The fluid consists of 1,4-benzoquinone, a derivative of hydroquinone, along with several hydrocarbons and caprylic acid, which serves as a solvent, wetting agent, and irritant (Eisner *et al.*, 2005; Eisner and Meinwald, 1966). The spray has an acrid odor, like bleach or formaldehyde. Similar to the effects of hydroquinone and its other derivatives, benzoquinone spray can damage the skin, eyes, nose, and mouth with prolonged exposure (Anderson, 1947; Estable, 1948; Mbiya *et al.*, 2016; Oglesby *et al.*, 1947; Roberts and Aptula, 2009). Grasshopper mice have been reported to avoid the benzoquinone spray by manipulating the beetles’ abdomen tips into the sand, where the spray is harmlessly discharged, and then incapacitate their prey by biting the beetle’s head (Cyr, 1972; Eisner and Meinwald, 1966; Parmenter and Macmahon, 1988). This behavior can be learned through experience: naïve, laboratory-reared mice bit both ends of defenseless stink beetles that had their glands blocked with wax, but stopped biting the abdomens of stink beetles after experiencing the spray (Cyr, 1972; Langley, 1981a). Other studies suggest that the mice cannot avoid the spray and that they have consistently adverse reactions to encountering benzoquinones

(Egoscue, 1960; Langley, 1994). In behavioral trials between grasshopper mice and beetles, grasshopper mice that are sprayed take twice as long to kill the beetles (Cyr, 1972). Similarly, grasshopper mice are more efficient at killing the beetles when the beetles' spray glands are blocked (Langley, 1981a).

Although it has been verified that grasshopper mice are one of the main predators of defended beetles (Bailey and Sperry, 1929; Slobodchikoff, 1987), it remains unclear how grasshopper mice are able to incapacitate these beetles and whether they employ adaptive behavior to do so. Contradictory conclusions about grasshopper mice predation on stink beetles may be misleading due to focusing on qualitative descriptions (Cyr, 1972; Egoscue, 1960; Eisner and Meinwald, 1966; Parmenter and Macmahon, 1988) and attack latency (Langley, 1994), rather than specific steps of the predation process (but see Langley, 1981a). Moreover, previous work does not describe how the researchers confirmed that the mice were sprayed by the beetles (Cyr, 1972; Egoscue, 1960; Langley, 1981a; Langley, 1994). Confirming whether mice are exposed to benzoquinone is essential for identifying if rodent behavior is a specific response to the defensive spray.

Furthermore, most grasshopper mouse predation studies attempt to identify the singular effects of individual prey defensive traits (e.g., scorpion venom vs. nonvenomous crickets), but this ignores the broader, ecological reality in which prey species use multiple armaments to defend themselves against predators. For example, arthropod size, movement, and defensive chemicals may contribute cumulatively to increasing a grasshopper mouse's prey handling time, providing more opportunities for the prey to escape and survive (e.g., Parmenter & Macmahon, 1988). Body size can also signal toxin dose and nutritional content. When toxin levels are equal among variably sized prey, predators may be more attracted to larger prey items because they indicate a higher caloric reward (Smith *et al.*, 2016). One study teased apart the effects of prey caloric content and grasshopper mouse handling time to find that juvenile grasshopper mice chose crickets over *E. obscura* beetles (Slobodchikoff *et al.*, 1987). This is likely because the difficulty and handling time of defended beetles produced a net caloric deficit for inexperienced mice; adult grasshopper mice had lower handling times and consumed both beetles and crickets (Slobodchikoff *et al.*, 1987). These results align with the predictions of optimal foraging theory: predators are expected to behave optimally by pursing prey whereby the benefit of its energetic value outweighs the cost of searching for and capturing food (MacArthur and Pianka, 1966). To determine whether grasshopper mice behavior ascribes to these predictions, a comprehensive understanding of how grasshopper mice assess their prey would greatly benefit from considering prey size and caloric content, in addition to their chemical defenses.

In summary, it is inconclusive whether grasshopper mice employ strategic predatory behavior to eat stink beetles or not. Resolving this may shed insight into the behaviors that facilitate the predator-prey relationship between grasshopper mice and stink beetles, while also broadening knowledge on the selective factors determining potential co-evolutionary behavioral adaptations between predators and defended prey.

This study used a comparative approach to investigate the adaptive predatory behavior of desert rodents and their predator-prey relationship with chemically defended *E. longicollis* beetles and crickets. Using feeding experiments, the predatory behavior of grasshopper mice, obligate carnivores, was compared with that of deer mice (Rodentia: Cricetidae), facultative insectivores, and granivorous pocket mice (Rodentia: Heteromyidae), which consume insects under conditions of high nutritional stress. *E. longicollis,* a widespread and common desert beetle species (Wise, 1981) sympatric with several rodents, is a well-suited prey item for testing the adaptive predatory behavior and risk assessment strategies of southern grasshopper mice, *Onychomys torridus*, compared to other rodent predators. The study addresses the following **hypotheses** and predictions: 1) **Predatory mice eat stink beetles**. In spite of their different diet classes, both deer mice and grasshopper mice are opportunistic feeders that overlap in food sources (Cyr, 1972). Insectivorous mice are predicted to consume more beetles and crickets than granivorous pocket mice. 2) **Rodents employ specific behaviors to subdue their prey**. Rodents which prey on noxious beetles may demonstrate specialized predatory behavioral adaptations targeted to subdue their prey. If predators incapacitate the beetles by grasping the first available body part, then it would be expected that the mice will bite the beetle's raised abdomen first when it performs a defensive headstand. However, if the spray sufficiently deters mice from biting the beetle's abdomen, the mice may intentionally attack the beetle's head. This study therefore sought to evaluate whether grasshopper mice adjust their attack orientation in response to the beetle's defense, as this will help determine whether their predatory behavior is a specific adaptation to avoid the prey's defense. 3) **Predators are sprayed by the beetles.** Rodents which encounter the spray are predicted to perform defensive maneuvers to evade or recover from the beetles spray. 4) **Beetle size, spray concentration, calories, and defensive headstands affect predators' handling of prey**. *E. longicollis* caloric content, spray concentration, body length, and the number of headstands they performed were evaluated to compare the interrelated costs and benefits of consuming beetles as prey. Larger beetles are expected to have a higher caloric content, but may contain a more concentrated chemical spray and thus take more time for mice to subdue.

## 2. Materials and Methods

### *2.1 Animal Collection*

Each rodent species was collected in May 2017 at the University of Arizona’s Santa Rita Experimental Range in the Santa Rita Mountains of Pima County, in south-central Arizona. Mice were captured in Sherman live traps placed approximately 15-20 m apart, at the entrances to small mammal burrows, the base of vegetation clumps, or next to rodent tracks. Traps were baited with a mix of dry cat food and sunflower seeds shortly before sunset and checked the following morning prior to sunrise. Non-target species were released immediately where they were trapped. Mice were weighed, sexed, and categorized as either adults or juveniles depending on their measured body lengths (nose tip to base of the tail) and/or dorsal coat color, which lightens with age for grasshopper mice (Horner *et al.*, 1965).

For the following experiments, 81 adult mice were grouped according to species and dietary niche (Table 1.1): grasshopper mice (obligate carnivore), deer mice (facultative insectivore), and pocket mice (granivore). The grasshopper mice all belonged to the same species (*Onychomys torridus*) (n=41). The deer mice sample was composed of 29 mice belonging to three separate, closely related species: the North American deermouse (*Peromyscus maniculatus*) (n=3), the white-footed mouse (*Peromyscus leucopus*) (n=12), and the cactus mouse (*Peromyscus eremicus*) (n=14). The pocket mice sample was comprised of 11 mice belonging to two separate, closely related species: Bailey’s pocket mouse (*Chaetodipus baileyi*) (n=3), and the desert pocket mouse (*Chaetodipus penicillatus*) (n=8). To prevent retesting, mice were marked with a non-toxic permanent blue marker on their right ear. Mice were given cat food and sunflower seeds after they completed each feeding trial and released later that same day where they were captured. No rodents were held in captivity for more than 18 hours.

Stink beetles representing a single species (*Eleodes longicollis*) (n = 140) were collected during several evening hours after sunset in the same habitat where the mice were trapped. Immediately after collection, beetles were housed in containers with coconut fiber substrate, water, and an *ad libitum* diet of cricket powder supplemented with vegetable matter. Surplus specimens not used in the behavioral experiments were brought back in plastic containers to Michigan State University for bomb calorimetry and mass spectrometry. Adult large-sized house crickets (*Acheta domesticus*), approximately 25 mm in length, were obtained from local pet stores and fed an *ad libitum* diet of cricket powder.

| Dietary Niche | Species | Sample Size (n) |
|---|---|---|
| Obligate carnivore | Grasshopper mouse | |
| | *Onychomys torridus* | 41 |
| Facultative insectivore | Deer mouse | |
| | *Peromyscus maniculatus* | 3 |
| | *Peromyscus leucopus* | 12 |
| | *Peromyscus eremicus* | 14 |
| Granivore | Pocket Mouse | |
| | *Chaetodipus baileyi* | 3 |
| | *Chaetodipus penicillatus* | 8 |

**Table 1.1:** Rodent species, dietary niche, and sample sizes for behavioral trials.

### *2.2 Behavioral Trials*

Behavioral trials were conducted in a plexiglass terrarium (approximately 55 cm L × 20 cm W × 30 cm H) with a substrate of commercial odorless, natural clay cat litter filled to a height of 3-4 cm. Trials were filmed under ambient light using a digital video camcorder (Canon XL1 mini DV) equipped with a 3X wide-angle zoom lens (Canon XL 3.4–10.2 mm). Behavioral trials were conducted shortly after sunrise on the same day mice were captured. Each mouse participated in two behavioral trials: one with a cricket and one with a stink beetle.

For all trials, an individual mouse was placed in the terrarium and given 5 minutes to habituate. At the end of the habituation period, either a cricket or stink beetle was dropped into the opposite side of the terrarium. Based on methods previously described by Rowe and Rowe (2006), feeding trials were terminated when one of the following conditions was met: (1) the mouse incapacitated and consumed the prey, (2) the mouse incapacitated the prey, but failed to consume it within 10 minutes or (3) the mouse retreated from the prey and showed no interest in it for 10 minutes. At the end of each feeding trial, the remains of the first prey item were removed from the terrarium. The mouse was then given the second insect prey. When a mouse completed both behavioral trials, the terrarium was cleaned with 50% ethanol and the cat litter replaced. The order in which mice received crickets or stink beetles was alternated between trials.

### *2.3 Video Analysis*

Video recordings of behavioral trials were analyzed by two observers (interrater reliability kappa = 0.94) using real-time and frame-by-frame playback modes with the behavioral decoding software Behavioral Observation Research Interactive Software (BORIS) (Friard and

Gamba, 2016). Rodent behavior was assessed based on operational definitions of grasshopper mice predatory behavior modified from Rowe & Rowe (2006) (Table 1.2).

The number of times that the rodents performed ten different behaviors were counted during each trial with each prey type. These included four predatory behaviors: the number of times a rodent inspected and attacked its prey, which served to measure the rodents' persistence in pursuing each prey item, as well as the number of times the rodent incapacitated and consumed its prey. Inspections were defined as occurrences in which the mouse placed its nose within 3 cm of the prey. The orientation of the attack was used as a descriptive variable to determine whether the mouse targeted three possible regions of the prey's body: the head region (which also includes the prothorax), abdomen, or abdomen tip. Six defensive behaviors were counted to assess rodent difficulty in handling the prey: face shoveling, face rubbing, digging, substrate kicking (when a mouse used its back feet to kick substrate at its prey), the number of times the rodents closed their eyes, and number of times they dropped the prey. Since it was impossible to observe beetle sprays either through video analysis or in-person observations, face shoveling was also counted as a proxy for noting whether mice were sprayed by a beetle. This behavior, along with face rubbing, has been described as representative of rodent defensive behavior from discomfort (Cyr, 1972; Langley, 1981a).

Total handling time for each predator with each prey type was measured in duration to the nearest tenth of a second and identified as the main measure used for comparing different rodent capabilities in handling different prey types. It was defined as the amount of time between when a rodent first attacked and incapacitated its prey. Any time in which the mouse had its back to the camera or the action was out of focus was defined as a period in which the rodent was unobservable.

Beetle prey response was analyzed by counting the number of defensive headstands. Headstands were defined as a body position incurred by an interaction with a mouse in which the beetle lowered its head to the substrate and raised the posterior tip of its abdomen at a minimum angle of 45°. Beetle length was determined by measuring the carapace from the tip of the abdomen to the base of the prothorax. Available beetle measurements (n = 132) were obtained by hand using vernier calipers, with the exception of 16 beetles whose measurements were retrieved from the videos using BORIS' geometric measurement tool.

| Behavior | Definition |
|---|---|
| Attack | Mouse attempts to capture a prey item by lunging with forepaws outstretched and mouth open. |
| Consumption | The mouse begins to eat the prey following incapacitation. |
| Digging (d) | The mouse uses its forepaws to push substrate under its body during or within one minute after an interaction with a prey item. |
| Drop | Mouse stops its attack and releases its grasp on the prey item, permitting the prey to move away from the mouse. |
| Eyes closed (d) | Mouse has at least one eye closed longer than a single blinking interval during or within one minute after an interaction with the prey item. |

**Table 1.2:** Operational definitions of the behavioral variables counted during interactions between desert rodents, crickets, and stink beetles. (d) indicates a defensive behavior performed by rodents towards prey.

Table 1.2 (cont'd)

| Behavior | Definition |
|---|---|
| Face rubbing (d) | The mouse rubs, scratches, and/or grooms its face with at least one paw within one minute after interacting with a prey item. |
| Face shoveling | Within one minute after interacting with a prey item (e.g., inspect or attack), the mouse lowers its head into the substrate while either stationary or moving forward, then pushes its head through the substrate. |
| Headstand | After interacting with a mouse, a beetle lowers its head to the substrate and raises the posterior tip of its abdomen at a minimum angle of 45°. |
| Incapacitate | The point after the final attack when at least 3 of the following variables are observed: mouse is sitting stationary on its hindquarters; prey item being held without a struggle; prey item held perpendicular to the ground; even, repetitive motion of the mouse's jaws as evidence of consumption; if dropped, movement of the prey item is minimal (e.g., no attempt to stand or escape); the prey's head is detached from its body. |
| Inspect | The mouse has its nose pointed towards the prey at a distance of 3 cm or less. |
| Substrate Kicking (d) | The mouse actively flings substrate behind itself during or within one minute after an interaction with a prey item. |

### *2.4 Bomb Calorimetry*

Bomb calorimetry was performed on adult *E. longicollis* beetles (n = 40, males (M) = 20, females (F) = 20). Beetles were sexed, had their length measured from prothorax to abdomen tip (mm), weighed, and then dried until they maintained constant mass at 60° C. Beetles were then reweighed to determine relative dry mass content. Dried beetles were ignited in a bomb calorimeter (model 1266; Parr Instruments Moline, IL USA) to obtain dry mass energy content (kJ/g). The caloric content of the live beetles, also known as the wet mass energy content, was obtained by determining the product of the dry mass energy content and total dry mass divided by the original wet mass of each beetle.

Beetle Caloric Value (kJ/g wet) = (dry mass energy content (kJ/g) * dry mass (g)) / wet mass (g)

### *2.5 Mass Spectrometry*

Mass spectrometry was performed on adult *E. longicollis* beetles (n = 19, M = 10, F = 9) that were sexed and measured in length from prothorax to abdomen tip (mm). Beetles were aggravated with forceps until they secreted 10 µL of spray into a microcentrifuge tube placed at the tip of their abdomen. Samples were immediately placed on ice and then stored at 4°C. Mass spectrometry analysis was performed on the samples using a p-benzoquinone standard (Sigma Aldrich, #PHR-1028-1G) and a Waters GCT Premier GC/time-of-flight mass spectrometer at the Mass Spectrometry and Metabolomics Core at Michigan State University.

### *2.6 Statistical Analysis*

For all analyses reported, I used R version 4.0.3 (R Core Team, 2020). To test the hypothesis that predatory mice consume stink beetles, I calculated the proportion of times each rodent consumed each prey type. To test the hypothesis that rodents employ specific behaviors to subdue their prey, I modeled the number of times rodents attacked and inspected their prey items, as well as the total number of defensive behaviors each species towards each prey type (see Table 1.2). For each behavior, data was fit to generalized or linear mixed effects models ((G)LMM) with mouse species, prey type, and their interaction as fixed effects. The individual mouse is included as a random intercept. The number of inspections and defensive behaviors were fit to Poisson GLMMs with the "glmer" function of the lme4 R package (Bates *et al.*, 2015). For the number of attacks, data was fit to a LMM with the "lmer" function of the lme4 R package. The "glht" function from the multcomp R package (Hothorn *et al.*, 2008) was used to perform generalized linear hypothesis test (glht) of pairwise comparisons of estimated marginal means with Holm's method. I report differences within a rodent species across both prey types as well as differences across rodent species for the same prey type. Additionally, an unpaired t-test was used to compare prey handling times across rodent species.

To evaluate whether grasshopper mice adjust their attack orientation in response to the beetle's defense, I modeled the number of times grasshopper mice attacked different regions of the beetles' bodies: the abdomen, the abdomen tip, and the head region. Data was fit to a negative binomial GLMM with the "glmer.nb" function of lme4 with body region as the sole fixed effect and individual mouse included as a random intercept. Tukey's multiple comparisons were performed using the "glht" function from the multcomp R package to compare differences between the three regions.

To test the hypothesis that predators are sprayed by the beetles and perform specific behaviors to evade the spray, I calculated the number of times each rodent performed face shoveling behavior during behavioral trials with both prey types. Additionally, to test the hypothesis that different characteristics of stink beetles affect predators' handling times of prey, I performed Pearson' correlation tests to compare the relationships between beetle spray concentration and beetle mass, as well as beetle length vs. several metrics: grasshopper mice handling time, number of grasshopper mice attacks, beetle spray concentration, and beetle caloric content. I then modelled grasshopper mouse handling time of beetles with grasshopper mouse attacks on different beetle body regions using linear models, one for each body region, and used number of attacks on the body region and beetle body length as independent variables. I used two-tailed t-tests to compare whether beetle spray concentration and caloric content differed by beetle sex and a paired t-test to examine differences in prey drops by grasshopper mice depending on prey species. I also conducted a one-way ANOVA to assess differences in beetle headstands among rodent predators which encountered headstand behavior.

## 3. Results

### *3.1 Predatory mice consume stink beetles*

During 81 behavioral trials in which individual mice were paired with beetles, neither deer mice nor pocket mice successfully consumed beetles (0%). In comparison, 37 out of 41 (90%) grasshopper mice incapacitated the beetles, only one of which did not then eat the beetle. The mean handling time of beetles by grasshopper mice was 69.2s (SD = 108.9s).

### *3.2 Rodents employ specific behaviors to subdue their prey*

During 81 behavioral trials in which the same mice given beetles were also given crickets, pocket mice did not consume crickets (0%), while 19 out of 29 deer mice (66%) and 40 out of 41 grasshopper mice (97.5%) successfully consumed crickets. An unpaired t-test comparing handling times across rodent species showed that grasshopper mice killed crickets (M = 6.7s, SD = 10.7s) much more quickly than deer mice killed crickets (M = 61.5s, SD = 79.1s) ($t_{18.3} = -3.00$, $p < 0.01$).

Rodent species differed in their average number of attack behaviors depending on prey type and mouse species (Figure 1.1). Grasshopper mice attacked beetles more than crickets ($t_{139} = 4.49$, $p < 0.001$). In contrast, there was no difference in the number of times beetles and crickets were attacked by deer mice ($t_{139} = -2.08$, $p > 0.05$) or pocket mice ($t_{139} = -0.60$, $p > 0.05$). The number of attacks performed on beetles by grasshopper mice was significantly greater than attacks on beetles by deer mice ($t_{139} = 6.84$, $p < 0.001$) and pocket mice ($t_{139} = 4.89$, $p < 0.001$). Neither deer mice nor pocket mice attacked beetles at all.

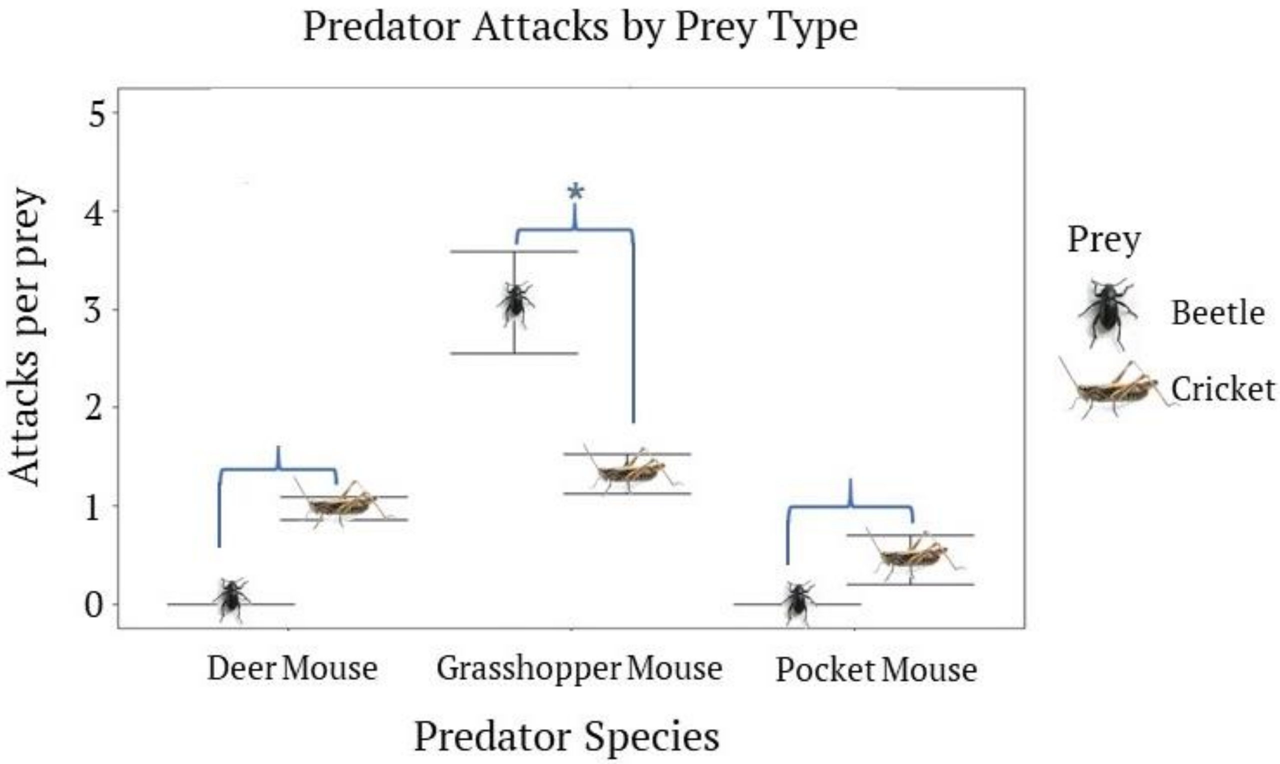

**Figure 1.1**: Grasshopper mice attack beetles more than crickets. Figure shows differences between prey types for rodent species and the average number of attacks on prey species by grasshopper mice, deer mice, and pocket mice during behavioral trials. Error bars denote ± 1 standard error. Asterisks denote significant differences between groups encompassed by the bracket (glht $p < 0.05$). Comparisons between rodent species are not shown. See text for details.

Grasshopper mice were the only rodents to attack the beetles. During beetle attacks ($n = 130$) in which the orientation was visible ($n = 114$), grasshopper mice attacked the head more than they attacked the beetles' abdomens (Tukey's HSD = 4.93, $p < 0.001$) and abdomen tips (Tukey's HSD = 5.47, $p < 0.001$). There was no significant difference between grasshopper mice attacks on abdomens and abdomen tips (Tukey's HSD = -0.87, $p > 0.05$).

All rodents showed defensive behaviors indicative of discomfort when encountering both prey types (Figure 1.2). Both grasshopper mice and deer mice performed more total defensive behaviors towards beetles than crickets (grasshopper mice, glht $z = 7.61$, $p < 0.001$; deer mice, glht $z = 6.17$, $p < 0.001$). In comparison, pocket mice performed more total defensive behaviors towards crickets than beetles (glht $z = -2.65$, $p < 0.05$). Overall defensive behaviors towards crickets differed by rodent species as well. Pocket mice reacted more towards crickets than deer mice (glht $z = -4.81$, $p < 0.001$) and grasshopper mice (glht $z = -6.98$, $p < 0.001$).

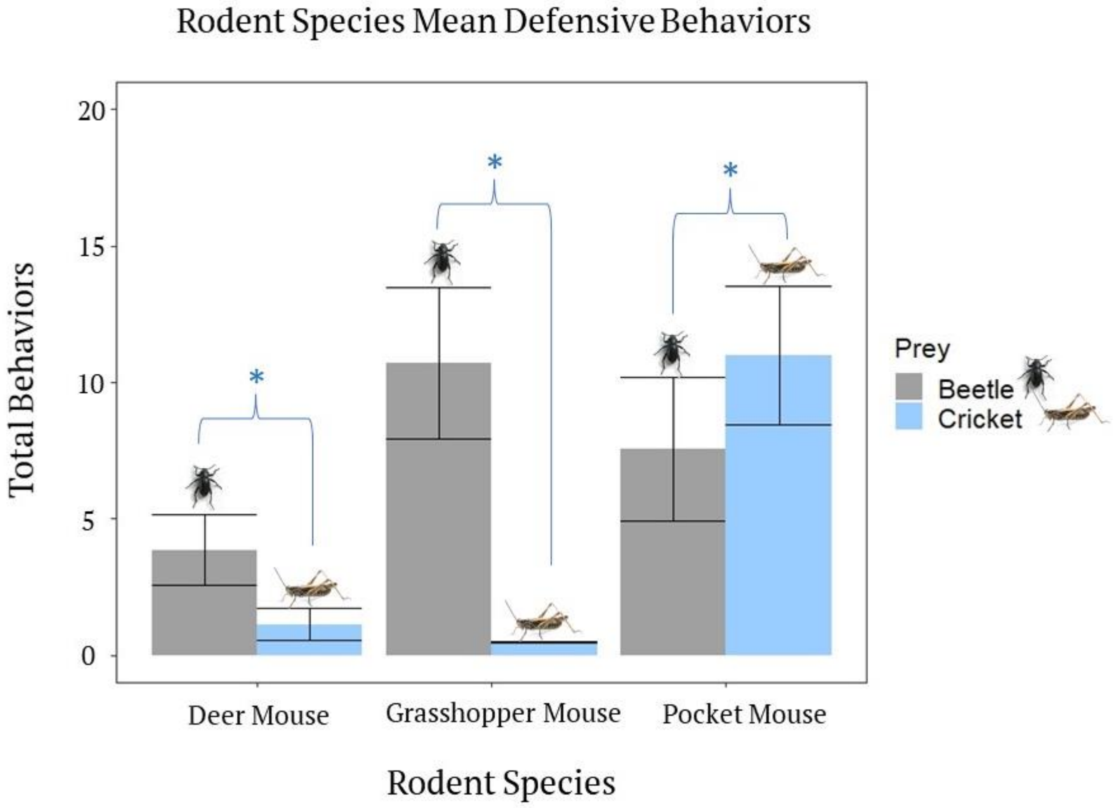

**Figure 1.2**: Rodent species differed in the total number of defensive behaviors performed towards each prey type. Figure shows the total number of defensive behaviors demonstrated towards beetles and crickets by grasshopper mice, deer mice, and pocket mice during behavioral

trials. Error bars denote ± 1 standard error. Asterisks denote significant differences between groups encompassed by the bracket (glht $p < 0.05$). Comparisons between rodent species are not shown, see text for details.

All rodents inspected beetles more than crickets (grasshopper mice, glht $z = 10.99$, $p < 0.001$; deer mice, glht $z = 11.09$, $p < 0.001$; pocket mice, glht $z = 3.42$, $p < 0.01$). There was no difference between the number of times pocket mice and deer mice inspected beetles (glht $z = -0.68$, $p > 0.05$), but beetles were inspected more by deer mice (glht $z = -3.00$, $< 0.05$) and pocket mice (glht $z = -2.90$, $< 0.05$) than by grasshopper mice. All species differed in the number of times they inspected crickets as prey; pocket mice inspected crickets more than deer mice (glht $z = -4.10$, $p < 0.001$) and grasshopper mice (glht $z = -6.33$, $p < 0.001$), and deer mice inspected crickets more than grasshopper mice (glht $z = -3.00$, $p < 0.05$).

Since grasshopper mice were the only rodents to attack and handle the beetles, they were the only rodents to drop them. Grasshopper mice rarely dropped crickets compared to how often they dropped beetles ($t_{40} = 5.31$, $p < 0.001$).

### *3.3 Predators are sprayed by the beetles*

Fifteen out of 41 grasshopper mice performed face shoveling behaviors during trials with beetles ($M = 3.95$, $SD = 8.23$). None of the grasshopper mice face shoveled when they encountered crickets. Neither deer mice nor pocket mice performed face shoveling when encountering either beetles or crickets.

### *3.4 Beetle size, spray concentration, calories, and defensive headstands affect predators' handling of prey*

The average length of beetles 25.14mm (SD = 4.28mm) was obtained by pooling available measurements of beetles used in the behavioral trials, mass spectrometry, and bomb calorimetry (n=132 beetles). The distribution of length appeared to be bimodal (Figure 1.3), suggesting that beetle length is disproportionately centered around 23mm and 29mm. Beetle length was positively correlated with grasshopper mouse total handling time ($r = 0.52$, $p < 0.05$) and the total number of attacks made by the mice ($r = 0.49$, $p < 0.05$). While individual crickets were not measured, large crickets are similar in length to the beetles (M = 24.51mm, SD = 4.67mm) used in the trials. A paired t-test showed that for the 36 out of 41 grasshopper mice that consumed both crickets and beetles, these mice killed crickets (M = 7.1s, SD = 11.2s) much more quickly than they killed beetles (M = 69.6s, SD = 110.4s) ($t_{35} = -3.40$, $p < 0.01$).

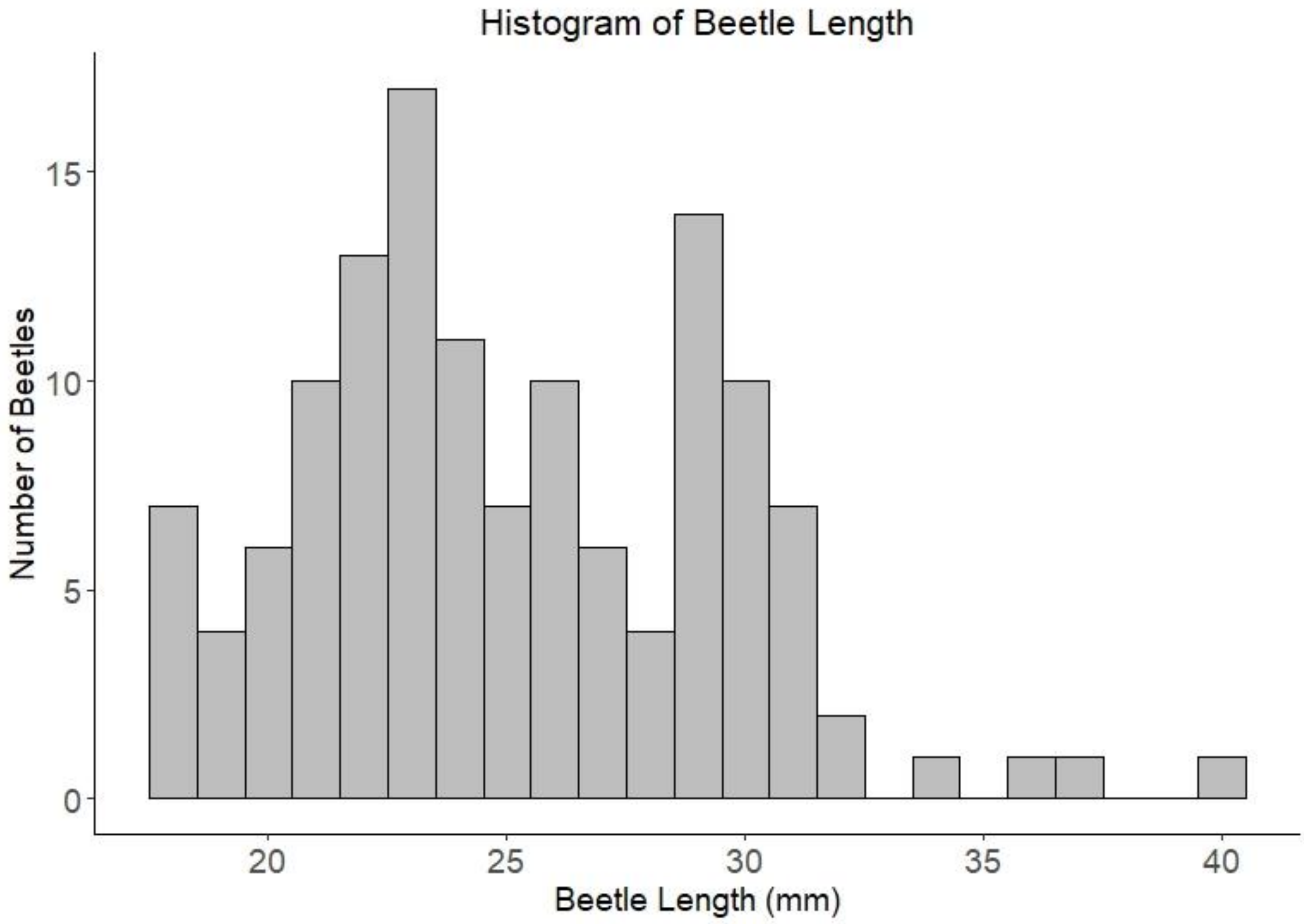

**Figure 1.3**: Beetle length follows a bimodal distribution. Histogram of beetle body length (n = 132) measured from the tip of the abdomen to the base of the prothorax.

When examining the relationship between grasshopper mouse handling time of beetles, the rodents' attack orientation, and beetle length, handling time was positively associated with the number of attacks on the head ($F_{1,29} = 9.14$, $p < 0.01$) and beetle length ($F_{1,29} = 4.48$, $p < 0.05$). Similarly, when examining attacks on the beetles' abdomen tips, handling time was positively associated with the number of attacks on the abdomen tips ($F_{1,29} = 9.18$, $p < 0.01$) and beetle length ($F_{1,29} = 4.48$, $p < 0.05$). However, when examining attacks on the abdomen, handling time was not related to abdomen attacks ($F_{1,29} = 0.09$, $p > 0.05$) or beetle length ($F_{1,29} = 3.42$, $p > 0.05$).

The average benzoquinone concentration of the beetles' spray (n = 19) was 110.44 mM (SD = 50.33 mM). The spray concentration was highly variable, ranging from 38.81 mM to 210.97 mM. Benzoquinone concentration appeared to follow a trend of being inversely correlated with total length ($r = -0.45$, $p < 0.10$), but was not correlated with total mass ($r = -0.32$, $p > 0.05$). There was no difference between spray concentration of males and females ($t_{17} = 0.45$, $p > 0.05$).

Regarding caloric content, *E. longicollis* beetles (n = 40) averaged 12.38 kJ/g (SD = 1.37 kJ/g). There was no relationship between caloric content and beetle length ($r = -0.31$, $p > 0.05$), caloric content and beetle mass ($r = -0.20$, $p > 0.05$), or between caloric content of males and females ($t_{38} = 1.48$, $p > 0.05$).

There was no difference in the number of headstands that beetles demonstrated for different rodent species, when evaluating only rodents that received headstands ($F_{2,27} = 0.75$, $p > 0.05$). However, most rodents did not induce beetle headstands – beetles only displayed defensive headstands for 4/11 pocket mice, 20/41 grasshopper mice, and 6/29 deer mice.

## 4. Discussion

To determine whether grasshopper mice employ adaptive, strategic behavior to consume stink beetles, this study used behavioral trials to compare predatory behavior among desert rodents on both chemically defended beetles and chemically undefended crickets. To further explore the predator-prey relationship between these species, beetles' size, spray concentration, caloric value, and defensive headstand behavior was considered in relation to rodent handling of prey. Taken together, the results showed that grasshopper mice are the most persistent and efficient predators among desert rodents, and comparative behaviors among rodents supported their different dietary classifications. This work therefore provides new species-specific information about the animals evaluated in the behavioral trials, as well as broader knowledge that can be applied for understanding the relationships between predators and chemically defended prey. The following highlights the results of each of the tested hypotheses and concludes with a discussion of how these species can be used as models for understanding prey choice within a larger ecological and evolutionary context.

### *4.1 Predatory mice eat stink beetles*

Grasshopper mice were the only rodent species to attack, incapacitate, and consume beetles. Behavioral variation among the rodents appeared to facilitate which predators were able to consume which prey. As previously described (Cyr, 1972; Parmenter & Macmahon, 1988), deer mice were much less effective in comparison to grasshopper mice. While they inspected beetles more than crickets (just like grasshopper mice), they never attacked or killed beetles and were much slower to kill and consume crickets than grasshopper mice. Pocket mice, in turn, were the most cautious and wary potential predators. They inspected crickets more than both grasshopper mice and deer mice, but ultimately chose to engage with them as potential predators less often. This led to their demonstrating more overall defensive behaviors towards crickets than the other rodents. Like deer mice, they never attacked beetles, and ultimately, they never successfully incapacitated either prey type. This suggests that behavioral adaptations are at, at least in part, a major role in determining prey choice.

### *4.2 Rodents employ specific behaviors to subdue their prey*

Attacks and attack orientation were specific behaviors that enabled grasshopper mice to subdue their prey. Grasshopper mice attacked beetles' heads more than their abdomens or abdomen tips. Head attacks occurred even when the beetle raised its head in a defensive headstand, which potentially positions the abdomen closer to the mouse's mouth. This nonrandom attack orientation therefore appears to be an intentional action by the grasshopper mice, as suggested by Langley (1981). However, its adaptive value is unclear. Attacking the head or abdomen tips did not appear to improve the efficiency of handling time. This was surprising, as a speedy decapitation would seemingly allow the mice to spend less energy subduing prey that was only injured, but not killed, by bites to other parts of its body. Nevertheless, head attacks, like all attacks, did help grasshopper mice obtain potentially profitable prey. The number of attacks was also correlated with the ability to take down larger prey. If beetles are more energetically profitable than crickets, the decision to exert energy through increased attacks and handling time to take down beetles may still be an adaptive strategy for the mice (*see 4.4*). Due to the diversity of their insect prey, desert rodents are well-suited models for testing optimal foraging theory, in which predators are expected to pursue prey that provides them with a net energetic profit (MacArthur and Pianka, 1966).

All three rodent species inspected beetles before deciding whether to attack them, which suggests inspection is not a specific adaptive behavior. Considering that inspections resulted in only grasshopper mice attacking beetles, these results could support the conclusion that grasshopper mice attack their prey without assessing its risk. However, inspection behavior, even in short intervals, indicates that grasshopper mice do take some time to assess prey item risk and reward, even if attacks are ultimately triggered by visual cues (Kemble and Lewis, 1982;

Langley, 1983a; 1989). Assessment time may help grasshopper mice choose whether to implement a specialized attack strategy suited for defended prey. As demonstrated in earlier work with scorpions (Rowe and Rowe, 2006) and studies that assessed juveniles' capacity to learn from experience with defended prey (Cyr, 1972; Langley, 1981), this suggests that grasshopper mice are also useful models for studying risk assessment, especially for prey choice studies. Additional work that builds on previous studies showing grasshopper mice rely on visual and olfactory cues (e.g., Whitman *et al.*, 1986a) to attack prey would provide new details about whether there are nuanced cues necessary to illicit different attack strategies for different prey types from grasshopper mice.

### *4.3 Predators are sprayed by the beetles*

The beetles' benzoquinone spray likely contributed to grasshopper mice's increased handling time of beetles compared to crickets, as grasshopper mice were the single rodent species to show evidence of being sprayed and to respond to the spray by taking time to perform face shoveling. Face shoveling was the only defensive behavior that grasshopper mice exhibited solely towards beetles (similarly to Cyr, 1972), supporting its use as a proxy for identifying whether mice were sprayed. In comparison to earlier reports describing grasshopper mice behavior (Eisner and Meinwold, 1966), they did not appear to avoid the spray by discharging the beetles' abdomen into the sand. It was not possible to visualize the beetle ejecting the spray. Anecdotally, however, some mice that encountered the spray also had fur that was stained and discolored by a brown residue.

Neither deer mice nor pocket mice displayed face shoveling with either prey type. Even though these rodents may not have been sprayed by the beetles directly, the benzoquinone fumes may have been disturbing enough to prevent them from attacking beetles as prey and strong enough to elicit other defensive behaviors. A comparison of rodent defensive behaviors showed that when the number of defensive behaviors was summed together for each rodent, grasshopper mice and deer mice responded more defensively to beetles than to crickets. Deer mouse defensive behavior was partly driven by the difficulty deer mice had in incapacitating their prey. Grasshopper mice defensive behaviors were aimed much more at beetles than crickets, as handling of crickets ended shortly after the first attack and they showed very few defensive behaviors in these trials; crickets were often immediately subdued and had no time to cause discomfort to the mouse. In comparison, crickets may have been more of a nuisance to omnivorous pocket mice, which responded more defensively towards crickets than beetles.

### *4.4 Beetle size, spray concentration, calories, and defensive headstands affect predators' handling of prey*

Beetles were much more challenging prey than crickets and this was likely due to multiple, interacting defensive traits: body length, body texture, and benzoquinone spray. Grasshopper mice attacked and spent considerably more time attempting to incapacitate beetles vs. crickets and large beetles vs. small beetles. Beetles appeared more difficult to grip than crickets and grasshopper mice dropped them more often than they dropped crickets. This may have been due to the difficulty in grasping the beetles' smooth, tough carapace, as well as the beetles' spiny, bristly legs that they kicked in the face of their predators. Larger beetles may have larger carapaces, legs, and potentially more power behind their attempts to escape from predators. The need to recapture beetles after dropping them was likely partially responsible for increasing grasshopper mice handling time of beetles.

Grasshopper mice's overall efficient handling times of crickets and handling of beetles appears to support the predictions of optimal foraging theory. Their behavior may be an adaptive trait allowing them to obtain more food using less time and energy than other rodents. As demonstrated by the results of bomb calorimetry, successfully hunting stink beetles yields a substantial reward for capable predators. *E. longicollis* is slightly higher in calories than *E. obscura*, which are chemically protected, as well as their non-secretory mimics, *Stenormopha marginata* and domestic crickets (Slobodchikoff *et al.*, 1987). While grasshopper mice's handling time of crickets appears to be fairly consistent between this and previous studies, their handling time of *E. longicollis* beetles was also about 8x shorter than their handling time of *E. obscura* (Slobodchikoff *et al.*, 1987). *E. obscura* beetles, however, may be more difficult to handle due to their greater size and potentially higher concentration of benzoquinone spray than *E. longicollis* (Tschinkel, 1975; A. Smith, personal communication). Additional bomb calorimetry and behavioral trials of multiple beetle species, including mimics of *Eleodes*, would be necessary to directly compare the caloric benefits of these prey with rodent handling times. Surprisingly, there was no association between calories and *E. longicollis* length, possibly indicating that differences in length were not substantial enough to generate differences in internal caloric content. Alternatively, this method may have been too crude to ascertain minute differences in calories in insect prey (Fulton *et al*., 2016). In comparison with the calories from available prey, however, the nutritious value of stink beetles suggests that any predator able to overcome the beetles' defense, regardless of its size, thus stands to benefit from a highly caloric meal in a resource-poor desert.

The variation in desert predator behavior has likely played a role in selection of beetle defense strategies, particularly spray concentration and body length. Beetle length measurements followed a bimodal distribution, in which most beetles fell into either a small (21-24 mm) or large (29-32 mm) length range. The beetles' spray had consistently high benzoquinone concentrations, in spite of high variability between individuals. There was no association between beetle spray concentration and body length. Small beetles thus acquire benefits from a defensive spray when facing pocket mice or insectivorous predators, like deer mice. Pain and potential tissue damage from the spray, combined with time and energy to consume the beetles, may therefore not be worth the risk for rodents that can rely on alternative food sources. Differences in spray concentration may function well to protect beetles from most rodent predators. Beetles appeared to respond to all potential prey equally – there was no difference in the number of headstands that beetles exhibited towards the different rodent species. However, in addition to grasshopper mice, *Eleodes'* spray appears insufficient to deter larger predators including ringtails (Trapp, 1978) and skunks, which are reported to use a specific behavior (like grasshopper mice) intended to subdue these prey: skunks use alternating front paws to roll the beetles in the sand, eyes closed, until the spray is extinguished into the substrate (Slobodchikoff, 1978).

While the spray may be ineffective against a few exceptional predators, beetle length may be a more viable defense during these encounters. A longer body length correlated with increased handling time of the beetles, even with rodent attacks oriented to the beetle's head, thereby benefitting beetles by providing them with time to escape as the mice grow fatigued and stop predation. In this study, mice that dropped beetles were able to recapture them because there were no available structures under which beetles could escape and hide. Under natural conditions, mice that turn away from the beetles to perform defensive behaviors like substrate kicking and face shoveling risk allowing their prey to escape. If refuge is available, larger beetles may have a greater chance of survival by taking advantage of distracted predators, especially less aggressive species like deer mice, and hiding somewhere inaccessible to the rodents (Parmenter and Macmahon, 1988). Since grasshopper mice had less difficulty incapacitating smaller beetles, regardless of their spray concentrations, they may actually serve to select against small beetles and contribute to the survival of larger beetles who can escape from their predators. Large, chemically defended beetles are thus well protected against a variety of possible predators.

Predators are not, of course, the only factor contributing to beetle size. Beetle size, in general, is constrained by availability of food at the larval stage (Karino *et al.*, 2004). Thus, competition for food may result in most *Eleodes* beetles fitting into the smaller size bracket (A. Smith, personal communication). Climate change and warming temperatures have also been identified as selecting for smaller sized beetles in the Caribidae family (Tseng *et al*., 2018). These consequences may be extremely complex if other beetle species are experiencing similar effects, as beetles make up 25% of all animal species and these changes may influence a large network of multispecies predator-prey interactions down the line.

### *4.5 Predatory Behavior and Dietary Niche*

Proclivity to attack, as well as differences in handling time, aligned with species-specific diet niche. Grasshopper mice's pursuit of both prey types aligns with their obligately carnivorous lifestyle. This study provides further support for the suggestion that grasshopper mice are capable of occupying a carnivorous dietary niche as a result of multiple adaptive, predatory traits, some of which are particularly suited for consuming toxic and chemically defended prey (Langley, 1981). Grasshopper mice eat highly venomous bark scorpions because the mice evolved resistance to the scorpions' neurotoxins (Rowe and Rowe, 2008) and when stung, use the venom's toxins to block voltage-gated pain transmission (Rowe *et al.*, 2013). The mice also have long toenails that help them grasp prey, high crowned teeth to deliver a forceful bite, and a fundic pouch in their stomach that protects them from abrasive chitin and helps them digest insects (Bailey and Sperry, 1929; Carleton and Eshelman, 1979; Horner *et al.*, 1965; Langley, 1994). Their enlarged jaws are stronger than deer mice and may be better equipped to break the fused carapace of stink beetles (Satoh and Iwaku, 2006; Williams *et al.*, 2009). The evolutionary benefit of combined adaptations would enable them to avoid competition for food with related rodents that would be harmed or killed during attempts to prey on the same arthropods (Carleton and Eshelman, 1979; Langley, 1994).

Pocket mice differed the most behaviorally from grasshopper mice and deer mice because as herbivores, they rely less on insects than seeds for their nutritional intake. Pocket mice are also less related to grasshopper mice and deer mice than those two species are to one another (Fabre *et al.*, 2012). Pocket mice may not have the predatory behaviors or morphology to efficiently take down insects for a net caloric profit. However, their high number of inspection behaviors demonstrates they may have at least considered the insects as prey, similar to other herbivorous animals like manatees (Courbis and Worthy, 2003) and white-tailed deer (Pietz and Granfors, 2000), which can be opportunistic predators.

Deer mice consumption of some, but not all crickets, supports their dietary classification as facultative insectivores. This variability may partly be due to variability in prey size and deer mice morphology. Depending on their nutritional status when trapped, the amount of time required for a deer mouse to handle a cricket, which is similar to the beetles' midrange length, and the energy to break apart its exoskeleton may have not been energetically profitable, considering that deer mice have weaker jaws than grasshopper mice and it takes them more time to eat crickets. Deer mice may have been more enthusiastic about hunting smaller-sized crickets if they were easier to hunt than the large sized crickets in this study.

Nevertheless, the lack of deer mice predation on beetles was surprising because it contrasted with numerous previous descriptions stating that deer mice attack stink beetles. This may partly be an effect of sample size and the relative contribution of predatory mice in these earlier works. In comparison to this study which used a large, recently caught rodent sample, Langley's studies examined only 11 deer mice and 10 northern grasshopper mice (1994) and 22 southern grasshopper mice (1981a), while Slobodchikoff tested 20 southern grasshopper mice (1987) and Cyr conducted comparable feeding trials with 6 field-caught deer mice, 4 field-caught grasshopper mice, and 4 lab-reared grasshopper mice (1972).

Variability in rodent behavior across different studies may also be an effect of sympatry between selected predators and prey, as well as differences in the toxin levels of the beetles themselves. For example, southern grasshopper mice may have evolved greater resistance to bark scorpion venom, another chemically defended prey item, than northern grasshopper mice because they overlap more geographically with this scorpion species (Rowe and Rowe, 2008). Related *Eleodes spp*. beetles can differ in their defensive sprays as well (Eisner and Meinwald, 1966); speculatively, *E. longicollis* may have a more aversive spray than other *Eleodes spp*. used in a former study with northern grasshopper mice (Parmenter and Macmahon, 1988) and in another work by Langley (1994), who did not identify beetles to the species level. Given the variability of defensive compounds among chemically defended prey, even within a small geographic scale (Fry, 2015), it is critical to compare only species trapped from the same location to avoid superficial or unequivocal comparisons between subspecies of grasshopper mice and beetles. These results comparing species from the same geographic space suggest that

*E. longicollis* defensive adaptations and desert rodent predation variability have the potential to be co-evolutionary traits between predators and prey.

Hunger level or a reluctance to pursue novel prey (Curio, 1976) were not likely to have contributed to behavioral variation between species during the feeding trials. All rodents were adult-aged and wild-caught and there is a high chance they previously encountered undefended prey, such as the crickets, and defended beetles, which are common to the Sonoran Desert. Deer mice did not appear to avoid beetles due to lack of hunger, as the same deer mouse that left a beetle alone would kill and consume crickets 66% of the time. Any hint of an acrid benzoquinone smell from the beetles may serve as a signal to experienced mice to avoid the beetles' spray through aversive conditioning. The differences in rodent behavioral frequencies are therefore more likely related to the rodents' dietary preferences and prey defenses.

## 5. Conclusion

Grasshopper mice and their relatives are well-suited as a comparative model system for investigating multiple factors contributing to the predictions of optimal foraging theory, including learning, risk assessment, and adaptive behavior. Predators must navigate trade-offs between time and energy required to hunt profitable prey, but grasshopper mice may be successful by mixing general efficiency with specialized predatory capabilities. Grasshopper mice have routinely demonstrated their ability to handle multiple prey types through speed and persistence. Yet, they can also overcome a suite of very diverse arthropod defenses, including toxins and aversive chemicals, through a combination of both physical and physiological adaptations. Additional studies that examine grasshopper mice for evidence of physiological adaptations targeted for overcoming additional specific arthropod defenses, as well as work that compares the energetic costs and benefits of foraging for these prey types, would clarify the extent to which grasshopper mice are predatory generalists or specialists. The diverse prey options, as well as variation in predatory behavior among desert rodents, makes this a feasible model system for future studies seeking to identify general behavioral patterns within this group and apply them more broadly to other taxa. Identifying the ultimate function of these mechanisms of behavior would contribute towards providing new insight into predator-prey relationships.

# REFERENCES

# REFERENCES

Anderson, B. (1947). Corneal and conjunctival pigmentation among workers engaged in manufacture of hydroquinone. *Archives of Ophthalmology* **38**, 812-826.

Bailey, V. & Sperry, C. (1929). Life history and habits of grasshopper mice, genus Onychomys. pp. 1-19: U.S. Department of Agriculture.

Bates, D., Maechler, M., Bolker, B. & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software, **67**(1), 1-48.

Carleton, M. D. & Eshelman, R. E. (1979). A Synopsis of Fossil Grasshopper Mice, Genus Onychomys, and Their Relationships to Recent Species. *Papers on Paleontology* **21**.

Courbis, S. S. & Worthy, G. A. (2003). Opportunistic carnivory by Florida manatees (Trichechus manatus latirostris). *Aquatic Mammals* **29**, 104-107.

Curio, E. (1976). *The ethology of predation*: Springer Science & Business Media.
Cyr, M. A. (1972). Predatory Behavior of the Grasshopper Mouse (Onychomys). In *Zoology*: University of California, Los Angeles.

Doyen, J. T. & Somerby, R. (1974). Phenetic similarity and Müllerian mimicry among darkling ground beetles (Coleoptera: Tenebrionidae). *The Canadian Entomologist* **106**, 759-772.

Egoscue, H. J. (1960). Laboratory and field studies of the northern grasshopper mouse. *Journal of Mammalogy* **41**, 99-110.

Eisenberg, J. & Leyhausen, P. (1972). The Phylogenesis of Predatory Behavior in Mammals 1. *Zeitschrift für Tierpsychologie* **30**, 59-93.

Eisner, T., Eisner, M. & Siegler, M. (2005). *Secret weapons: defenses of insects, spiders, scorpions, and other many-legged creatures*: Harvard University Press.

Eisner, T. & Meinwald, J. (1966). Defensive secretions of arthropods. *science* **153**, 1341-1350.

Estable, J. J. (1948). The ocular effect of several irritant drugs applied directly to the conjunctiva. *American journal of ophthalmology* **31**, 837-844.

Fabre, P.-H., Hautier, L., Dimitrov, D. & Douzery, E. J. (2012). A glimpse on the pattern of rodent diversification: a phylogenetic approach. *BMC evolutionary biology* **12**, 88.

Friard, O. & Gamba, M. (2016). BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. *Methods in Ecology and Evolution* **7**, 1325-1330.

Fry, B. (2015). *Venomous reptiles and their toxins: evolution, pathophysiology and biodiscovery*: Oxford University Press.

Fulton, S.A., Dodd, L. E. & Rieske, L. K. (2016). Evaluating the Energetic Value of Lepidoptera Using Bomb Calorimetry. *Mammoth Cave Research Symposia*. Paper 12. http://digitalcommons.wku.edu/mc_reserch_symp/11th_Research_Symposium_2016/Day_two/12

Horner, B. E., Taylor, J. M. & Padykula, H. A. (1965). Food habits and gastric morphology of the grasshopper mouse. *Journal of Mammalogy* **45**, 513-535.

Jackson, R. & Pollard, S. (1996). Predatory behavior of jumping spiders. *Annual review of entomology* **41**, 287-308.

Karino, K., Natsuki, S. & Chiba, M. (2004). Larval nutritional environment determines adult size in Japanese horned beetles Allomyrina dichotoma. *Ecological Research* 19, 663-668.

Kemble, E. D. & Lewis, C. (1982). Effects of vibrissal amputation on cricket predation in northern grasshopper mice (Onychomys leucogaster). *Bulletin of the Psychonomic Society* **20**, 275-276.

Langley, W. (1981a). The effect of prey defenses on the attack behavior of the southern grasshopper mouse (Onychomys torridus). *Zeitschrift für Tierpsychologie* **56**, 115-127.

Langley, W. (1981b). Failure of food-aversion conditioning to suppress predatory attack of the grasshopper mouse, Onychomys leucogaster. *Behavioral and Neural Biology* **33**, 317-333.

Langley, W. M. (1983a). Relative importance of the distance senses in grasshopper mouse predatory behaviour. *Animal Behaviour* **31**, 199-205.

Langley, W. M. (1983b). Stimulus control of feeding behavior in the grasshopper mouse. *Zeitschrift für Tierpsychologie* **62**, 291-306.

Langley, W. M. (1987). Specializations in the predatory behavior of grasshopper mice (Onychomys leucogaster and O. torridus): A comparison with the golden hamster (Mesocricetus auratus). *Journal of Comparative Psychology* **101**, 322.

Langley, W. M. (1989). Grasshopper mouse's use of visual cues during a predatory attack. *Behavioural processes* **19**, 115-125.

Langley, W. M. (1991). Relationship between attack and feeding in the insect predatory behavior of grasshopper mice. *Aggressive Behavior* **17**, 275-284.

Langley, W. M. (1994). Comparison of predatory behaviors of deer mice (Peromyscus maniculatus) and grasshopper mice (Onychomys leucogaster). *Journal of Comparative Psychology* **108**, 394.

Langley, W. M. & Knapp, K. (1982). Importance of olfaction to suppression of attack response through conditioned taste aversion in the grasshopper mouse. *Behavioral and Neural Biology* **36**, 368-378.

MacArthur, R. H. & Pianka, E. R. (1966). On optimal use of a patchy environment. *The American Naturalist* **100**, 603-609.

Mbiya, W., Chipinda, I., Simoyi, R. H. & Siegel, P. D. (2016). Reactivity measurement in estimation of benzoquinone and benzoquinone derivatives' allergenicity. *Toxicology* **339**, 34-39.

Oglesby, F., Sterner, J. & Anderson, B. (1947). Quinone Vapors and their Harmful Effects. II. Plant Exposures associated with Eye Injuries. *Journal of Industrial Hygiene and Toxicology* **29**, 74-84.

Parmenter, R. R. & Macmahon, J. A. (1988). Factors limiting populations of arid-land darkling beetles (Coleoptera: Tenebrionidae): predation by rodents. *Environmental Entomology* **17**, 280-286.

Pietz, P. J. & Granfors, D. A. (2000). White-tailed deer (Odocoileus virginianus) predation on grassland songbird nestlings. *The American Midland Naturalist* **144**, 419-422.

Roberts, D. W. & Aptula, A. O. (2009). Does the extreme skin sensitization potency of benzoquinone result from special chemistry? *Contact dermatitis* **61**, 332-336.

Rowe, A. H. & Rowe, M. P. (2006). Risk assessment by grasshopper mice (Onychomys spp.) feeding on neurotoxic prey (Centruroides spp.). *Animal Behaviour* **71**, 725-734.

Rowe, A. H. & Rowe, M. P. (2008). Physiological resistance of grasshopper mice (Onychomys spp.) to Arizona bark scorpion (Centruroides exilicauda) venom. *Toxicon* **52**, 597-605.

Rowe, A. H., Xiao, Y., Rowe, M. P., Cummins, T. R. & Zakon, H. H. (2013). Voltage-gated sodium channel in grasshopper mice defends against bark scorpion toxin. *science* **342**, 441-446.

Satoh, K. & Iwaku, F. (2006). Jaw muscle functional anatomy in northern grasshopper mouse, Onychomys leucogaster, a carnivorous murid. *Journal of Morphology* **267**, 987-999.

Slobodchikoff, C. (1978). Experimental studies of tenebrionid beetle predation by skunks. *Behaviour* **66**, 313-322.

Slobodchikoff, C. (1987). Aversive conditioning in a model-mimic system. *Animal Behaviour* **35**, 75-80.

Slobodchikoff, C., Vaughan, T. A. & Warner, R. M. (1987). How prey defenses affect a predator's net energetic profit. *Journal of Mammalogy* **68**, 668-671.

Smith, K. E., Halpin, C. G. & Rowe, C. (2016). The benefits of being toxic to deter predators depends on prey body size. *Behavioral Ecology*, arw086.

Team, R. C. (2020). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Hothorn, T., Bretz, F. & Westfall, P. (2008). Simultaneous Inference in General Parametric Models. Biometrical Journal **50**(3), 346--363.

Trapp, G. (1978). Comparative behavioral ecology of ringtail and gray fox in southwestern Utah. *Carnivore* **1**, 3-32.

Tschinkel, W. R. (1975). A comparative study of the chemical defensive system of tenebrionid beetles. Defensive behavior and ancillary features. *Annals of the Entomological Society of America* **68**, 439-453.

Whitman, D. W., Blum, M. S. & Jones, C. G. (1986a). Olfactorily mediated attack suppression in the southern grasshopper mouse toward an unpalatable prey. *Behavioural processes* **13**, 77-83.

Whitman, D. W., Blum, M. S. & Jones, C. G. (1986b). Prey-specific attack behaviour in the southern grasshopper mouse, Onychomys torridus (Coues). *Animal Behaviour*.

Williams, S. H., Peiffer, E. & Ford, S. (2009). Gape and bite force in the rodents Onychomys leucogaster and Peromyscus maniculatus: Does jaw-muscle anatomy predict performance? *Journal of Morphology* **270**, 1338-1347.

Wise, D. H. (1981). Seasonal and yearly patterns in the densities of darkling beetles (Coleoptera: Tenebrionidae) in a montane community. *Environmental Entomology* **10**, 350-358.

# CHAPTER 2: RODENT SENSITIVITY TO BENZOQUINONE: GRASSHOPPER MICE AS A MODEL SPECIES FOR CHEMOSENSATION

## Abstract

Animals use chemosensation to evaluate food for its nutritional value and rely on the sense of bitter taste as a signal of harmful content. Stink beetles (*Eleodes longicollis*) deter many predators by producing a bitter-tasting benzoquinone spray, but predatory grasshopper mice (*Onychomys torridus*) consume these beetles in spite of exposure to the defensive spray. To determine whether grasshopper mice are sensitive to benzoquinone, the drinking behavior of grasshopper mice and house mice (*Mus musculus*) was evaluated in a two-bottle choice test involving multiple low concentrations of benzoquinone. Both grasshopper mice and house mice were equally gustatorily sensitive to benzoquinone and preferred water to the chemical dilutions. In comparison to related rodents, however, grasshopper mice demonstrated unique amino acid substitutions in a benzoquinone binding receptor, TRPA1 (transient receptor potential cation channel A1), suggesting there may be evidence for a genetic or physiological mechanism other than gustatory sensitivity that underlies their ability to consume benzoquinone. Understanding predators’ behaviors in response to chemical deterrents like benzoquinone can reveal new insight into the chemosensory system and the way it mediates sensitivity to aversive taste.

## 1. Introduction

Chemosensation is the detection of chemicals, such as by olfaction and gustation, and is fundamental to all living things (Spielman *et al.*, 2019). These senses affect feeding behaviors by allowing animals to find and evaluate food for nutritional or harmful content. In particular, bitter taste sensation functions to promote survival through the detection of noxious and potentially toxic compounds (Garcia, 1975; Shi *et al.*, 2003; Spielman *et al.*, 2019). Yet, research that aims to investigate the underlying mechanisms of sensitivity to aversive taste often relies on laboratory model species such as mice and rats (Davis *et al.*, 1986; Davis *et al.*, 1988; Sclafani and Glendinning, 2003); these organisms may be less suited to address these questions than specialized rodents, such as carnivorous grasshopper mice (*Onychomys torridus*), which demonstrate more natural variation in their behavioral responses to defended prey with bitter-tasting chemicals, such as stink beetles (*Eleodes longicollis*) (see Chapter 1). Grasshopper mice therefore have the potential to be informative nontraditional model species for studies of chemosensation and for comparative work seeking to identify the genetic underpinnings of chemosensation in other species. However, investigations of the ways by which grasshopper mice consume most bitter-tasting *Eleodes* beetles have been limited to behavioral observations of predator-prey interactions (Cyr, 1972; Langley, 1994; Parmenter and Macmahon, 1988; see Chapter 1).

Bitter quinone secretions are one of the main chemicals employed by *Eleodes* beetles, as well as other noxious arthropod prey (Eisner *et al.*, 2005), and are specifically targeted to irritate the vertebrate sensory system (Conner *et al.*, 2007) of prospective carnivores. One common form of defensive quinones produced by arthropods is benzoquinone (also known as para-quinone, p-benzoquinone, or 1,4-benzoquinone), which is employed as an antipredator defense against small mammals by bombardier beetles (Aneshansley *et al.*, 1969), millipedes (De Capitani *et al.*, 2011; Eisner *et al.*, 2005), flour beetles (Lis *et al.*, 2011), in addition to various *Eleodes* species of tenebrionid stink beetles (Doyen and Somerby, 1974; Eisner and Meinwald, 1966; Tschinkel, 1975). In spite of its noxious taste and smell, benzoquinone does not completely protect these arthropods from several exceptional mammalian predators. Coatis (*Nasua* spp) (Weldon *et al.*, 2006) and skunks (*Mephitis mephitis*) (Slobodchikoff, 1978) have been reported to use their forepaws to roll millipedes and stink beetles, respectively, to discharge the spray and avoid its contact, while grasshopper mice consume stink beetles with voracity (Cyr, 1972; Langley, 1994; Parmenter and Macmahon, 1988; see Chapter 1).

These predators are highly unusual because the behavioral rejection of bitter-tasting substances is widespread and conserved from invertebrates, like mollusks and *Drosophila* (Reinhard, 2010; Sahley *et al.*, 1981) to vertebrates across the entire range of dietary habits (Dong *et al.*, 2009; Garcia, 1975). The adaptive value of bitter taste appears to vary with dietary exposure; herbivores, which frequently encounter bitter chemical compounds produced by plants (Wittstock and Gershenzon, 2002) are generally more tolerant of toxins and have a higher bitter threshold than carnivores (Glendinning, 1994; Lunceford and Kubanek, 2015). Herbivorous red and roe deer, for instance, are deterred by extremely bitter denatonium (Wright and Milne, 1996) at concentrations five times higher than omnivorous laboratory rodents (Davis *et al.*, 1986; Davis *et al.*, 1988; Langley *et al.*, 1987). Grasshopper mice’s tolerance for noxious prey seemingly contradicts the expectation that carnivores are highly sensitive to bitter taste.

Understanding the behavioral reactions of predators, such as grasshopper mice, towards the chemical deterrents used by their prey can reveal new insight into the underlying mechanisms that drive sensitivity to aversive taste (Blair *et al.*, 2016; Ibarra and Blair, 2013). This study tests whether grasshopper mice have reduced gustatory sensitivity to benzoquinone produced by *E. longicollis* stink beetles. As shown previously (see Chapter 1), *E. longicollis* produces benzoquinone spray at high levels of concentrations within an expansive range (mean = 110 mM, SD = 50.33 mM), but grasshopper mice consume the arthropods in spite of being sprayed in their mouth, nose, and eyes. In comparison, closely related deer mice (*Peromyscus* spp.) did not consume any beetles. The behavioral differences between the two closely related species suggest that grasshopper mice may have reduced sensitivity or some other physiological mechanism that protects them against the irritating properties of benzoquinone and allows them to consume this prey without adverse effects, potentially similar to how they have evolved resistance to scorpion neurotoxin and the pain signals it transmits (Rowe and Rowe, 2008; Rowe *et al.*, 2013). Evaluating the grasshopper mice's gustatory responses to benzoquinone in their diet may also aid in identifying the genetic underpinnings of their adaptive predatory behavior.

Benzoquinone is an electrophilic compound that reacts with cysteines and lyseines in numerous proteins (Lamé *et al.*, 2003), including a polymodal noxious chemical sensor known as transient receptor potential ankyrin 1 (TRPA1) (Andersson *et al.*, 2011; Ibarra and Blair, 2013). TRPA1 is a $Ca^{2+}$ permeable nonselective cation channel that is highly conserved across the animal kingdom, expressed in both non-neuronal and neuronal cell types (including in the dorsal root ganglia (DRG) and trigeminal ganglion (TG) sensory neurons), and is activated by extreme temperatures, reactive chemicals, and a wide variety of stimuli associated with cell damage (Bandell *et al.*, 2004; Bautista *et al.*, 2006; Jordt *et al.*, 2004; Macpherson *et al.*, 2007; Macpherson *et al.*, 2005; for a review see Talavera *et al.*, 2020).

Variable concentrations of electrophilic chemical stimuli affect TRPA1's activation. Benzoquinone has been shown to activate 30% of cultured neurons from mouse DRG at 0.001

mM (Andersson *et al.*, 2011), as well as heterologously expressed human TRPA1 at low nanomolar concentrations up to 0.001 mM; at this level, benzoquinone also causes desensitization of the channel (Ibarra and Blair, 2013). Cysteine amino acid residues appear to play a crucial role in both processes. Electrophilic substances like benzoquinone are reported to activate TRPA1 by covalently modifying cysteine residues on the channel's cytosolic surface, while desensitization is likely produced by interactions with cysteine residues in inner or hydrophobic regions of the channel (Ibarra and Blair, 2013; Nilius *et al.*, 2012; Paulsen *et al.*, 2015; Wang *et al*., 2012). Other pungent compounds, like AITC (mustard oil), similarly interact with TRPA1's cysteine residues on the N-terminus (Hinman *et al.*, 2006; Ibarra and Blair, 2013) as well as several basic residues in the C-terminus involved in voltage gating and chemical sensitivity (Samad *et al.*, 2011).

It is currently unknown whether grasshopper mice have modified benzoquinone receptors, like TRPA1, that render these mice undaunted by high concentrations of benzoquinone in stink beetles' spray. However, in comparison to house mice (*Mus musculus*), grasshopper mice show reduced sensitivity to formalin (Rowe *et al.*, 2013), another TRPA1 agonist (McNamara *et al.*, 2007). Therefore, grasshopper mice may have modifications in their TRPA1 channels that affect their gustatory sensitivity and influence their prey choice.

To evaluate if grasshopper mice have the potential for a physiological or genetic mechanism that enables their predation of benzoquinone defended beetles, their gustatory sensitivity to benzoquinone was investigated using a two-bottle choice test and their TRPA1 gene sequence was examined for sites that could confer differences in benzoquinone sensitivity in order to address two **hypotheses**: **1) grasshopper mice are less sensitive to benzoquinone and will drink more benzoquinone than water compared to house mice (*M. musculus***

**C57BL/6)**. A two-bottle choice test is frequently used to evaluate rodents' sensitivity and consumption of different taste solutions (e.g., Bachmanov *et al.*, 1996; Sclafani and Glendinning, 2003; Eagle *et al.*, 2016). If grasshopper mice consume stink beetles because they are less sensitive to benzoquinone, they may not be able to detect it at low concentrations and therefore drink equal amounts of benzoquinone solution as water. Grasshopper mice were tested along with a laboratory strain of house mice which are not specialist feeders, as a comparative outlier and less closely related species within the rodent family (Scholl *et al.*, 2017). Both species were offered a choice between water and a solution of benzoquinone diluted within and above the range of concentrations previously tested with TRPA1. These dosages were chosen because monitoring the rodents' reaction to increasing concentrations, beginning with those known to activate TRPA1 (Ibarra and Blair, 2013; Macpherson *et al.*, 2007; Wang *et al.*, 2012) and building towards those they encounter during natural interactions with stink beetles, could potentially pinpoint which concentrations, if any, trigger species-specific responses to benzoquinone. Second, it was expected that **2) grasshopper mice have reduced sensitivity to benzoquinone due to cysteine variants in their TRPA1 gene sequences that differ from related rodents.** The grasshopper mouse TRPA1 gene sequence was compared to TRPA1 orthologs from multiple related species and examined for cysteine substitutions, especially within the N-terminus, as well as for variants in C-terminus amino acid residues implicated in chemical sensitivity. Variation in TRPA1 homology between grasshopper mice, deer mice, and house mice may reveal important amino acid mutations driving functional and behavioral differences in chemosensation across these species.

## 2. Methods

### *2.1 Animal care and housing*

This study used ten adult lab-raised grasshopper mice (female = 6, male = 4) and ten adult house mice (female = 5, male = 5) of the C57BL/6 strain purchased as retired breeders (> 7 months old) from Charles River Labs (Wilmington, MA). All mice were housed individually at Michigan State University using the Campus Animal Resources facility mouse cages located in a single room on a 12-hour light/dark cycle. The top of each cage had a divided wire rack lid: one section, which held the two bottles used during the choice test, and one section which held standard rodent diet (Teklad 8940, Envigo) that the mice received *ad libitum*. Cages were cleaned and bedding changed once per week.

### *2.2 Two-bottle Choice Test*

The two-bottle choice test followed a protocol similar to previously described two-bottle choice set-up and methodology (e.g., Bachmanov *et al.*, 1996; Eagle *et al.*, 2016) with modifications to its duration and the apparatus design in order to fit the rodents' cages (Eisthen, Rowe, and Rowe, unpublished). The drinking tubes used for the experiment were devised from 25 ml serological pipettes cut down to approximately 20 ml. One end of the tube was closed with a rubber stopper while the other end was closed with a stainless-steel sipper spout. The spouts extended into the cage from the wire rack and had a hole from which the mouse could lick fluid, similar to the sipper spout used in their regular drinking water bottle. Each bottle was placed on either the right or left side of the drinking bottle section of the wire rack and filled to the 20 ml mark with either reverse osmosis (RO) water or a specific concentration of benzoquinone solution. To avoid the effects of a location preference, tube placement was switched daily, ensuring that mice had to relearn the contents of each bottle every day. Every day during the light-period, fluid consumption for each mouse was recorded to the nearest 0.1 ml by subtracting the remaining volume of liquid from its initial value the previous day. These initial readings were taken after the tube locations were switched in order to account for potential spillage incurred by jostling the tubes.

The experiment took place over the course of 6 weeks and tested rodent sensitivity to benzoquinone (Sigma-Aldrich, Catalog number B10358) diluted in RO water at the following concentrations: 0.0001 mM, 0.01 mM, 0.1 mM, 1 mM, 3mM, and 10 mM. Concentrations were chosen based on an earlier range of chemical concentrations used to test TRPA1 activation (Ibarra and Blair, 2013; Macpherson *et al.*, 2007). Mice received concentrations each week in ascending order and had exposure to each concentration for six consecutive days. On the 7th day of the week, the mice received a rest day in which both tubes were filled with only RO water.

Mice had previously been used in this experimental paradigm. However, there was a possibility that the lowest concentration of benzoquinone was incorrect due to human error while diluting the benzoquinone in water. Since it was not possible to ascertain the quantity of benzoquinone in the trials that were previously completed, all experimental trials were repeated on the same subjects four months later. The results from these later trials were used for analysis.

### *2.3 Multispecies TRPA1 gene comparison*

To investigate the grasshopper mouse TRPA1 gene for possible structural modifications or substitutions related to potential benzoquinone activation, I performed a multispecies TRPA1 protein alignment.

I first downloaded the coding region of TRPA1 from multiple closely related species from the NCBI database and aligned them using the CLUSTALW algorithm in Geneious (version 9.1.8) (https//www.geneious.com). These species included grasshopper mouse (*Onychomys torridus*, XP_036035908.1), deer mouse (*Peromyscus maniculatus*, XM_006974737.1), white-footed mouse (*Peromyscus leucopus*, XM_028864648.2), Chinese hamster (*Cricetus griseus*, XP_027254622.1), golden hamster (*Mesocricetus auratus,* XP_021080916.1), prairie vole (*Microtus ochrogaster*, XM_005362351.2), house mouse (*Mus musculus*, NM_177781.5), kangaroo rat (*Dipodomys ordii*, XP_012883871.1) and as an outgroup, human (*Homo sapiens*, NP_015628.2).

In an effort to confirm the grasshopper mouse TRPA1 sequence on NCBI, I performed PCR and Sanger sequencing of an additional tissue sample retrieved from a grasshopper mouse originally caught in an area sympatric with stink beetles. I first extracted mRNA from one grasshopper mouse dorsal root ganglion tissue using TRIzol (Ambion) following the manufacturer's protocol. I subsequently converted mRNA to cDNA using SuperScript III First Strand Synthesis Reverse Transcriptaste PCR kit (Invitrogen). The reverse transcription reaction for cDNA synthesis was primed with Oligo $d(T)_{20}$. I then designed primers to amplify the grasshopper mouse TRPA1 gene in four overlapping pieces and performed PCR according to the guidelines of New England Biolabs' (NEB) Q5 DNA polymerase kit. I ran the amplified fragments on a 0.8% w/v agarose gel, excised the appropriate sized bands, purified the DNA using a PCR Clean-Up System (Promega) and sent the purified products to GENEWIZ for sequencing. Aligning the NCBI TRPA1 sequence with the one produced by GENEWIZ showed that when both sequences were aligned with the other species orthologues, they were nearly identical (Pairwise% Identity: 99.2%; Pairwise Positive% Identity (BLSM62): 98.5%), with the exception of an unresolved 5 prime end and an additional 10 amino acids throughout the sequence. Since lack of similarity appeared to be due only to potential issues with generating a complete sequence and not a result of differences in the sequences themselves, I used NCBI's grasshopper mouse sequence in subsequent analyses.

I examined all sequences in the multispecies protein alignment generated in Geneious for their number of cysteine amino acids and any cysteine variants or amino acid substitutions, with a specific focus on cysteines previously shown to activate TRPA1 (Ibarra and Blair, 2013; Macpherson *et al.*, 2007; Wang *et al*., 2012). I also checked for homology among eight positively charged regions first identified in the human TRPA1 C-terminus as possible interactive sites for electrophilic molecules that modulate TRPA1 (Samad *et al.*, 2011). These sites in the human TRPA1 sequence are: K969, A975, K988, K989, K1048, K1052, K1092 and A1099.

### *2.4 Statistical Analyses*

For all analyses reported, I used R version 4.0.3 (Team, 2020). To evaluate the results of the two-bottle choice test, the amount of either benzoquinone or water consumed during six trials was averaged for each individual mouse at each given concentration. This average amount was used as the response variable and fit to a linear mixed effects model with function "lmer" in package lme4 (Bates *et al.*, 2015). The Akaike information criterion (AIC) was used for model selection. Species, the concentration of benzoquinone, and the type of fluid consumed (benzoquinone or water) were used as fixed effects, and individual mouse was included as a random effect intercept. I performed orthogonal contrasts to compare mean differences for significant interactions.

## 3. Results

### *3.1 Two Bottle Choice Test*

To evaluate the two-bottle choice test, the best performing model included the two-way interaction of solution type and concentration of benzoquinone and the species effect without interactions. As such, I compared differences between the amount of benzoquinone and water consumed by both species at each concentration and between different concentrations.

Differences in the amount of benzoquinone compared to water consumed by mice varied according to concentration levels (Figure 2.1). Mice drank more water than benzoquinone at four concentrations: 0.0001 mM ($t_{208} = -2.39$, $p < 0.05$), 1 mM ($t_{208} = -3.25$, $p < 0.01$), 3 mM ($t_{208} = -15.32$, $p < 0.001$) and 10 mM ($t_{208} = -19.22$, $p < 0.001$). There was no difference between benzoquinone and water consumption at 0.01 mM ($t_{208} = 0.26$, $p > 0.05$) or 0.1 mM ($t_{208} = -0.34$, $p > 0.05$).

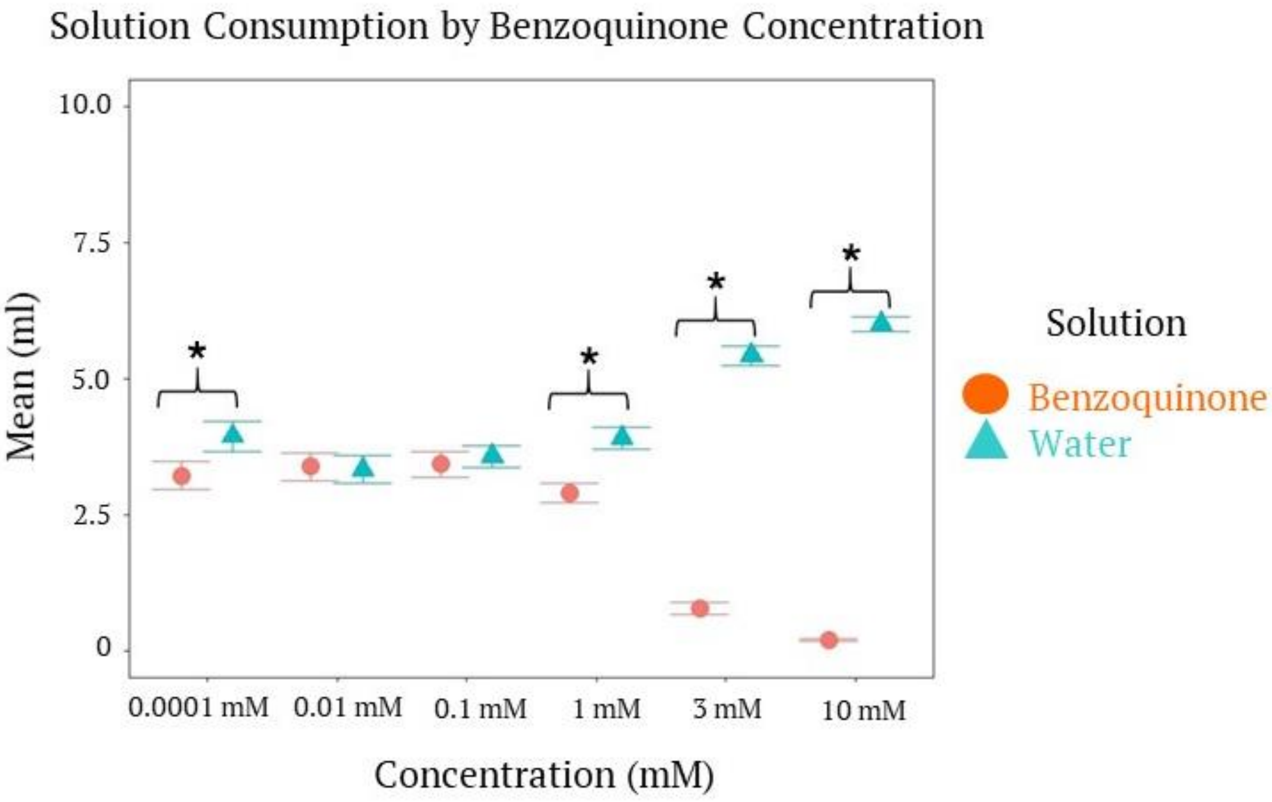

**Figure 2.1**: Mice drink less benzoquinone as the concentration increases. Figure shows the average amount of benzoquinone and water consumed by both species of mice. Error bars denote ± 1 standard error. Asterisks denote significant differences between the groups encompassed by the bracket (linear contrasts $p < 0.05$).

### *3.2 TRPA1 Sequence*

A multispecies protein alignment showed that the selected mammalian TRPA1 orthologous sequences are highly conserved (Pairwise% Identity: 89.7% ; Pairwise% Positive (BLSM62): 94.9%). High sequence similarity was found between closely related grasshopper mice and deer mice (Pairwise% identity: 97.8%; Pairwise% Positive (BLSM62): 99.4%), as well as between more phylogenetically distant grasshopper mice and house mice (Pairwise% identity: 91.8%; Pairwise% Positive (BLSM62): 95.9%)

The grasshopper mouse TRPA1 sequence had only 29 cysteine amino acids, in comparison to house mice which have 31. Of the 31 cysteines previously described and tested in relation to mouse TRPA1 activation (Wang *et al*. 2012), 25 were conserved across all rodent species orthologs. Four cysteines (C3, C98, and C231 in the grasshopper mouse TRPA1 N-terminus and C1028 in the grasshopper mouse TRPA1 C terminus), varied between grasshopper mice and house mice TRPA1 sequences, although three of these were conserved between grasshopper mice, deer mice, and varying other rodent species (Figure 2.2). One cysteine variant, C231, was unique to grasshopper mice. This same site was occupied by a threonine for house mice and serine for both species of deer mice surveyed.

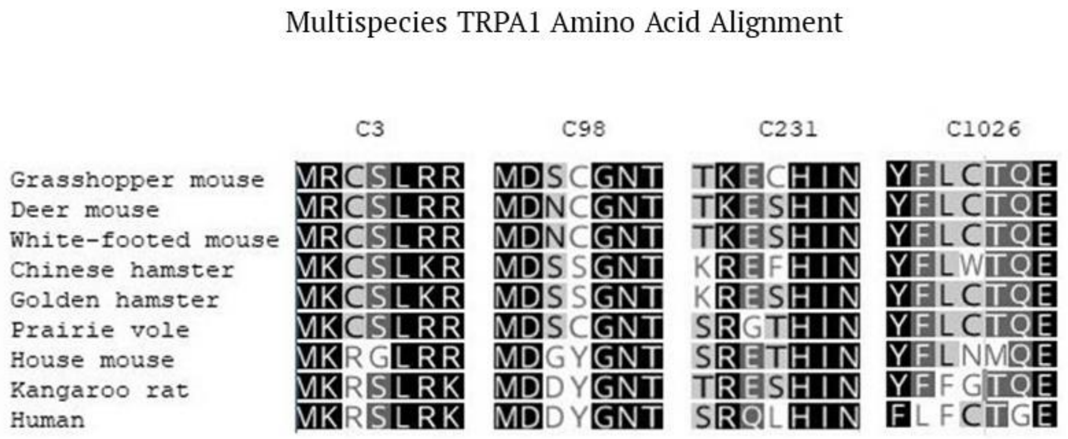

**Figure 2.2:** Partial multispecies amino acid sequence alignment shows cysteine amino acid changes at four sites among closely related mammalian TRPA1 orthologs. Cysteines are numbered according to their location on the grasshopper mouse TRPA1 sequence. Gray shading highlights similarities in conservation of the amino acids between species as determined by Geneious software.

Eight positively charged regions first identified in the human TRPA1 C-terminus as possible interactive sites for electrophilic molecules that modulate TRPA1 (Samad *et al.*, 2011) were also found to be homologous across all rodent species: K969, A975, K988, K989, K1048, K1052, K1092, and A1099. More than 80 additional amino acid sites not previously associated with TRPA1 activation were nonhomologous between grasshopper mice, deer mice, and house mice.

## 4. Discussion

The purpose of this study was to evaluate whether **1) grasshopper mice are less sensitive to benzoquinone and will drink more benzoquinone than water compared to house mice (*M. musculus* C57BL/6)** and whether **2) grasshopper mice have reduced sensitivity to benzoquinone due to cysteine variants in their TRPA1 gene sequences that differ from related rodents.** The two-bottle choice test showed that grasshopper mice and house mice were equally gustatorily sensitive to benzoquinone. Both of them found benzoquinone to be more distasteful at higher concentrations. In contrast, the results from the multispecies alignment of TRPA1 genetic sequences showed that grasshopper mice have different cysteine variants compared to house mice. The lack of differences in aversion between the species in the two-bottle choice test was unexpected because grasshopper mice consume benzoquinone defended prey more than house mice, have modifications in their TRPA1 receptors that may mediate benzoquinone differently than house mice, and have previously been shown (Rowe *et al.*, 2013) to have reduced sensitivity to other noxious chemicals like formalin.

### *4.1 Two-Bottle Choice Test Comparisons*

Grasshopper mice's aversion to the benzoquinone concentrations used in the two-bottle choice test was surprising because grasshopper mice willingly consume benzoquinone-defended beetles in their natural environment at concentrations ten times higher on average than 10 mM (see Chapter 1). Yet at 10 mM, the concentration of benzoquinone may have been sufficiently aversive to entirely inhibit drinking of benzoquinone solution; data showing minimal consumption amounts may have been due to liquid dripping when mice inspected and jostled the tubes. If grasshopper mice find 10 mM of benzoquinone aversive enough to inhibit its consumption, how do they overcome its noxiousness when they are exposed to beetle spray at higher concentrations?

There are several explanations as to why grasshopper mice may pursue benzoquinone defended prey. First, the beetles present a high caloric reward (see Chapter 1 for caloric results). The caloric value may be more or less motivating depending on the grasshopper mouse's food options and its likelihood of encountering other caloric prey, or whether it has simultaneous options to choose from, such as between a nonpalatable and palatable substance in the two-bottle choice test. A noxious benzoquinone solution without nutrients may not have provided sufficient incentive for consumption during the two-bottle choice test. Postingestive sensing of nutrients is an independent mechanism apart from taste signaling (Fernandes *et al.*, 2020) and is capable of changing feeding behavior (Sclafani, 2001). For example, mice that are unable to taste sweet substances due to lacking sensitivity in a different TRP channel, TRP5, can develop a preference for sucrose concentrations based on caloric content (De Araujo *et al.*, 2008). Considering that the results of the two-bottle choice test indicate that mice find high concentrations of benzoquinone to be aversive, additional testing could analyze different pairs of nutrients and benzoquinone concentrations to determine at which point caloric value may override the cost of noxious taste.

Caloric value may be more motivating than noxious taste if benzoquinone's noxious taste is a "false alarm" that tricks most prospective predators or is, at the least, harmless to grasshopper mice. *Manduca sexta* caterpillars have developed just such an avoidance of false signals through habituation to harmless, bitter-tasting plant compounds (Glendinning *et al.*, 2001). Grasshopper mice may similarly habituate to the bitter taste in order to meet their nutritional requirements. Moreover, the mice do not appear to show aversive conditioning towards stink beetles (Cyr, 1972; Langley, 1981). Alternatively, benzoquinone's bitterness may be a true signal that warns of its ability to cause tissue damage or affect fitness, especially since benzoquinone is toxic to mammalian cells and has at least short-term irritative effects (De Capitani *et al.*, 2011; Eisner *et al.*, 2005). In this case, grasshopper mice may have developed another style of physiological protection against tissue damage. Considering their longer handling times of beetles compared to undefended prey and evidence of discomfort (Chapter 1), this protection may be incomplete, just as how some protected garter snakes (*Thamnophis sirtalis*) are rendered immobile after eating toxic newts (*Taricha granulosa*) (Brodie III and Brodie Jr, 1990).

The predictions of optimal foraging theory may provide further support for this explanation, as a potential relationship between palatability and caloric reward may be more effectual in motivating grasshopper mice predation than for other rodent species. Optimal foraging theory predicts that organisms approximate optimal strategies to maximize their success at catching prey that affords them an energetic net profit. As a result, caloric input is expected to outweigh the cost of searching for and finding food, as well as the risks of exposure or losing opportunities to find other sources of calories (MacArthur and Pianka, 1966). Stink beetles may not be calorically profitable for rodents other than grasshopper mice. Grasshopper mice have multiple morphological adaptations, including long toenails to grasp prey and stronger jaws than deer mice, that enable speedy incapacitation of their prey (Bailey and Sperry, 1929; Carleton and Eshelman, 1979; Horner *et al.*, 1965; Langley, 1994; Satoh and Iwaku, 2006; Williams *et al.*, 2009). Juvenile grasshopper mice begin to pursue defended beetles as they become more efficient hunters and gain a net caloric profit (Slobodchikoff *et al.*, 1987). Adult grasshopper mice are also much faster predators than adult deer mice (Langley, 1994; Chapter 1), which suggests they have less energetically costly handling times. Armed with both morphological adaptations and acute predatory behavior, grasshopper mice may be better equipped and therefore more susceptible than other rodents to develop a preference for high caloric prey in spite of its unpalatability. Other prospective predators, like deer mice or house mice, may be deterred by the spray if there is no caloric profit, or if they are physiologically unprotected from insect benzoquinone and the chance that it can generate harmful tissue damage.

On a similar note, a second explanation for differences in rodent behavior is that experience with stink beetles may affect their preference for benzoquinone. Since the two-bottle choice test was designed to test innate sensitivity to benzoquinone, only mice naïve to insect prey were tested. Mice that have encountered benzoquinone and learned to associate it with caloric reward may show more interest and drink more of the benzoquinone solution.  For instance, both rats and mice fitted with catheters will drink more of one flavored solution over another when it is paired with a simultaneous intragastric nutrient infusion (Ramirez, 1994; Sclafani and Glendinning, 2003). Rats which associate high caloric content with a flavored drinking solution will then prefer food with the same flavor, even when given a choice between two food items with identical calories (Bolles *et al.*, 1981). This conditioning may explain the high rate of beetle predation by wild grasshopper mice (see Chapter 1), since these mice were very likely to have previous exposure to stink beetles and may have learned to associate its chemical cues with nutrients. In contrast, lab mice in the two-bottle choice test could not yet have developed this association. Testing wild mice experienced with stink beetles in a benzoquinone two-bottle choice test would help answer whether experience with stink beetles and their nutrient content results in decreased gustatory aversion to high concentrations of benzoquinone.

Third, contradicting reports of stink beetle predation by wild deer mice (Parmenter and Macmahon, 1988) and lab-raised deer mice (Langley, 1994) with a recent study describing aversion to stink beetles by wild deer mice (Chapter 1) may also be due to the effects of hunger on taste and chemical sensitivity. Similarly, the rodents' physiological states may have affected their sensitivity to benzoquinone during the two-bottle choice test when compared with the hunger levels experienced by grasshopper mice that consume beetles in nature. The mice used in the two-bottle choice test were provided food *ad libitum* throughout the experiment. Satiated animals are less likely to consume substances they normally find distasteful, while hungry animals have been shown to feel decreased aversion to bitter foods or food they usually dislike (Siep *et al.*, 2009, reviewed in Fu *et al.*, 2021). For example, energy deficient *Drosophila* are more likely to risk eating potentially toxic food as a result of enhanced sugar sensitivity and reduced bitter sensitivity during starvation (Inagaki *et al.*, 2014; Kain and Dahanukar, 2015). It is likely that wild mice are hungrier than those raised in the lab; state-dependent differences may have thereby contributed to their finding benzoquinone less aversive, potentially through a mechanism which downregulated their bitter taste sensitivity. Furthermore, downregulated bitter sensitivity that motivates dietary risk-taking may be an especially beneficial trait for facultatively carnivorous grasshopper mice that infrequently encounter prey. Omnivorous deer mice and house mice, in contrast, rely on a more expansive diet and may be able to afford being more selective in their food choices. Future work comparing the extent to which bitter taste is downregulated across rodent species with different dietary niches and different state-dependent hunger levels would be informative for understanding their behavioral responses to chemically defended prey.

Fourth, variation in rodent behaviors may be explained by their reliance on multiple cues in different experimental settings and differences in the ways rodent species perceive these cues. The two-bottle choice test was limited to investigating gustatory sensitivity, but in nature, predators rely on multimodal sensory cues to identify prey (e.g., Lawson *et al.*, 2019). As a result, sensory receptors in the rodents' eyes and olfactory tissue may also play an integral role in their decision to pursue beetles after encountering benzoquinone. Mice frequently close their eyes in response to the benzoquinone fumes and spray (see Chapter 1). Grasshopper mice are also known to rely heavily on olfaction, especially at close range and for deciding whether to attack prey (Whitman *et al.*, 1986). If sensitivity is lower in the eyes and nose of grasshopper mice than deer mice, the benzoquinone spray may be less noxious, and subsequently less aversive for them. The noxious effects of vinegarroon secretions, for example, are most effective when they contact sensitive respiratory, visual, or sensory systems of potential predators; feeding trials with vinegarroons and spadefoot toads (*Scaphiopus couchii*) revealed that the spray is an ineffective deterrent when restricted to inside the predator's mouth (Schmidt *et al.*, 2000). Differential expression of sensory receptors, like TRPA1, across tissue in the eye, nose, and mouth could also mitigate the effects of benzoquinone, especially if employed with defensive behaviors that reduce rodents' contact with the spray. This may involve olfactory suppression specifically in response to benzoquinone, since chemical cues from other types of defended prey, like *Taeniopoda eques* grasshoppers, are sufficiently aversive to grasshopper mice that they choose not to attack (Whitman *et al.*, 1986). Testing benzoquinone sensitivity in different rodent species' eyes and nose may reveal that gustatory sensitivity plays a less integral role in their reactions to stink beetles.

Finally, sensitivity to benzoquinone may also send signals other than pain. It is possible that benzoquinone may have an anti-nociceptive effect at higher concentrations than those tested in the two-bottle choice test, although currently there is no evidence for this occurring via oral digestion. Nonetheless, injections of benzoquinone in mouse spinal cord trigger TRPA1 while mimicking the chemically similar breakdown products and pain-blocking effects of acetaminophen (Andersson *et al.*, 2011). Future work would be required to determine if beetle concentrations of benzoquinone trigger an analgesic effect when contracted in a grasshopper mouse's eyes, nose, or mouth.

While both species of mice found the highest benzoquinone concentrations to be most aversive, they also preferred water over the lowest concentration tested, 0.0001 mM. This suggests that the rodents are able to detect benzoquinones at nanomolar concentrations. Since the rodents showed no preference for water over benzoquinone at 0.01 or 0.1 mM, it is possible that these concentrations created a physiological reaction similar to the Schweppes effect. This term refers to a preference for low concentrations of a deterrent chemical, similar to how humans enjoy low concentrations of quinine hydrochloride in the eponymously named tonic water (Glendinning, 1993). Variation in preference for low concentrations of bitter-tasting substances is demonstrated by numerous animal species (including *Peromyscus* spp.) and has intraspecific variation. Aversion to benzoquinone was also not gender specific for mice in this study, another stipulation of the Schweppe's effect (Glendinning, 1993). The indifference to benzoquinone solution at 0.01 and 0.1 mM may be the result of averaging together consumption amounts for individual mice that vary in preference and aversion to low concentrations of this chemical. In studies testing preferences for acidic solutions in spider monkeys (*Ateles geoffroyi*) and macaques (*Macaca nemestrina*), a similar inverted "U-shaped" pattern emerged in which the primates drank greater quantities of mid-range concentrations of acidic tastants but were indifferent or averse to the lowest and highest concentrations, respectively (Laska, 2000; Laska *et al.*, 2000). While the two-bottle choice test never identified concentrations of benzoquinone that mice consumed on average more than water, conducting additional tests with benzoquinone concentrations ranging from 0 to 0.1 mM may tease out whether there are consistent, individual preferences for benzoquinone and the range of concentrations that mice prefer or find neutral compared to water.

### *4.2 TRPA1 Sequence Comparisons*

Since TRPA1 is very conserved across the animal kingdom, it is not surprising that sequence similarity was high for all species surveyed. This included most of the N-terminus cysteines and C-terminus interactive sites that are likely essential for TRPA1 functionality. However, four cysteines were nonhomologous between house mice and the more closely related deer mice and grasshopper mice. Selection for any coding changes in a highly conserved channel that are unique to one or a few related species may signal an important modification that facilitates species-specific behavioral differences. For example, the single cysteine variant unique to grasshopper mice is a top target for future mutagenesis work investigating the relationship between form and function in TRPA1. When TRPA1 is knocked out entirely, only

5% of neurons cultured from mouse DRG tissue responded to 0.001 mM benzoquinone (Andersson *et al.*, 2011). Behavioral tests with grasshopper mice C231 knock-outs that maintain TRPA1's function may help determine whether this amino acid plays a role in their reduced sensitivity to formalin compared to deer mice or house mice. This site is also of interest because it is occupied by different amino acids between different rodent species, potentially indicating that it is less conserved across all vertebrates. These four variable positions may present potential sites in the TRPA1 gene driving sensitivity variation for prospective electrophilic agonists. Other TRP channel mutations, such as the sites nonhomologous between grasshopper, deer, and house mice, may also affect function and channel conformation (Ibarra and Blair, 2013), but would need to be explored further since most TRPA1 research has focused on cysteine activation (Macpherson *et al.*, 2007; Wang *et al.*, 2012).

A possible relationship between rodents' TRPA1 sequences and gustatory sensitivity to benzoquinone is unclear without additional information from physiological work. Based on the results of the two-bottle choice test, there appears to be no association between TRPA1 sequence homology and gustatory sensitivity in grasshopper mice and house mice. Both species were equally sensitive to benzoquinone, even though their TRPA1 genes had amino acid variants predicted to alter the effects of benzoquinone reception. Physiological work to determine the function of cysteine residue differences between the species would be a required next step for identifying links between genes and behavior.

A possible relationship between rodents' TRPA1 sequences and their predation of stink beetles is similarly ambiguous. Grasshopper mice and deer mice appear to have the most homologous TRPA1 sequences surveyed in this study and both have been reported to consume benzoquinone-squirting prey (Cyr, 1972; Langley, 1994). Nonetheless, no deer mice attacked or consumed stink beetles in more recent behavioral trials (see Chapter 1) and deer mice have been described as much more wary and reluctant predators of stink beetles when they are encountered (Cyr, 1972). These contradictory behavioral reports therefore prevent the possibility of drawing conclusions about the role of TRPA1 amino acid variants in shaping differences in grasshopper and deer mice predatory behavior.

TRPA1 structural homology may be more closely related to phylogenetic, rather than behavioral patterns. Deer mice and grasshopper mice are more genetically similar than house mice and grasshopper mice (Fabre *et al.*, 2012). These similarities may extend to single amino acid substitutions in the TRPA1 sequence without any consequences for gene functionality.

While conclusions about the effects of TRPA1's genetic code remain unclear, TRPA1 may mediate differences in rodent sensitivity to benzoquinone through variation in expression, especially considering that genes are known to be differentially expressed in DRG and TG neurons (Lopes *et al.*, 2017; Megat *et al.*, 2019) and TRPA1 is expressed in both the DRG and TG (Bautista *et al.*, 2005). These neuron groups are highly specialized and respond to noxious stimuli, but a number of TG neurons have been described as having specific chemosensitive properties related to olfaction and the gustatory system (Gerhold and Bautista, 2009; Lopes *et al.*, 2017; Viana, 2011). They also primarily innervate the head where grasshopper mice mainly encounter benzoquinone, while DRG neurons mostly innervate the rest of the body. Capsaicin, a chemical which activates a different TRP receptor, TRPV1, was found to trigger behavioral responses more strongly in mouse TG than DRG due to preferential signaling pathways that differ between the neuronal groups (Megat *et al.*, 2019). Similar chemical pathways may mediate benzoquinone in grasshopper mice TRPA1 receptors. For instance, if grasshopper mice have reduced TRPA1 expression in their TG compared to deer mice, then benzoquinone's effects may not be sufficient to inhibit predation. As described earlier, behavioral work testing grasshopper mice tissue sensitivity in the eyes and nose would complement TRPA1 differential expression work. Examining the TRPA1 sequence alongside its expression levels would thus be a promising starting point for confirming its role as a candidate gene underlying grasshopper mice predatory behavior.

TRPA1 is also not the only possible target for benzoquinone. Additional benzoquinone receptors may work alone or interact with TRPA1 to mediate rodent predation of stink beetles. Benzoquinone has been found to target at least eight other proteins in pulmonary bronchial epithelial cells that appear to play a role in distributing benzoquinone into a cell's mitochondrion and nucleus (Lamé *et al.*, 2003). Like TRPA1, these proteins, such as Galectin-1 and HSP60, are expressed in multiple tissue types, although changes to these genes may be constrained by conservation of their roles in supporting cellular functionality. None of these genes have been implicated in pain signaling, but species-specific changes to their genetic sequence or expression that do not impede basic function may provide additional evidence for an underlying mechanism driving tolerance or insensitivity to benzoquinone.

## 5. Conclusion

Grasshopper mice's pursuit of benzoquinone-defended prey, in spite of their sensitivity and aversive response to the chemical at low concentrations, offers numerous applications for translating lab results to the natural world. This positions grasshopper mice as model species that can be informative for ecological, evolutionary, and biomedical research. Most studies seeking to understand postingestive effects vs. palatability of food focus on artificial flavors, unnatural forms of intake, and limited food choices (Bolles *et al.*, 1981; Inagaki *et al.*, 2014; Laska, 2000;

Sclafani, 2001; Sclafani and Glendinning, 2003). The ability to compare a species' aversion to a chemical solution in spite of tolerance (and potentially preference) for food containing that chemical in the wild is an ideal opportunity for learning more about how state-dependence, postingestive effects, and foraging costs drive prey choice, prey defenses, and behavioral and morphological adaptations related to predator-prey interactions.

The mechanism behind grasshopper mice's predatory capabilities may be a complex interaction of genetic structure, expression, and input from gustatory, olfactory, and chemical detecting chemosensory systems. Determining the extent to which these factors influence prey choice and chemical tolerance may be accomplished through comparative studies of other predators that consume defended prey. This can be done by comparing grasshopper mice to related rodents that lacks those behaviors within a model system of desert rodent species, as demonstrated here. But identifying the patterns of chemosensory mechanisms underlying consumption of noxious prey would also benefit from comparing grasshopper mice with predators that employ similar hunting strategies. Behavioral observations describing coati predation of millipedes (Weldon *et al.*, 2006), skunk predation of beetles (Slobodchikoff, 1978), spadefoot toad predation of vinegaroons (Schmidt *et al.*, 2000), or sea turtle and sunfish consumption of venomous jellyfish (Breen *et al.*, 2017) have stopped short of searching for reduced chemical sensitivity in these animals. Identifying more physiological mechanisms in species that regularly eat an array of noxious food may help determine whether these predators employ specialized protections against targeted prey, potentially as a result of a co-evolutionary arms race, or rely on an underlying genetic flexibility that renders them resistant to diverse chemical defenses. Grasshopper mice, for instance, consume venomous tarantulas and centipedes without adverse effects, but only the mechanism by which they are immune to scorpion toxin is currently understood.

The ability to pinpoint new genes that motivate predation in additional carnivorous or omnivorous species will complement current understanding of the genetic mechanisms behind adaptive feeding behavior in herbivores, such as unique ribonucleases that increase the efficiency of digestion in leaf-eating colobine monkeys (Zhang *et al.*, 2002) or expanded cytochrome P450 genes that allow koalas to detoxify eucalyptus (Johnson *et al.*, 2018). Pain and chemical sensitivity are essential because they warn animals of possible tissue damage; seemingly paradoxical species that demonstrate immunity to multiple toxic and chemical compounds, without the loss of basic pain function, provide remarkable opportunities for understanding how selective forces work within the constraints of biology to shape adaptive traits.

# REFERENCES

# REFERENCES

Andersson, D. A., Gentry, C., Alenmyr, L., Killander, D., Lewis, S. E., Andersson, A., Bucher, B., Galzi, J.-L., Sterner, O. & Bevan, S. (2011). TRPA1 mediates spinal antinociception induced by acetaminophen and the cannabinoid Δ 9-tetrahydrocannabiorcol. *Nature communications* 2, 1-11.

Aneshansley, D. J., Eisner, T., Widom, J. M. & Widom, B. (1969). Biochemistry at 100 C: explosive secretory discharge of bombardier beetles (Brachinus). *science*, 61-63.

Bachmanov, A. A., Tordoff, M. G. & Beauchamp, G. K. (1996). Ethanol consumption and taste preferences in C57BL/6ByJ and 129/J mice. *Alcoholism: Clinical and Experimental Research* **20**, 201-206.

Bailey, V. & Sperry, C. (1929). Life history and habits of grasshopper mice, genus Onychomys. pp. 1-19: U.S. Department of Agriculture.

Bandell, M., Story, G. M., Hwang, S. W., Viswanath, V., Eid, S. R., Petrus, M. J., Earley, T. J. & Patapoutian, A. (2004). Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. *Neuron* **41**, 849-857.

Bates, D., Maechler, M., Bolker, B. & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software, **67**(1), 1-48.

Bautista, D. M., Jordt, S.-E., Nikai, T., Tsuruda, P. R., Read, A. J., Poblete, J., Yamoah, E. N., Basbaum, A. I. & Julius, D. (2006). TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell* **124**, 1269-1282.

Bautista, D. M., Movahed, P., Hinman, A., Axelsson, H. E., Sterner, O., Högestätt, E. D., Julius, D., Jordt, S.-E. & Zygmunt, P. M. (2005). Pungent products from garlic activate the sensory ion channel TRPA1. *Proceedings of the National Academy of Sciences* **102**, 12248-12252.

Blair, N. T., Philipson, B. I., Richards, P. M., Doerner, J. F., Segura, A., Silver, W. L. & Clapham, D. E. (2016). Naturally produced defensive alkenal compounds activate TRPA1. *Chemical senses* **41**, 281-292.

Bolles, R. C., Hayward, L. & Crandall, C. (1981). Conditioned taste preferences based on caloric density. *Journal of Experimental Psychology: Animal Behavior Processes* **7**, 59.

Breen, P., Cañadas, A., Cadhla, O. Ó., Mackey, M., Scheidat, M., Geelhoed, S. C., Rogan, E. & Jessopp, M. (2017). New insights into ocean sunfish (Mola mola) abundance and seasonal distribution in the northeast Atlantic. *Scientific reports* **7**, 1-9.

Brodie III, E. D. & Brodie Jr, E. D. (1990). Tetrodotoxin resistance in garter snakes: an evolutionary response of predators to dangerous prey. *Evolution* **44**, 651-659.

Carleton, M. D. & Eshelman, R. E. (1979). A Synopsis of Fossil Grasshopper Mice, Genus Onychomys, and Their Relationships to Recent Species. *Papers on Paleontology* **21**.

Conner, W. E., Alley, K. M., Barry, J. R. & Harper, A. E. (2007). Has vertebrate chemesthesis been a selective agent in the evolution of arthropod chemical defenses? *The Biological Bulletin* **213**, 267-273.

Cyr, M. A. (1972). Predatory Behavior of the Grasshopper Mouse (Onychomys). In *Zoology*: University of California, Los Angeles.

Davis, S. F., Cunningham, L. A., Burke, T. J., Richard, M. M., Langley, W. M. & Theis, J. (1986). A preliminary analysis of the suppressive effects of denatonium saccharide. *Bulletin of the Psychonomic Society* **24**, 229-232.

Davis, S. F., Hoskinson, K. J., Wilder, K. A., Sander, J. A., Larsen, R. K. & Knapp, M. (1988). A cross-species analysis of the aversiveness of denatonium saccharide and quinine. *Bulletin of the Psychonomic Society* **26**, 419-422.

De Araujo, I. E., Oliveira-Maia, A. J., Sotnikova, T. D., Gainetdinov, R. R., Caron, M. G., Nicolelis, M. A. & Simon, S. A. (2008). Food reward in the absence of taste receptor signaling. *Neuron* **57**, 930-941.

De Capitani, E. M., Vieira, R. J., Bucaretchi, F., Fernandes, L. C., Toledo, A. S. & Camargo, A. C. (2011). Human accidents involving Rhinocricus spp., a common millipede genus observed in urban areas of Brazil. *Clinical toxicology* **49**, 187-190.

Dong, D., Jones, G. & Zhang, S. (2009). Dynamic evolution of bitter taste receptor genes in vertebrates. *BMC evolutionary biology* **9**, 1-9.

Doyen, J. T. & Somerby, R. (1974). Phenetic similarity and Müllerian mimicry among darkling ground beetles (Coleoptera: Tenebrionidae). *The Canadian Entomologist* **106**, 759-772.

Eagle, A. L., Mazei-Robison, M. & Robison, A. J. (2016). Sucrose preference test to measure stress-induced anhedonia. *Bio-protocol* **6**, e1822-e1822.

Eisner, T., Eisner, M. & Siegler, M. (2005). *Secret weapons: defenses of insects, spiders, scorpions, and other many-legged creatures*: Harvard University Press.

Eisner, T. & Meinwald, J. (1966). Defensive secretions of arthropods. *science* **153**, 1341-1350.

Fabre, P.-H., Hautier, L., Dimitrov, D. & Douzery, E. J. (2012). A glimpse on the pattern of rodent diversification: a phylogenetic approach. *BMC evolutionary biology* **12**, 88.

Fernandes, A. B., da Silva, J. A., Almeida, J., Cui, G., Gerfen, C. R., Costa, R. M. & Oliveira-Maia, A. J. (2020). Postingestive modulation of food seeking depends on vagus-mediated dopamine neuron activity. *Neuron* **106**, 778-788. e776.

Fu, O., Minokoshi, Y. & Nakajima, K.-i. (2021). Recent Advances in Neural Circuits for Taste Perception in Hunger. *Frontiers in Neural Circuits* **15**, 1.

Garcia, J. (1975). The Evolution of Bitter and the Acquisition of Toxiphobia John Garcia and Walter G. Hankins Departments of Psychology and Psychiatry University of California at Los Angeles. In *Olfaction and Taste V: Proceedings of the Fifth International Symposium Held at the Howard Florey Institute of Experimental Physiology & Medicine, University of Melbourne, Australia, October 1974,* p. 39: Academic Press.

Gerhold, K. A. & Bautista, D. M. (2009). Molecular and cellular mechanisms of trigeminal chemosensation. *Annals of the New York Academy of Sciences* **1170**, 184.

Glendinning, J. I. (1993). Preference and aversion for deterrent chemicals in two species of Peromyscus mouse. *Physiology & behavior* **54**, 141-150.

Glendinning, J. I. (1994). Is the bitter rejection response always adaptive? *Physiology & behavior* **56**, 1217-1227.

Glendinning, J. I., Brown, H., Capoor, M., Davis, A., Gbedemah, A. & Long, E. (2001). A peripheral mechanism for behavioral adaptation to specific “bitter” taste stimuli in an insect. *Journal of Neuroscience* **21**, 3688-3696.

Hinman, A., Chuang, H.-h., Bautista, D. M. & Julius, D. (2006). TRP channel activation by reversible covalent modification. *Proceedings of the National Academy of Sciences* **103**, 19564-19568.

Horner, B. E., Taylor, J. M. & Padykula, H. A. (1965). Food habits and gastric morphology of the grasshopper mouse. *Journal of Mammalogy* **45**, 513-535.

Ibarra, Y. & Blair, N. T. (2013). Benzoquinone reveals a cysteine-dependent desensitization mechanism of TRPA1. *Molecular pharmacology* **83**, 1120-1132.

Inagaki, H. K., Panse, K. M. & Anderson, D. J. (2014). Independent, reciprocal neuromodulatory control of sweet and bitter taste sensitivity during starvation in Drosophila. *Neuron* **84**, 806-820.

Johnson, R. N., O’Meally, D., Chen, Z., Etherington, G. J., Ho, S. Y., Nash, W. J., Grueber, C. E., Cheng, Y., Whittington, C. M. & Dennison, S. (2018). Adaptation and conservation insights from the koala genome. *Nature genetics* **50**, 1102-1111.

Jordt, S.-E., Bautista, D. M., Chuang, H.-h., McKemy, D. D., Zygmunt, P. M., Högestätt, E. D., Meng, I. D. & Julius, D. (2004). Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature* **427**, 260-265.

Kain, P. & Dahanukar, A. (2015). Secondary taste neurons that convey sweet taste and starvation in the Drosophila brain. *Neuron* **85**, 819-832.

Lamé, M. W., Jones, A. D., Wilson, D. W. & Segall, H. J. (2003). Protein targets of 1, 4-benzoquinone and 1, 4-naphthoquinone in human bronchial epithelial cells. *Proteomics* **3**, 479-495.

Langley, W. (1981). Failure of food-aversion conditioning to suppress predatory attack of the grasshopper mouse, Onychomys leucogaster. *Behavioral and Neural Biology* **33**, 317-333.

Langley, W. M. (1994). Comparison of predatory behaviors of deer mice (Peromyscus maniculatus) and grasshopper mice (Onychomys leucogaster). *Journal of Comparative Psychology* **108**, 394.

Langley, W. M., Theis, J., Davis, S. F., Richard, M. M. & Grover, C. A. (1987). Effects of denatonium saccharide on the drinking behavior of the grasshopper mouse (Onychomys leucogaster). *Bulletin of the Psychonomic Society* **25**, 17-19.

Laska, M. (2000). Gustatory responsiveness to food-associated sugars and acids in pigtail macaques, Macaca nemestrina. *Physiology & behavior* **70**, 495-504.

Laska, M., Salazar, L. T. H., Luna, E. R. & Hudson, R. (2000). Gustatory responsiveness to food-associated acids in the spider monkey (Ateles geoffroyi). *Primates* **41**, 213-221.

Lawson, R. R., Fogarty, D. T. & Loss, S. R. (2019). Use of visual and olfactory sensory cues by an apex predator in deciduous forests. *Canadian Journal of Zoology* **97**, 488-494.

Lis, L., Bakula, T., Baranowski, M. & Czarnewicz, A. (2011). The carcinogenic effects of benzoquinones produced by the flour beetle. *Polish Journal of Veterinary Sciences* **14**.

Lopes, D. M., Denk, F. & McMahon, S. B. (2017). The molecular fingerprint of dorsal root and trigeminal ganglion neurons. *Frontiers in molecular neuroscience* **10**, 304.

Lunceford, B. E. & Kubanek, J. (2015). Reception of aversive taste. *Integrative and comparative biology* **55**, 507-517.

Macpherson, L. J., Dubin, A. E., Evans, M. J., Marr, F., Schultz, P. G., Cravatt, B. F. & Patapoutian, A. (2007). Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. *Nature* **445**, 541-545.

Macpherson, L. J., Geierstanger, B. H., Viswanath, V., Bandell, M., Eid, S. R., Hwang, S. & Patapoutian, A. (2005). The pungency of garlic: activation of TRPA1 and TRPV1 in response to allicin. *Current Biology* **15**, 929-934.

McNamara, C. R., Mandel-Brehm, J., Bautista, D. M., Siemens, J., Deranian, K. L., Zhao, M., Hayward, N. J., Chong, J. A., Julius, D. & Moran, M. M. (2007). TRPA1 mediates formalin-induced pain. *Proceedings of the National Academy of Sciences* **104**, 13525-13530.

Megat, S., Ray, P. R., Tavares-Ferreira, D., Moy, J. K., Sankaranarayanan, I., Wanghzou, A., Lou, T. F., Barragan-Iglesias, P., Campbell, Z. T. & Dussor, G. (2019). Differences between dorsal root and trigeminal ganglion nociceptors in mice revealed by translational profiling. *Journal of Neuroscience* **39**, 6829-6847.

Nilius, B., Appendino, G. & Owsianik, G. (2012). The transient receptor potential channel TRPA1: from gene to pathophysiology. *Pflügers Archiv-European Journal of Physiology* **464**, 425-458.

Parmenter, R. R. & Macmahon, J. A. (1988). Factors limiting populations of arid-land darkling beetles (Coleoptera: Tenebrionidae): predation by rodents. *Environmental Entomology* **17**, 280-286.

Paulsen, C. E., Armache, J.-P., Gao, Y., Cheng, Y. & Julius, D. (2015). Structure of the TRPA1 ion channel suggests regulatory mechanisms. *Nature* **520**, 511-517.

Ramirez, I. (1994). Stimulation of fluid intake by carbohydrates: interaction between taste and calories. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **266**, R682-R687.

Reinhard, J. (2010). Taste: invertebrates. *Encyclopedia of animal behavior* **3**, 379-385.

Rowe, A. H. & Rowe, M. P. (2008). Physiological resistance of grasshopper mice (Onychomys spp.) to Arizona bark scorpion (Centruroides exilicauda) venom. *Toxicon* **52**, 597-605.

Rowe, A. H., Xiao, Y., Rowe, M. P., Cummins, T. R. & Zakon, H. H. (2013). Voltage-gated sodium channel in grasshopper mice defends against bark scorpion toxin. *science* **342**, 441-446.

Sahley, C., Gelperin, A. & Rudy, J. W. (1981). One-trial associative learning modifies food odor preferences of a terrestrial mollusc. *Proceedings of the National Academy of Sciences* **78**, 640-642.

Samad, A., Sura, L., Benedikt, J., Ettrich, R., Minofar, B., Teisinger, J. & Vlachova, V. (2011). The C-terminal basic residues contribute to the chemical-and voltage-dependent activation of TRPA1. *Biochemical Journal* **433**, 197-204.

Satoh, K. & Iwaku, F. (2006). Jaw muscle functional anatomy in northern grasshopper mouse, Onychomys leucogaster, a carnivorous murid. *Journal of Morphology* **267**, 987-999.

Schmidt, J. O., Dani, F. R., Jones, G. R. & Morgan, E. D. (2000). Chemistry, ontogeny, and role of pygidial gland secretions of the vinegaroon Mastigoproctus giganteus (Arachnida: Uropygi). *Journal of Insect Physiology* **46**, 443-450.

Scholl, B., Pattadkal, J. J., Rowe, A. & Priebe, N. J. (2017). Functional characterization and spatial clustering of visual cortical neurons in the predatory grasshopper mouse Onychomys arenicola. *Journal of neurophysiology* **117**, 910-918.

Sclafani, A. (2001). Post-ingestive positive controls of ingestive behavior. *Appetite* **36**, 79-83.

Sclafani, A. & Glendinning, J. I. (2003). Flavor preferences conditioned in C57BL/6 mice by intragastric carbohydrate self-infusion. *Physiology & behavior* **79**, 783-788.

Shi, P., Zhang, J., Yang, H. & Zhang, Y.-p. (2003). Adaptive diversification of bitter taste receptor genes in mammalian evolution. *Molecular biology and evolution* **20**, 805-814.

Siep, N., Roefs, A., Roebroeck, A., Havermans, R., Bonte, M. L. & Jansen, A. (2009). Hunger is the best spice: an fMRI study of the effects of attention, hunger and calorie content on food reward processing in the amygdala and orbitofrontal cortex. *Behavioural brain research* **198**, 149-158.

Slobodchikoff, C. (1978). Experimental studies of tenebrionid beetle predation by skunks. *Behaviour* **66**, 313-322.

Slobodchikoff, C., Vaughan, T. A. & Warner, R. M. (1987). How prey defenses affect a predator's net energetic profit. *Journal of Mammalogy* **68**, 668-671.

Spielman, A. I., Yan, W. & Brand, J. G. (2019). Chemosensory systems. *eLS*.

Talavera, K., Startek, J. B., Alvarez-Collazo, J., Boonen, B., Alpizar, Y. A., Sanchez, A., Naert, R. & Nilius, B. (2020). Mammalian transient receptor potential TRPA1 channels: from structure to disease. *Physiological reviews* **100**, 725-803.

Team, R. C. (2020). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Tschinkel, W. R. (1975). A comparative study of the chemical defensive system of tenebrionid beetles. Defensive behavior and ancillary features. *Annals of the Entomological Society of America* **68**, 439-453.

Viana, F. (2011). Chemosensory properties of the trigeminal system. *ACS chemical neuroscience* **2**, 38-50.

Weldon, P. J., Cranmore, C. F. & Chatfield, J. A. (2006). Prey-rolling behavior of coatis (Nasua spp.) is elicited by benzoquinones from millipedes. *Naturwissenschaften* **93**, 14-16.

Whitman, D. W., Blum, M. S. & Jones, C. G. (1986). Olfactorily mediated attack suppression in the southern grasshopper mouse toward an unpalatable prey. *Behavioural processes* **13**, 77-83.

Williams, S. H., Peiffer, E. & Ford, S. (2009). Gape and bite force in the rodents Onychomys leucogaster and Peromyscus maniculatus: Does jaw-muscle anatomy predict performance? *Journal of Morphology* **270**, 1338-1347.

Wittstock, U. & Gershenzon, J. (2002). Constitutive plant toxins and their role in defense against herbivores and pathogens. *Current opinion in plant biology* **5**, 300-307.

Wright, I. & Milne, J. (1996). Aversion of red deer and roe deer to denatonium benzoate in the diet. *Forestry: An International Journal of Forest Research* **69**, 1-4.

Zhang, J., Zhang, Y.-p. & Rosenberg, H. F. (2002). Adaptive evolution of a duplicated pancreatic ribonuclease gene in a leaf-eating monkey. *Nature genetics* **30**, 411-415.

# CHAPTER 3: SPERM COMPETITION, SEXUAL SELECTION, AND THE DIVERSE REPRODUCTIVE BIOLOGY OF OSTEOGLOSSIFORMES

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## Abstract

Osteoglossiformes are an order of “bony tongue” fish considered the most ancient living order of teleosts. This review seeks to consolidate known hypotheses and identify gaps in the literature regarding the adaptive significance of diverse reproductive traits and behavior of osteoglossiforms within the context of sperm competition and the wider lens of sexual selection. Many of the unusual traits observed in osteoglossiforms indicate low levels of sperm competition; most species have unpaired gonads and mormyroids are the only known vertebrate species with aflagellate sperm. Several osteoglossiform families have reproductive anatomy associated with internal fertilization but perform external fertilization, which may be representative of the evolutionary transition from external to internal fertilization and putative tradeoffs between sperm competition and the environment. They also employ every type of parental care seen in vertebrates. Geographically widespread and situated as one of the oldest teleost groups, Osteoglossiforms present an effective study system for understanding how sperm competition and sexual selection have shaped the evolution of teleost reproductive behavior, sperm and gonad morphology, fertilization strategies, courtship and paternal care, and sexual conflict. We suggest that the patterns seen in osteoglossiform reproduction are a microcosm of teleost reproductive diversity, potentially signifying the genetic plasticity that contributed to the adaptive radiation of teleost fishes.

## 1. Introduction

The approximately 26,000 known species of teleost fish occupy a wide range of aquatic habitats (Froese and Pauly, 2019) and exhibit a remarkable array of reproductive strategies (Helfman *et al.*, 2009). It is presently unclear which factors contribute to the evolution of such numerous reproductive strategies within teleosts (Smith and Wootton, 2016). Within teleosts, Osteoglossiformes ("bony tongue") are one of the oldest orders and are a microcosm of teleost reproductive diversity. They exhibit variation in reproductive anatomy, gamete morphology, and reproductive behavior, all suggesting flexibility early in teleost evolution (Figure 3.1). Osteoglossiforms are distributed worldwide and are comprised of five families: Osteoglossidae (15 species in South America, Africa, Asia, and Australia), Notopteridae (10 species in Africa and South-East Asia), Pantodontidae (one species in Africa), and two families of weakly electric fish, Mormyridae (>200 species, Africa) and Gymnarchidae (one species, Africa). In accordance with osteoglossiforms' cosmopolitan distribution and their ancestral position within teleosts, insights into the evolution of their reproductive diversity may lead to new understanding about the factors underlying the evolution of reproduction in teleosts (Figure 3.2).

Several reviews have considered osteoglossiform reproductive behavior (Britz, 2004; Hopkins, 1986; Karino, 2009; Kolm, 2009; Yanwirsal, 2013) and sperm morphology (Jamieson, 1991; Mattei, 1988; 1991; Mattei *et al.*, 2018; Pitnick *et al.*, 2009). The purpose of this review is to integrate and contextualize current understanding of reproductive anatomy, gametic morphology and reproductive behavior in light of sperm competition and sexual selection. Section 2 assesses the role of sperm competition and sexual selection in contributing to osteoglossiform reproductive anatomy with a focus on their unusual unpaired gonads and sexually dimorphic traits. Section 3 discusses how sperm competition may select for aflagellate, monoflagellate, and biflagellate sperm in osteoglossiforms, and whether this gamete morphology imposes specific sperm-egg interactions. Section 4 considers how morphological traits, sperm competition, and sexual selection interact and contribute to adaptive reproductive behavior during courtship and parental care. Section 5 details the challenges of differentiating the effects of sperm competition and sexual selection from environmental constraints and includes suggestions for studies to fill current gaps in our knowledge on these topics. This article concludes with recommendations for combining research on osteoglossiform reproduction with genomics for the benefit of understanding teleost diversity.

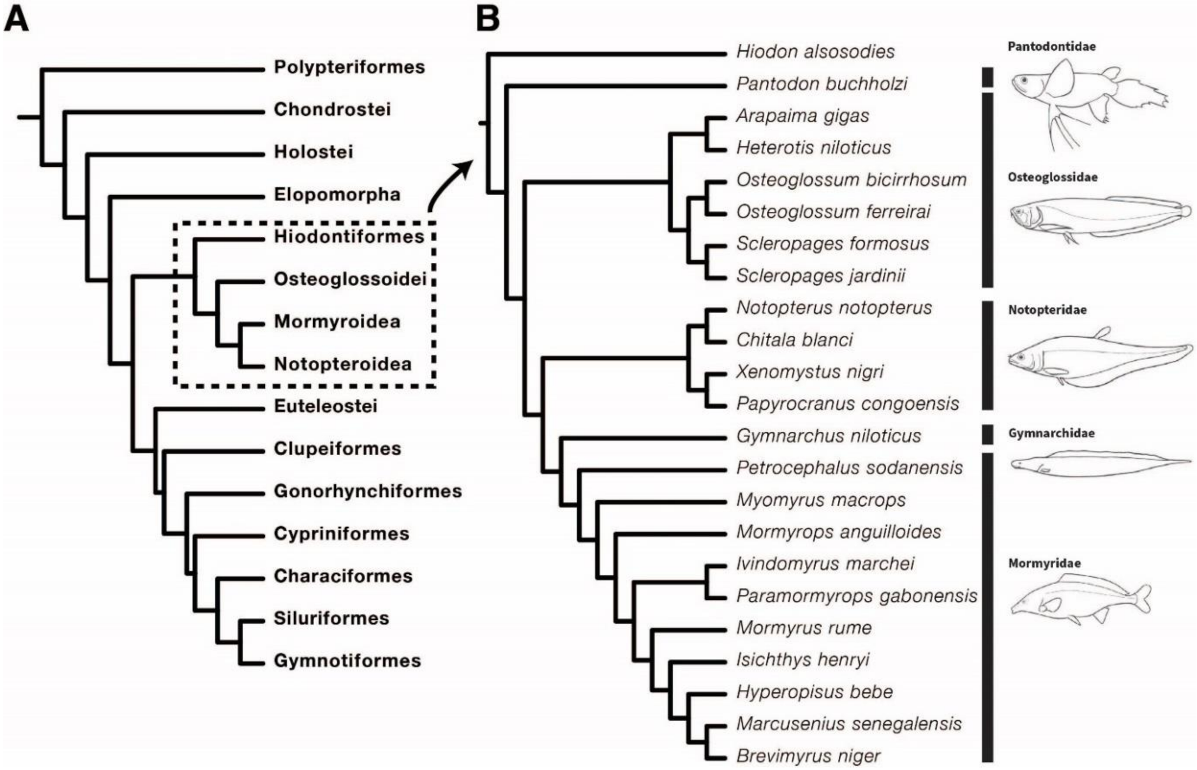
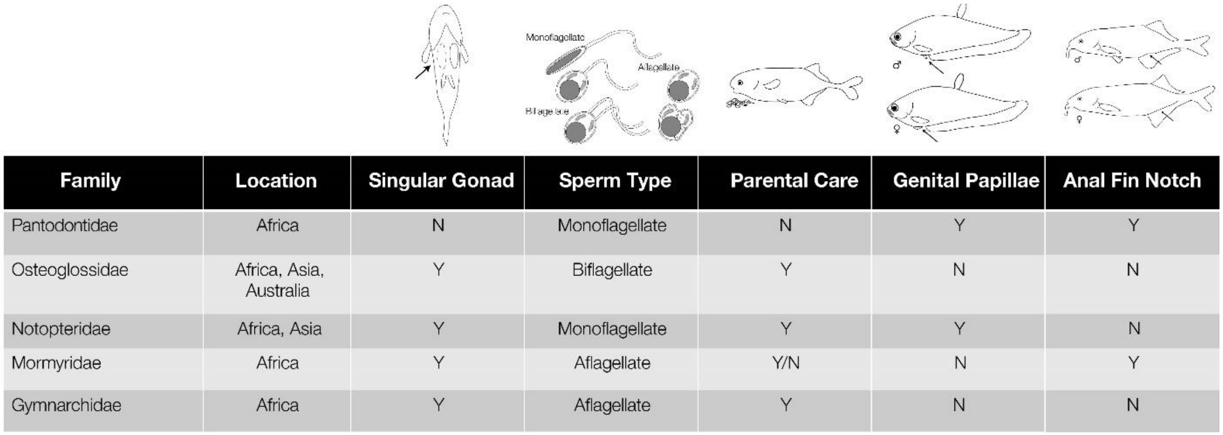


**Figure 3.1:** (a) Phylogeny of all major teleost clades (adapted from Nelson et al., 2016). (b) Detailed phylogeny of Osteoglossiformes, including selected names and illustrations of representative species from the families Pantodontidae, Osteoglossidae, Notopteridae, Gymnarchidae and Mormyridae. Representative images of each group are from Nelson et al. (2016), reproduced with permission from publisher.



| Family | Location | Singular Gonad | Sperm Type | Parental Care | Genital Papillae | Anal Fin Notch |
|---|---|---|---|---|---|---|
| Pantodontidae | Africa | N | Monoflagellate | N | Y | Y |
| Osteoglossidae | Africa, Asia, Australia | Y | Biflagellate | Y | N | N |
| Notopteridae | Africa, Asia | Y | Monoflagellate | Y | Y | N |
| Mormyridae | Africa | Y | Aflagellate | Y/N | N | Y |
| Gymnarchidae | Africa | Y | Aflagellate | Y | N | N |

**Figure 3.2:** Summary of the geographic distribution and reproductive traits in various families of Osteoglossiformes. Y = yes, N = no, see text for supporting references. Images depict reproductive traits from representative species.

### *1.1 Sperm Competition*

Sperm competition theorists largely agree that ejaculate expenditure is determined by sexual selection in accordance with economic principles (Parker and Pizzari, 2010). A major sperm competition theory models sperm competition as a raffle, in which each male has a probability of fertilization that is dependent on the number of sperm he has contributed, compared to his competitors (Parker, 1990). Under conditions in which there is less competition among males either pre-fertilization (e.g., internal fertilization or close proximity of a mating pair) or post-fertilization (e.g., females mate only once), a single male would be expected to provide sperm of lower quantity and quality. In situations where sperm competition risk is high, sperm competition has the potential to act as a type of quality control and favor high volumes of efficient, error-free sperm. This strategy has limitations, however; while producing sperm is not considered to be as energetically expensive as producing eggs, sperm and ejaculate fluid is not low-cost (for a review see Pitnick et al., 2009). Thus, sperm competition suggests that males will not produce high quality sperm unless the benefits exceed the costs of production.

Sperm competition and sexual selection are cyclically linked from the cellular to behavioral levels of biological organization (Parker, 2014). Females contribute more resources than males to gamete production and often invest further in offspring survival by providing parental care. Because their fitness is limited by their ability to produce gametes, females typically become the choosy sex. Male fitness is limited instead by the availability of the opposite sex. This leads to sexual selection, in which males compete with other males for mating opportunities with the opposite sex. When males experience strong sexual selection, sperm competition is expected to be high.

## 2. Anatomical Traits

The diversity of teleost body plans presents a challenge for identifying rules of selection on fish reproduction. The relationship between reproductive anatomy and overall body anatomy can be confounded by similarities with other species due to relatedness, foraging mode, and hydrodynamic and environmental concerns on body shape (Montgomerie and Fitzpatrick, 2009). Nonetheless, general patterns in osteoglossiform morphology, as a subset of teleosts, can serve as useful indicators of selective factors, such as sperm competition and sexual selection, that shape reproductive traits in fishes. This section focuses on two anatomical traits in osteoglossiforms, unpaired gonads and sexual dimorphism, and discusses the relationship of these features with parental care and divergent fertilization strategies.

### *2.1 Singular Gonads*

Although most teleost fish have paired gonads, most osteoglossiforms have a single gonad. The one known exception is *Pantodon buccholzi*, in which females have both left and right developed ovaries (Nysten, 1962). A single gonad on the left side of the body has been observed in adult Osteoglossidae (Fontanele, 1948; Lake and Midgley, 1970; Lüling, 1964; Merrick and Schmida, 1984; Moreau, 1982), Notopteridae (Argumedo, 2009; Dalela *et al.*, 1976; Nyonje, 2006; Yanwirsal *et al.*, 2017), Mormyridae (Adjibade *et al*., 2020; Iles, 1960; Nawar, 1959; Schugardt and Kirschbaum, 2004; Scott, 1973), and Gymnarchidae (Opadokun and Ajani, 2015). Unpaired gonads are synapomorphic for the Osteoglossiformes, whereas paired ovaries are likely a plesiomorphic trait of Osteoglossomorpha (Britz, 2004; Yanwirsal *et al.*, 2017).

Although singular gonads are relatively uncommon, identifying the selective pressures that led to their development in other species may be fruitful for understanding how they evolved in osteoglossiforms. In teleosts, the evolution of an unpaired testis appears to be a rare occurrence. Nonetheless, a single median ovary is observed in most viviparous teleosts, such as poecillids (Scrimshaw, 1945) and the marbled swamp eel (Synbranchiformes: Symbranchidae) (Ravaglia and Maggese, 2002), as well as both oviparous and viviparous goodeids (Uribe *et al.*, 2012). Fertilization can take place in the ovarian cavity or follicles, with the ovary providing a nutritional role for internally gestating young (Aranzábal *et al.*, 2009).

Ovarian asymmetry is also associated with viviparity in other ancestral groups of fishes. Coelacanths tend to have asymmetric ovaries in which the right ovary is functional and the left is sterile (Millot *et al.*, 1978). Many sharks and rays also have asymmetric ovaries (Wourms, 1977). Nurse sharks (*Ginglymostoma cirratum)* only develop one ovary on the right side of the body (Castro, 2000). Amazonian freshwater stingrays (*Potamotrygon wallacei*) have two functional ovaries, but the left ovary is 55 times larger than the right ovary (da Silva *et al.*, 2017). The presence of ovarian asymmetry in both Chondrichthyes and Osteichthyes suggests that this ancestral trait may go as far back as the Gnathosomes.

Asymmetric gonads are found in other vertebrate species (Yu, 1998), including platypus, which have a functioning ovary only on the left side (Grützner *et al.*, 2008), as well as contrasting directional asymmetry found in frogs (Liu *et al.*, 2011; Zhou *et al.*, 2011) and birds (Friedmann, 1927). Males in many avian species have a smaller right testis than the left (Lake, 1981). For example, the left testis of the male zebra finch is about 50% larger than the right testis (Birkhead *et al.*, 1998). Females typically only develop a left ovary and oviduct, whereas the right ovary regresses and degenerates after hatching (Wakamatsu *et al.*, 2000). This reversed pattern of asymmetry between males and females implies that the loss of a functional gonad is not homologous between the two sexes in birds (Ligon, 1997). It is hypothesized that the right ovary is vestigial in females and this loss is an adaptation to reduce weight for flight (Guraya, 2013; Zhang *et al.*, 2014). However, considering that ovarian asymmetry exists in non-flying platypus and ratite birds, it is also possible that coordination of egg production through a single oviduct provides protection for fragile eggs (Guioli *et al.*, 2014). In birds, it takes time for the eggshell to develop between ovulation and when the egg is laid, and it has been suggested for raptors that multiple eggs in close proximity within the mother's abdomen could reduce their survival (Guioli *et al.*, 2014; Walter, 1979). If a potential relationship exists between single gonads and egg protection, it is possible that this may be a practical cause of single gonads in other egg producing species, including amphibians or fish.

The cause and mechanisms of directional asymmetry in most avian males is unknown, but male coucals (Cuculiformes: Centropodinae) exhibit atrophied or complete loss of the left testis (Rand, 1933). Several studies suggested that coucals may develop only one functional testis as the byproduct of a physiological mechanism that decreases testosterone and promotes paternal care (Goymann *et al.*, 2004b; Ligon, 1997). But, this idea was subsequently rejected due to male coucals exhibiting testosterone levels similar to those of socially monogamous male birds with two testes, as well as presenting high levels of androgens overall (Goymann *et al.*, 2004a; Goymann and Wingfield, 2004; Goymann *et al.*, 2004b; Voigt and Goymann, 2007).

### *2.2 Sexual dimorphism*

Sexual dimorphism in osteoglossiforms appears to be primarily functional for reproduction and, similar to many fishes, related to key differences that define internal from external fertilization strategies (Evans and Meisner, 2009; Meisner, 2005). A notched anal fin is attributed to the males of *Pantodon* (Hjerresen, 1937) and several mormyrid species (Brown *et al.*, 1996; Iles, 1960; Kirschbaum, 1987; Nawar, 1959; Pezzanite and Moller, 1998; Svensson, 1933), and Notopteridae have sexually dimorphic genital papillae (Weitkamp, 2005; Yanwirsal, 2013; Yanwirsal *et al.*, 2017). Interestingly, these species exhibit behaviors attributed to both external and internal fertilizers, even though sexually dimorphic anal fins, elongated genital papillae, and elongated spermatozoa are all traits associated with internal insemination in species belonging to Characiformes, Siluriformes, Osmeriformes, Ophiidiformes, Atheriniformes, Beloniformes, Cryprinodontiformes, Scorpaeniformes, and Perciformes (Burns *et al.*, 1995; Evans and Meisner, 2009; Jamieson, 1989; Koya *et al.*, 2002; Luo *et al.*, 2011; Spadella *et al.*, 2012).

*P. buccholzi* is the only osteoglossiform species with all three of these traits and also the only one hypothesized to perform internal fertilization; visual observations are still required for definitive confirmation. Males have paired orifices adjacent to the anal fin that lead to a pouch holding a complex copulatory organ (Lastein and Van Deurs, 1973). The cone shaped organ is made of connective tissue that forms two folded structures situated in two pouches, each covered by a bony plate that protrudes during spawning and that appears to function to internally inseminate the female (Hjerresen, 1937; Lastein and Van Deurs, 1973). In combination with their unusual sperm phenotype (see section 3.2), *P. buccholzi* is the only member of this order with anatomy wholly suited for insemination, exhibiting reproductive anatomy similar to that of several internally fertilizing species belonging to Galaxiiidae (Pusey and Stewart, 1989), Aphyonidae (Nielsen, 1984), and Bythidae (Suarez, 1975), among others (Evans and Meisner, 2009). Among the nine teleost orders listed above that contain internally inseminating species with modified anal fins and/or elongated genital papillae (Evans and Meisner, 2009), those species which instead perform external fertilization often have modified reproductive features that do not form a true intromittent organ, but rather allow the male to direct sperm towards the female. These modifications can range from elongated and thickened anal fins with hooks or spines, to anal fins whose rays have been fused to create a pseudopenis. There are, however, additional notable exceptions and gaps within whole family studies of teleosts in which one or more defining features of sexual dimorphism and internal fertilization are lacking (e.g., Grier *et al.*, 1990). This makes it impossible, thus far, to link the presence of these traits to a specific reproductive strategy without evidence of the reproductive mode in question.

The notopterids *Notopterus notopterus*, *Chitala ornata* (Asia), and *Xenomystus nigri* (Africa) exhibit sexually dimorphic genital papillae which differ by species. *N. notopterus* and *C. ornata* males have a narrow, reddish genital papilla that is longer than the pelvic fin, while the *N. notopterus* females have a broader, whitish papilla that is shorter than the pelvic fin (Yanwirsal, 2013), whereas the *C. ornata* females have an elongated papilla that is wider than the anal opening (Castro *et al.*, 2019). The female's papilla appears to grow and is larger during the courtship and spawning phases (Yanwirsal, 2013). In *X. nigri*, the female's genital papilla is surprisingly longer than the pelvic fin, whereas the male's genital papilla is shorter and located underneath the pelvic fin (Nyonje, 2006; Yanwirsal, 2013). These features are likely adaptive for egg deposition: tubular papillae, when swollen, can help fish propel eggs away from their body and contribute to precise positioning of the eggs on the substrate (Castro *et al.*, 2019; Martin and Page, 2015).

Mormyrids represent an anomaly among osteoglossiforms in that they are known external fertilizers that share the anatomical and behavioral traits of species that reproduce via internal fertilization. Several species have a sexually dimorphic anal fin notch (Brown *et al.*, 1996; Iles, 1960; Kirschbaum, 1987; Nawar, 1959; Pezzanite and Moller, 1998; Svensson, 1933) which, when accompanied by a behavioral sequence, may help males deliver immotile sperm (see section 3) to the female. The specific function of the anal fin notch is unknown, but it could simply provide structural flexibility, allowing the males to bend their body at a typically rigid midpoint. In mormyrids, the male is thought to cup his fin around the female's genital opening, creating a confined fertilization space in which the female releases her eggs (Brown *et al.*, 1996; Iles, 1960; Pezzanite and Moller, 1998). Similar spawning behavior has been observed in other distantly related fish species that have a modified anal fin (Britz and Bartsch, 1998). Although the cupping behavior has never been directly observed during spawning, a reflexive anal fin cupping motion can be stimulated through tactile stimulation in *Pollimyrus isidori* (Kirschbaum, 1987), *Campylmormyrus compressirostris*, *Campylmormyrus rhynchophorus*, and

*Campylmormyrus tamandua* (Feulner *et al.*, 2009). The notch may assume its original form when the testes regress after the breeding period (Iles, 1960).

### *2.3 Singular Gonads and Sexual Dimorphism as they relate to Sperm Competition*

Initially, osteoglossiforms' loss of a second gonad from an equally sized gonad pair appears to be a handicap to reproduction due to a potential loss in gamete production (Frey and Goymann, 2009). Nonetheless, it is not currently known whether a single gonad doubles gamete production as compensation for the loss of the pair. The absence of a paired gonad can also be viewed as loss of a "back up" organ. Redundancy within a general bilateral body plan affords continued reproductive success to any individual that damages or loses one gonad in a set.

Exceptions to the "paired gonad" pattern may demonstrate ways in which a single gonad may actually be adaptive, or at least neutral, for osteoglossiforms. Species that perform parental care typically have low sperm competition and are expected to have a smaller relative testis size (for review see Montgomerie and Fitzpatrick, 2009; Pitnick *et al.*, 2009), but not specifically the evolution of singular gonads. Osteoglossiforms demonstrate a positive association between parental care and single testis development from an anatomical perspective, indicating that there may be some relationship between these traits other than *via* a hormonal mechanism for the purpose of facilitating paternal care. This association could also be mediated differently in fish than it is in birds such as the coucals. Most osteoglossiforms have one gonad and most species demonstrate parental care; *Pantodon*, which has paired gonads, lacks parental care. Nonetheless, parental care is also lacking in a majority, but not all mormyrid species (Budgett, 1901a; Crawford *et al.*, 1986; Diedhiou *et al.*, 2007; Heymer and Harder, 1975; Kirschbaum and Schugardt, 2002), even though all mormyrids have single gonads.

Many osteoglossiform males demonstrate a low gonadosomatic index (GSI) (Adite *et al.*, 2006; Adjibade *et al.*, 2020; Hussain *et al.*, 2015; Khallaf and Authman, 2012; Opadokun and Ajani, 2015). Nonetheless, it is unclear how these values compare to other teleosts, because not all GSI reports consider cyclical fluctuations in gonad size. GSI values could be due to osteoglossiforms' singular testis being of comparatively smaller size than either one or both gonads in other teleosts. Nevertheless, low GSIs are associated with conventional male strategies (Neff *et al.*, 2003) and decreased sperm competition in fish (Peterson and Warner, 1998), amphibians (Jennions and Passmore, 1993), and birds (Birkhead and Moller, 1992), suggesting that osteoglossiforms' GSI values provide further support for low competition in these species as well.

Sexual dimorphism is often associated with sperm competition in that competition between males for access to females leads to the evolution of male alternative reproductive tactics (MARTs) (Taborsky, 1998) and secondary sex characteristics such as body size, male armaments to outcompete other males, or ornaments to impress choosy females (Darwin, 1871). MARTs have been associated with intra-male variation in sperm traits; sneaker males tend to have higher performing sperm than conventional males (Kustra and Alonzo, 2020), such as in the more motile sperm of male sneaker *Heterololigo bleekeri* squids (Hirohashi *et al.,* 2016). Nonetheless, there is currently no evidence of intra-male variation in mating tactics or sperm traits within any osteoglossiform species. Variation in secondary sex characteristics, and the adaptative nature of their functionality for survival and reproduction, represent raw material during intraspecific choice contests which may select for more extreme sexual dimorphic traits (West-Eberhard, 1983). Yet, osteoglossiforms have no notable differences in male and female colors or ornamentation (Bian *et al.*, 2016; Moreau, 1982; Yanwirsal, 2013). Most osteoglossiforms, like many fishes, also have similarly sized males and females. Further evidence of other secondary sex characteristics mediated by sexual selection may not comprise human-visible cues (Karino, 2009), such as the sexually dimorphic signals produced by electric fish; these signals may also be responsible for their rapid radiation (Carlson and Arnegard, 2011; Hopkins, 1986; Rabosky *et al.*, 2013).

In conclusion, the reduction in gonad number and sexually dimorphic structures associated with functional value, rather than secondary sex traits, indicate that osteoglossiforms have reproductive adaptations consistent with internal fertilization, even though most of them employ external fertilization. Internal fertilization often creates a mating environment with low sperm competition. Subsequently, these traits and fertilization strategies together point to low sperm competition as a driving force behind their adaptive functionality, and suggests that osteoglossiforms may be positioned midway on the evolutionary path from external to internal fertilization.

## 3. Sperm Morphology

Sperm morphology is often a reliable indicator of sperm competition because the cell itself is under such strong selection for achieving fertilization. Sperm ultrastructure has been extensively studied since the 1960s and described in over 280 species of fish (Mattei, 1991). Among fish, there are more than 70 derived sperm character states relative to a shared vertebrate ancestor (Jamieson, 1991). The order Osteoglossiformes present a unique system for examining the relationship between sperm competition and morphology since it claims monoflagellate, biflagellate, and aflagellate sperm types.

### *3.1 Monoflagellate sperm – Pantodontidae and Notopteridae*

*P. buccholzi* sperm is exceptionally elongated with a notably developed midpiece and extensive, modified mitochondria; it is morphologically more similar to the sperm of reptiles, birds, and snakes than other teleost fish (Van Deurs, 1975; Van Deurs and Lastein, 1973). Taken into consideration with the male copulatory organ, this sperm phenotype further suggests that this species reproduces by internal fertilization (Lastein and Van Deurs, 1973). The ultrastructure of a single species from Notopteridae has been identified: *Papyrocranus afer,* the only other notopterid species besides *X. nigri* found in Africa, has a simple, monoflagellate sperm that resembles the ancestral sperm of Neopterygii (Mattei, 1970).

### *3.2 Biflagellate sperm - Osteoglossidae*

The sperm ultrastructure of only one osteoglossid species, *Heterotis niloticus*, has been described and it has biflagellate sperm (Mattei *et al.*, 2018). Biflagellate sperm is relatively uncommon: it has been observed in flatworms (Bakhoum *et al.*, 2017; Orido, 1988), polychaetes (Franzén, 1982), freshwater clams (Komaru and Konishi, 1996), acoelomorphs (Barneah *et al.*, 2007), one amphibian family (Mainoya, 1981), and 31 fish species across seven orders (Montgomerie and Fitzpatrick, 2009). Most of these fish species perform external fertilization, but biflagellate sperm is found in at least one internally fertilizing species, the ocean pout (Perciformes: Zoarcidae) (Yao *et al.*, 1995). These fishes are distantly related, and without a clear phylogenetic pattern underlying the evolution of biflagellate sperm, it appears that this sperm morphology independently evolved many times (Montgomerie and Fitzpatrick, 2009).

### *3.3 Aflagellate sperm: Mormyridae and Gymnarchidae*

All mormyroid (Gymnarchidae and Mormyridae) sperm studied thus far (*Gymnarchus niloticus, Hyperopisus bebe, Mormyrus rume*, *Gnathonemus senegalensis*, *Gnathonemus niger*, and *Petrocephalus bovei*) have an aflagellate morphology (Mattei *et al.*, 1972). This is the only known origin of aflagellate sperm in vertebrates (Mattei *et al.*, 1972), although there are reports of aflagellate sperm in two deep-sea Myctophidae (order: Myctophiformes) that require further confirmation (Jamieson, 1991), especially considering that the internal structure of these species' sperm is more similar to monoflagellated sperm (Young *et al.*, 1987). The internal architecture of mormyrid spermatozoa resembles other teleost sperm more closely than the sperm of *G. niloticus* (Mattei *et al.*, 1967; Mattei *et al.*, 1972). In addition to lacking an acrosome (absent in all teleosts, see section 5), mormyrid sperm specifically lack flagella and submembrane microtubules. They possess a rounded nucleus with two centrioles at its base, and some mitochondria in an abundant vesicular cytoplasm (Mattei, 1991). Although this structure appears to negate the possibility of the sperm being motile, the immotility of live spermatozoa has yet to be confirmed.

In contrast, *G. niloticus* has an aflagellate spermatozoan that has the most anomalous phenotype of all osteoglossiforms (Mattei *et al.*, 2018). Due to its resemblance to basic metazoan sperm, it appears likely that *G. niloticus* secondarily evolved motility after the loss of flagella (Mattei, 1988). The uncondensed nucleus is at the center of the cell, there is a large amount of cytoplasm, and the plasma membrane is lined internally with a network of microtubules which contribute to its mobility via amoeboid motion (Mattei *et al.*, 1967). Mattei noted that the internal structure is so aberrant that it barely ascribes to the typical definition of spermatozoa (Mattei, 1988).

### *3.4 Sperm Morphology as it Relates to Sperm Competition*

Monoflagellate sperm are known as a “primitive” sperm type and observed in the majority of teleost fishes; these sperm are made of a small round, nucleus followed by two centrioles, a midpiece with several mitochondria, and a flagella with a typical 9 + 2 axoneme pattern that provides movement and propulsion (Jamieson *et al.*, 1999). The basic sperm model is typical of externally fertilizing species and tends to be shorter and less divergent than those found in internally fertilizing species (Franzén, 1956). Sperm modifications, such as the extended midpiece found in *Pantodon*, are often adaptive in response to sperm competition and functional demands of their environment, specifically the female reproductive tract in internally fertilizing species (Lüpold and Pitnick, 2018; Pitnick *et al.*, 2009). Notopterids demonstrate similar breeding behavior to species with low sperm competition, suggesting that they also experience low sperm competition (Montgomerie and Fitzpatrick, 2009). Although motile flagella are common to all living things, they come at an energetic cost (Pitnick, 1996). Males that experience higher levels of sperm competition may benefit from spending energy on motile sperm, but species without strong sperm competition may be at a greater advantage by adopting modified sperm without flagella (Morrow, 2004).

Aflagellate sperm is associated with low sperm competition (Morrow, 2004) and internal fertilization (for arthropods see Dallai *et al.*, 1973). However, mormyrids and Gymnarchidae reproduce by external fertilization, which is typically associated with high sperm competition. These fishes may use behavioral strategies to reduce the risk of sperm competition: mormyrid males may minimize the risk of multipaternity by facilitating contact between sperm and eggs through ventral coupling during courtship (see section 2.2). *G. niloticus* and two mormyrid species are known to build and guard nests, which is potentially another way to reduce competition among males. Nonetheless, these behaviors are not shared by all aflagellate mormyrid species.

It is presently unclear if aflagellism is adaptive. In the laboratory, studies on *Mormyrus kannume* macerated testis material was combined with eggs to simulate fertilization (Iles, 1960), and reported low fertilization success rates of 10-25%, though it is likely that simulated fertilization may not be an accurate representation of natural fertilization success. Alternatively, it is possible that mormyroids experience low sperm competition. In that case, sperm competition would not be high enough to drive selection for costly, motile sperm at the expense of other traits required for reproduction. Speculatively, the energetics of electrogenesis and electroreception may require metabolic trade-offs (Salazar *et al.*, 2013), and the ability to reproduce without producing sperm flagella may confer a metabolic fitness advantage (Montgomerie and Fitzpatrick, 2009). Evidence for low sperm competition in mormyrids is consistent with low GSI of about 0.5%, in *M. kannume* (Iles, 1960; Khallaf and Authman, 2012). Nonetheless, it is important to consider other life history traits, such as mating rate, that can conflate with GSI results (Parker and Pizzari, 2010). A cross-metazoan metanalysis of flagellar motility demonstrated an overall relationship between sperm competition and the presence of aflagellate sperm, primarily in invertebrate taxa (Morrow, 2004). Considering the monoflagellated notopterids are the mormyroids' nearest sister family, it is possible that relaxed selective pressure by sperm competition is common to both families and has led to directional selection in which the ancestral flagellated sperm type transitioned to immotile, aflagellate sperm (Montgomerie and Fitzpatrick, 2009).

It is unclear whether biflagellate sperm confer motility or fertility advantages (Pitnick *et al.*, 2009), and there is an overall absence of information on sperm behavior and fertilization dynamics in these systems (Montgomerie and Fitzpatrick, 2009). If biflagellate sperm are advantageous when sperm competition is high, it may be expected that additional flagella confer greater competitive abilities, potentially by enhancing motility, improving maneuverability, or excluding other sperm from entering the micropyle (Montgomerie and Fitzpatrick, 2009). Males from various species of cardinal fish (Kurtiformes: Apogonidae), which experience low sperm competition, have ejaculate that contain 50-80% biflagellate sperm among monoflagellated sperm (Fishelson *et al.*, 2006; Lahnsteiner, 2003). Neither biflagellate nor monoflagellate sperm appear to be maladaptive for cardinal fish, since both are produced within a single male, and biflagellate sperm may hold a competitive advantage by being more effective at vertical, undulatory motions that help it enter the egg micropyle of this species (Fishelson *et al.*, 2006). Nonetheless, biflagellate sperm has also been found in species, such as the plainfin midshipman (Batrachoidiformes: Batrachoididae), that experience high sperm competition (Brantley and Bass, 1994). To summarize, although the adaptive value of specific types of sperm morphology remains unclear, sperm competition and the fertilization environment play a major role in selecting sperm form. The relationship between sperm competition and specific sperm morphology will continue to require examination on a case-by-case basis before enough life history information is available to draw clade wide conclusions, especially when considering rarer morphologies like biflagellate sperm.

## 4. Reproductive Behavior & Parental Care

This section discusses osteoglossiform courtship behavior, parental care, and the potential relationships between their reproductive behavior, sperm competition, and sexual selection. Current knowledge about osteoglossiform reproductive behavior is extremely limited, with the exception of mormyrid electrical signaling. Courtship and fertilization events for osteoglossiforms have rarely been witnessed in the wild, infrequently in the lab, and never with advanced imaging techniques. Observations of courtship and fertilization, combined with identifying operational sex ratios, would provide essential information for evaluating the degree of polyandry and competition among males. If sperm competition levels are measurable, then it may be possible to correlate them with the evolution of various morphological adaptations and fertilization strategies.

### *4.1 Osteoglossidae*

Within Osteoglossidae, *Scleropages leichardtii* form direct pairs, in which males and females stay together for several days leading up to the spawning event at night (Merrick and Green, 1982). *S. leichardtii* males are known to participate in at least two spawning events and pair with a new female within several weeks (Merrick and Green, 1982). In contrast, *Osteoglossum bicirrhosum* are serially monogamous and form pairs every breeding season (Queiroz, 2008), indicating low risk of multipaternity and sperm competition. Nonetheless, another osteoglossid species, *Arapaima gigas*, constructs nests (Lüling, 1964), a behavior associated with low sperm competition, and also exhibits multipaternity (Verba *et al.*, 2014), a feature associated with high sperm competition.

Females from the *Osteoglossum* and *Scleropages* genera produce large, yolky eggs. *S. leichardtii* was reported to produce 30-130 eggs 10 mm in diameter (Lake and Midgley, 1970), whereas *S. formosus* was described as producing 30-80 eggs 14-18 mm in diameter (Scott and Fuller, 1976). *O. bicirrhosum* produces 23-220 yellow or orange eggs 11-12 mm in diameter that are non-adhesive and sink to the substrate (Yanwirsal, 2013; Yanwirsal *et al.*, 2017). Other osteoglossid eggs are much smaller, such as those of *H. niloticus*, whose eggs are about 2.5 mmm, adhere to each other when they sink to the bottom of the nest, and have a grooved micropyle (Daget, 1957). Similarly, the greenish, ovoid eggs of *A. gigas* are around 3 mm wide and stick together to form an egg mass (Fontanele, 1948). Reports of *A. gigas* clutch sizes are ambiguous (Britz, 2004), but females may contribute about 1,000 fry per year (Núñez *et al.*, 2011). Osteoglossids exhibit diverse parental care: males in multiple *Scleropages* species and both *Osteoglossum* species are mouthbrooders (Queiroz, 2008; Scott and Fuller, 1976). Only in *S. leichardtii* have females been identified as the mouthbrooding parent (Merrick and Green, 1982). *O. bicirrhosum* males also form aggregations called "hatcheries" during the parental care season; this may be tied to cooperative breeding, as suggested by some broods exhibiting multipaternity in this species and in *A. gigas* (Verba *et al.*, 2014). As noted previously, large, shallow nests for protecting eggs are built by adult male *A. gigas* (Lüling, 1964; Val and de Salvo Souza, 1990) and *H. niloticus* (Adite *et al.*, 2006; Budgett, 1901b). It is unclear if there is a predominant guardian in *H. niloticus* (Daget, 1957; Moreau, 1974), but Adite et. al (2006) reported that both parents transported larvae in their mouths to another location when the original nest was disturbed.

### *4.2 Pantodontidae*

Pantodontids likely undergo internal fertilization, and their courtship behavior involves males and females swimming close together. This behavior is similar to that of externally fertilizing osteoglossiforms; only their internal anatomy indicates that their courtship does not terminate in external fertilization. Females are reported to produce a range of 33-153 buoyant eggs with a 2.3 mm diameter; each egg has a micropyle 10 μm in diameter located in the middle of an 80 µm wide, ridged, funnel shaped micropylar pit (Britz, 2004). *P. buccholzi* does not perform parental care (Britz, 2004). By reproducing through internal fertilization, *P. buccholzi* males may experience such low levels of sperm competition that further male investment may add little benefit for ensuring reproductive success.

*Pantadon's* taxonomic position has been debated for decades (reviewed in Hilton and Lavoué, 2018) and its derived traits indicate that it may be better classified as a sister group to other osteoglossiforms (Lavoué, 2016). *Pantodon* shares an absence of paternal care with *Hiodon alosoides*, an ancient, freshwater osteoglossomorph (Britz, 2004). The eggs of both species contain a large oil globule that allows them to be buoyant; semipelagic eggs like these are typically found in more ancestral marine species (Ahlstrom and Moser, 1980; Battle and Sprules, 1960). Together with its reproductive behavior, *Pantodon's* paired gonads and modified gametes suggest that it is more phylogenetically distant from other osteoglossiforms. However, more morphological and molecular data are needed to resolve its position within Osteoglossomorpha (Hilton and Lavoué, 2018).

### *4.3 Mormyroidea*

Courtship behavior has been documented for several species of mormyrids (Bratton and Kramer, 1989; Kirschbaum, 1987; Landsman, 1993; Machnik and Kramer, 2008; Werneyer and Kramer, 2006; Wong and Hopkins, 2007; Yanwirsal, 2013). Mormyrid species often demonstrate male choice, in which males recognize female electric organ discharges (EODs) (Arnegard *et al.*, 2006; Nagel *et al.*, 2018). In addition to recognizing EOD waveforms, males and females actively interact by modifying their EOD discharge rate to produce stereotyped electrical displays called sequences of pulse intervals (SPIs) (Bratton and Kramer, 1989; Wong and Hopkins, 2007). Several motor behaviors typically occur alongside electrical signaling during courtship and some species, such as *Brienomyrus brachyistius*, exhibit sex-specific courting behaviors (Wong and Hopkins, 2007). Although males may often begin the courtship phase with antagonistic reactions to the female's approach, the male appears to tolerate the female's presence more than usual compared to times outside of courtship (Bratton and Kramer, 1989; Kirschbaum, 1987; Werneyer and Kramer, 2006; Wong and Hopkins, 2007). Observations of immediate spawning after pairing of *Marcusenius macrolepidotus* suggest that motor courtship behavior may not be as necessary as electrical signals for inducing spawning in some species (Werneyer and Kramer, 2006; Wong and Hopkins, 2007).

Mormyrid species typically do not demonstrate parental care, with the exception of a few species known to build nests. Parental care has been recorded in *Stomatorhinus* (Heymer and Harder, 1975) and nestbuilding *P. isidori*, *Pollimyus adspersus* (Diedhiou *et al.*, 2007; Kirschbaum and Schugardt, 2002) and *H. bebe* (Budgett, 1901b). *P. isidori* males will build multiple nests made of algae or macrophytes within their territory (Crawford *et al.*, 1986). They will also continue to defend the nest for 1-2 weeks after spawning, even against the mother. *P. isidori* and *P. adspersus* are the only two species that produce non-adhesive eggs (Kirschbaum and Schugardt, 2002), a feature common to nestbuilding Osteoglossid species. Mormyrid eggs are much smaller than those of nestbuilding Osteoglossids, with the exception of *H. niloticus*, but mormyrid females are also smaller in body size. *P. isidori* females are described as producing 28-132 eggs that are 2 mm in diameter (Kirschbaum, 1987), whereas the quantity of eggs produced by *M. kannume* (Kirschbaum and Schugardt, 1995), *Camplyomormyrus cassaicus* (Schugardt and Kirschbaum, 1998), and *Hippopotamyrus pictus* (Kirschbaum and Schugardt, 2002) typically number in the hundreds and are about 2-3 mm in size. A substrate with holes for attaching eggs is preferred by *H. pictus*, and a rocky substrate is preferred by *M. kannume* (Kirschbaum & Schugardt, 2002). These substrate specializations may reflect differences in reproductive behavior. Parental care was specifically determined to be nonexistent in *M. macrolepidoptus* (Werneyer and Kramer, 2006) and *M. rume* (Kirschbaum and Schugardt, 1995).

The monotypic Gymnarchidae also exhibit nestbuilding. After spawning during the rainy season, *G. niloticus* builds large floating elliptical nests about 1 meter in diameter that contain about 1000 large, amber colored eggs that are 10 mm in diameter, similar to the large-sized eggs of nestbuilding Osteoglossids (Budgett, 1901a; Budgett, 1901b; Svensson, 1933). The nest is built in shallow water and is made of dense vegetation that projects from the surface around the periphery. Adults actively patrol the nests (Hopkins, 1986), which can contain advanced stages of larvae over 3 weeks old (Budgett, 1901b). This behavior similarly exemplifies an indication of high male parental investment that likely reduces levels of sperm competition.

### *4.4 Notopteridae*

Breeding behaviors for notopterids are similar to those of mormyrids: in the lab, *N. notopterus* fish appear to choose individual territories, and males create a spawning site by removing gravel and cleaning the area with their mouth (Yanwirsal *et al.*, 2017). During the courtship phase, the male approaches and swims alongside a potential female partner, which is followed by multiple interactions in which both fish swim towards and away from one another. This phase ends when the female approaches the male and the male leads the female to his spawning site, where the female lays the eggs and he fertilizes them on the substrate (Yanwirsal *et al.*, 2017). Eggs are slightly larger than those of mormyrids, but smaller than most osteoglossids. *N. notopterus* females have been described as laying anywhere from 15 to 225 adhesive eggs per spawning events and eggs are about 3.8-4 mm in diameter (Yanwirsal *et al.*, 2017). *N. notoperus* eggs also appear to have a micropyle with a spiraling pattern of ridges on the egg's surface (Mookerjee and Mazumdar, 1946; Yanwirsal *et al.*, 2017). Older reports of notopterid egg numbers describe *Chitala chitala* females as laying 300-500 adhesive eggs with a 5.2 mm diameter (Southwell and Prashad, 1919) and *C. ornata* as laying several thousand adhesive eggs with a 4 mm diameter (Smith, 1933).

Among notopterids, it is known that male *N. notopterus* (Pinxteren, 1974; Yanwirsal, 2013) and males of both C*hitala* species (Smith, 1933) perform parental care and guard freshly spawned eggs. *X. nigri* was assumed to lack parental care (Siraad, 1999) and little information is available about *Papyrocranus* species reproduction. *N. notopterus* males guard and aggressively defend the eggs against the female and other males (Yanwirsal *et al.*, 2017).

### *4.5 Reproductive Behavior and Parental Care as it Relates to Sperm Competition*

Most osteoglossiforms are morphologically similar between the sexes, which may indicate that sexual selection has not had a large effect on the evolution of their morphology (see section 2.3). Nonetheless, sexual selection likely plays a much larger role in their courtship displays, in which females appear to choose their mates based on courtship activity (e.g., Merrick & Green, 1982). Courtship may serve as a signal of males' ability to contribute to offspring survival and fitness through parental care or signify a male's specific physiological and genetic advantages. Rather than rely heavily on morphological cues, females may prefer to choose mates depending on courtship frequency, type, and length of duration.

Like osteoglossids, mormyrids do not physically display showy secondary sexual features, but they have complicated courtship behavior involving choosy males and diverse electrical signaling. In combination with their aflagellate sperm and modified anal fins (see section 2.3), mormyrid behavior further underscores the likelihood that they experience low levels of sperm competition. Females mate with more than one male in a breeding season, but they do so monogamously and sequentially (Iles, 1960; Morrow, 2004).

The diversity of teleost family structures, ranging from no care to paternal, maternal, and biparental care, has led to fish, especially among the Cichlidae, serving as exemplary systems for investigating the evolutionary origins of parental care (Gonzalez-Voyer *et al.*, 2008). However, the pathway by which uniparental or biparental care evolved from the ancestral state of no care remains unresolved (Kolm, 2009). Osteoglossiforms demonstrate all parental care strategies within a single order, including evidence of cooperative breeding. Therefore, like cichlids, they may be helpful for examining evolutionary questions of parental care at a more compact phylogenetic scale. The greater geographic spread of osteoglossiforms may also allow them to be useful for comparative analysis with cichlid parental care.

Males provide most of the parental care in osteoglossiforms. As demonstrated empirically in other teleosts, their investment is shaped by the risk of multipaternity and sperm competition (Neff, 2003), as well as parental care serving as a sexual selection trait for female choice (Kolm, 2009). With the exception of most mormyrid species, osteoglossiforms appear to demonstrate a relationship between reduced sperm competition and parental care.

## 5. Future Research Directions

Sperm form must adapt to the environment and within the context of sperm competition, males need to balance selection for sperm traits against environmental variance (for review see Snook, 2005). Most empirical work on this topic is concerned with the female reproductive tract as an active environment that can induce sperm motility, namely in arthropods (Alberti, 2000; Dallai *et al.*, 1973; Dallai *et al.*, 1992). For internally fertilizing fish, increased sperm length is associated with an improved ability to navigate the viscosity of the female reproductive tract (Fitzpatrick *et al.*, 2009). In externally fertilizing fish, some work has focused on the importance of appropriate environmental conditions conducive for external fertilization. For instance, increased sperm length has been associated with substrate-fertilizing species, potentially because the length provides benefits that allow sperm to overcome difficulties navigating the substrate to locate an egg (Balshine *et al.*, 2001). Pipefish (Syngnathiformes: Syngnathidae) were found to have immotile sperm that earn motility upon contact with a combination of seawater and ovarian fluid (Ah-King *et al.*, 2006). Seven teleost species demonstrated sperm motility that responded to changes in osmolality, whereas two other fishes exhibited sperm that became motile in response to ionic changes in the water (Morisawa and Suzuki, 1980). Experiments aimed at describing sperm motility and fertilization mechanisms must accurately replicate natural conditions at the risk of studying adaptive traits in an environment lacking the selective forces responsible for their existence (Lüpold and Pitnick, 2018).

Mimicking natural fertilization conditions will be especially important for future work that seeks to describe the fertilization mechanism of African weakly electric fish. *G. niloticus,* whose sperm displays amoeboid movement, may have regained motility by coopting “ancestral amoeboid movement” (Dallai *et al.*, 1973). Nonetheless, *G. niloticus* also faces the difficulty of transferring amoeboid sperm as an external fertilizer; the mechanism by which amoeboid sperm achieve fertilization in freshwater without being dispersed by the current remains unknown. In mormyrids, it will first be necessary to determine whether the sperm are truly immotile before investigating possible mechanisms by which they are transported in their environment. It is important to note, however, that while claims of absolute immotility may be cause for suspicion, mormyrid sperm may simply lack the underlying structural features necessary for motility.

Replicating accurate environmental conditions will also be necessary for investigations of sperm-egg interactions and coevolution. There is presently no research into the presence of chemoattractants or sperm receptor proteins that could facilitate the union of sperm and egg in osteoglossiforms. Comparative studies of the egg structure would greatly improve current understanding of coevolution between sexes of species, particularly in those species that reproduce with aflagellate sperm. Assessing ovarian and seminal fluid is also vital for understanding the complementary roles of post-copulatory sexual selection and sperm competition. Ovarian fluid was found to affect the outcome of sperm competition in ocellated wrasse (Labriformes: Labridae); the presence of female ovarian fluid favored sperm velocity over sperm number, enabling increased fertilization by paternal males in spite of intense sperm competition with other male types (Alonzo *et al.*, 2016). Similarly, increased concentrations of ovarian fluid are associated with increased sperm motility, speed, and linearity of sperm movement in Arctic charr (Salmoniformes: Salmonidae) (Turner and Montgomerie, 2002). More research on cryptic choice in external fertilizers like osteoglossiforms will also provide insight into their fertilization mechanisms. Considering the diverse and unusual combinations of reproductive traits in osteoglossiforms, subsequent studies may elucidate novel methods by which cryptic choice or sexual conflict act to promote fertilization. Moreover, osteoglossiforms employ nearly all possible mating strategies, making them an advantageous system for exploring the evolutionary basis and consequences of sexual conflict theory.

Sexual conflict may lead to coevolution in which females evolve to resist male mating attempts that may be harmful to the female (Parker, 1979). This can be examined at the gametic level by comparing adaptations between eggs and sperm. Initial research on sexual conflict in osteoglossiforms should investigate features often associated with sexual selection, such as biased sex ratios in the population and polyandrous mating strategies. A second aspect could be a more comprehensive study of egg morphology. Like all teleosts, the sperm of osteoglossiforms lack an acrosome (Jamieson, 1991; Mattei, 1970). This absence is associated with the presence of an egg micropyle, the only entrance within the egg which permits a single sperm to penetrate the oocyte and enables attachment of the sperm to the egg's plasma membrane. Once fertilization is achieved, the egg swells, causing the sperm-guiding ridgelines to disappear as a means to prevent additional incoming sperm from fertilizing the egg (Amanze and Iyengar, 1990). The eggs of notopterid *N. notopterus* have been described as having numerous external ridges around the micropyle (Mookerjee and Mazumdar, 1946; Yanwirsal *et al.*, 2017), similar to eggs of the osteoglossids *H. niloticus* and Pantodontids (Britz, 2004; Daget, 1957). The chorion of mormyrid *P. isidori*, however, has been described as smooth (Diedhiou *et al.*, 2007). The vastly different sperm morphology of these species indicates that further research is necessary for determining if there are any similarities in their sperm-egg interactions. Visualization and SEM studies of osteoglossiform eggs is strongly recommended in order to investigate for the presence of any structural features (e.g., the radial micropylar furrows of *Pantodon, H. nilotus*, and *N. notopterus* eggs) that may facilitate sperm entry (Britz, 2004).

Finally, the study of genetic basis and changes in genetic architecture that have led to extreme specializations among osteoglossiforms has yet to be explored. The teleost specific whole genome duplication event (TS-WGD), which occurred early in the teleost lineage, is a potential source of genetic plasticity, whereby gene function may have been reallocated to lead to the evolution of some successful reproductive strategies, such as the development of egg buoyancy required to survive in the open ocean (Finn and Kristoffersen, 2007). Nonetheless, whole genome duplication events do not always lead to radiation, and the TS-WGD does not satisfactorily explain why some teleost orders have exceptionally higher reproductive diversity than others (Glasauer and Neuhauss, 2014). The degree of interaction between genetic material and additional biological and environmental processes that shape teleost reproductive adaptability remains largely unknown (Desjardins and Fernald, 2009; Wootton and Smith, 2014). Given that genomes have now been sequenced for multiple osteoglossiform species (*Scleropages formosus*, Austin *et al.*, 2015; Bian *et al.*, 2016; *Paramormyrops kingsleyae*, Gallant *et al.*, 2017; *Arapaima gigas*, Vialle *et al.*, 2018; *Mormyrus lacerda*, NCBI Accession: JAABNX010000000; *Mormyrus iriodes*, NCBI Accession: JAAGVU000000000; *Brevimyrus niger*, NCBI Accession: JAABNY000000000; *Gymnarchus niloticus*, NCBI Accession: JAACJT010000000) these resources could be leveraged to understand the genetic basis of some of the traits observed in osteoglossiforms and enable comparative studies with other teleosts.

Situated at the base of Teleostei, osteoglossiforms have long been appreciated for understanding early evolution of teleosts (Hilton and Lavoué, 2018). The development of their diverse reproductive anatomy also highlights early plasticity integral for successive adaptations across the teleost group. An increasingly integrative examination of osteoglossiform reproduction is both feasible and has potential for uncovering a comprehensive understanding spanning genomics to behavior. Morrow (2004) noted a research trend that remains relevant to date: “curiously, sperm morphology and ultrastructure is often the first (and sometimes the last) aspect of an organism’s reproductive biology that is examined.” Relating this information to behavioral traits and observations about mating systems will help untangle the selective forces that drive and connect anatomical, physiological, and behavioral adaptations. Other than examining sexually dimorphic electrical signaling in mormyrids, little research has examined osteoglossiform reproductive strategies for signposts of sexual selection. Yet, osteoglossiforms embody morphological traits with clear form and function relationships that are opportune for studies seeking to sort intrasexual from intersexual selection. In this manner, identifying general patterns in osteoglossiform morphology may help clarify the rules governing sperm competition, whereas applying an understanding of those rules to osteoglossiform reproductive behavior and morphology may explain their diversity. Considering that osteoglossiforms are the most ancient living teleost order, evaluating their reproductive diversity may shed key insights into the role of reproductive adaptations in large-scale teleost speciation.

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# REFERENCES

# REFERENCES

Adite, A., Winemiller, K. & Fiogbe, E. (2006). Population structure and reproduction of the African bonytongue Heterotis niloticus in the Sô River-floodplain system (West Africa): implications for management. *Ecology of Freshwater Fish* **15**, 30-39.

Adjibade, K. N., Adite, A., Arame, H., Chikou, A. & Abou, Y. (2020). Aspects of life-history strategy of Marcusenius senegalensis (Pisces: Osteoglossiformes: Mormyridae; Steindachner, 1870) from Niger River in Northern Benin. *International Journal of Forest, Animal and Fisheries Research* **4**(1), 1-13.

Ah-King, M., Elofsson, H., Kvarnemo, C., Rosenqvist, G. & Berglund, A. (2006). Why is there no sperm competition in a pipefish with externally brooding males? Insights from sperm activation and morphology. *Journal of fish biology* **68**, 958-962.

Ahlstrom, E. H. & Moser, H. G. (1980). Characters useful in identification of pelagic marine fish eggs. *California Cooperative Oceanic Fisheries Investigations Reports* **21**, 121-131.

Alberti, G. (2000). Chelicerata. In *Progress in Male Gamete Ultrastructure and Phylogeny* (Jamieson, B. G. M., ed.), pp. 311-388. New York: John Wiley & Sons, Ltd.

Alonzo, S. H., Stiver, K. A. & Marsh-Rollo, S. E. (2016). Ovarian fluid allows directional cryptic female choice despite external fertilization. *Nature communications* **7**, 12452.

Amanze, D. & Iyengar, A. (1990). The micropyle: a sperm guidance system in teleost fertilization. *Development* **109**, 495-500.

Aranzábal, M. C. U., Grier, H. J., De la Rosa Cruz, G. & Alarcón, A. G. (2009). Modifications in ovarian and testicular morphology associated with viviparity in teleosts. In *Reproductive Biology and Phylogeny of Fishes (Agnathans and Bony Fishes): Phylogeny, Reproductive System, Viviparity, Spermatozoa* (Jamieson, B. G. M., ed.), p. 85. Boca Raton, FL: CRC Press.

Argumedo, E. G. T. (2009). *Arawana Azul, manual para manejo de reproductores en cautiverio*. Florencia, Caqueta: Asociacion del Acuicultores del Caqueta (ACUICA).

Arnegard, M. E., Jackson, B. S. & Hopkins, C. D. (2006). Time-domain signal divergence and discrimination without receptor modification in sympatric morphs of electric fishes. *Journal of Experimental Biology* **209**, 2182-2198.

Austin, C. M., Tan, M. H., Croft, L. J., Hammer, M. P. & Gan, H. M. (2015). Whole genome sequencing of the Asian arowana (Scleropages formosus) provides insights into the evolution of ray-finned fishes. *Genome biology and evolution* **7**, 2885-2895.

Bakhoum, A. J., Miquel, J., Ndiaye, P. I., Justine, J.-L., Falchi, A., Bâ, C. T., Marchand, B. & Quilichini, Y. (2017). Advances in spermatological characters in the Digenea: review and proposal of spermatozoa models and their phylogenetic importance. In *Advances in Parasitology*, pp. 111-165. London: Elsevier.

Balshine, S., Leach, B. J., Neat, F., Werner, N. Y. & Montgomerie, R. (2001). Sperm size of African cichlids in relation to sperm competition. *Behavioral Ecology* **12**, 726-731.

Barneah, O., Brickner, I., Hooge, M., Weis, V. M. & Benayahu, Y. (2007). First evidence of maternal transmission of algal endosymbionts at an oocyte stage in a triploblastic host, with observations on reproduction in Waminoa brickneri (Acoelomorpha). *Invertebrate Biology* **126**, 113-119.

Battle, H. I. & Sprules, W. M. (1960). A description of the semi-buoyant eggs and early developmental stages of the goldeye, Hiodon alosoides (Rafinesque). *Journal of the Fisheries Board of Canada* **17**, 245-266.

Bian, C., Hu, Y., Ravi, V., Kuznetsova, I. S., Shen, X., Mu, X., Sun, Y., You, X., Li, J. & Li, X. (2016). The Asian arowana (Scleropages formosus) genome provides new insights into the evolution of an early lineage of teleosts. *Scientific reports* **6**, 1-17.

Birkhead, T. R., Fletcher, F. & Pellatt, E. J. (1998). Testes asymmetry, condition and sexual selection in birds: an experimental test. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **265**, 1185-1189.

Birkhead, T. R. & Moller, A. P. (1992). Sperm competition in birds. Evolutionary causes and consequences. *Academic Press, San Diego, CA. 1992.*

Brantley, R. K. & Bass, A. H. (1994). Alternative male spawning tactics and acoustic signals in the plainfin midshipman fish Porichthys notatus Girard (Teleostei, Batrachoididae). *Ethology* **96**, 213-232.

Bratton, B. O. & Kramer, B. (1989). Patterns of the electric organ discharge during courtship and spawning in the mormyrid fish, Pollimyrus isidori. *Behavioral Ecology and Sociobiology* **24**, 349-368.

Britz, R. (2004). Egg structure and larval development of Pantodon buchholzi (Teleostei: Osteoglossomorpha), with a review of data on reproduction and early life history in other osteoglossomorphs. *Ichthyological Exploration of Freshwaters* **15**, 209-224.

Britz, R. & Bartsch, P. (1998). On the reproduction and early development of Erpetoichthys calabaricus, Polypterus senegalus, and Polypterus ornatipinnis (Actinopterygii: Polypteridae). *Ichthyological Exploration of Freshwaters* **9**, 325-334.

Brown, B., Benveniste, L. & Moller, P. (1996). Basal expansion of anal-fin rays: a new osteological character in weakly discharging electric fish (Mormyridae). *Journal of fish biology* **49**, 1216-1225.

Budgett, J. S. (1901a). The habits and development of some West African fishes. *Proc. Cambridge Philos. Soc.* **11**, 102-104.

Budgett, J. S. (1901b). On the Breeding-habits of some West-African Fishes, with an Account of the External Features in Development of Protopterus annectens, and a Description of the Larva of Polypterus lapradei. *The Transactions of the Zoological Society of London* **16**, 115-136.

Burns, J. R., Weitzman, S. H., Grier, H. J. & Menezes, N. A. (1995). Internal fertilization, testis and sperm morphology in glandulocaudinae fishes (Teleostei: Characidae: Glandulocaudinae). *Journal of Morphology* **224**, 131-145.

Carlson, B. A. & Arnegard, M. E. (2011). Neural innovations and the diversification of African weakly electric fishes. *Communicative & Integrative Biology* **4**, 720-725.

Castro, J. I. (2000). The biology of the nurse shark, Ginglymostoma cirratum, off the Florida east coast and the Bahama Islands. *Environmental Biology of Fishes* **58**, 1-22.

Castro, J. M. C., Gonzales, J. C. B. & Camacho, M. V. C. (2019). Sexual dimorphism of invasive knifefish (Chitala ornata) in Laguna de bay, Philippines. *Philippine Journal of Systematic Biology* **13**, 105-111.

Crawford, J. D., Hagedorn, M. & Hopkins, C. D. (1986). Acoustic communication in an electric fish, Pollimyrus isidori (Mormyridae). *J. Comp. Physiol.* **159**, 297-310.

da Silva, M. I., de Oliveira, M. I. B., da Costa, O. T. F. & Duncan, W. P. (2017). Morphology and morphometry of the ovaries and uteri of the amazonian freshwater stingrays (Potamotrygonidae: Elasmobranchii). *The Anatomical Record* **300**, 265-276.

Daget, J. (1957). Mémoires sur la biologie des poissons du Niger moyen. III. Reproduction et croissance d' Heterotis niloticus Ehrenberg. *Bulletin de l'Institut Francais de'Afrique Noire, Ser. A* **1**, 295-323.

Dalela, R., Rani, M. & Verma, S. (1976). Comparative study of morpho-histology of gonads of three teleost fishes. *Gegenbaurs morphologisches Jahrbuch* **122**, 570-585.

Dallai, R., Baccetti, B., Bernini, F., Bigliardi, E., Burrini, A. G., Giusti, F., Mazzini, M., Pallini, V., Renieri, T., Rosati, F., Selimi, G. & Vegni, M. (1973). New models of aflagellate arthropod spermatozoa. In *Proceedings of the Second International Symposium* (Afzelius, B. A., ed.), pp. 279-287. Wenner-Gren Center, Stockholm, UK: Pergamon Press.

Dallai, R., Xué, L. & Yin, W.-Y. (1992). Flagellate spermatozoa of Protura (Insecta, Apterygota) are motile. *International Journal of Insect Morphology and Embryology* **21**, 137-148.

Darwin, C. (1871). *The descent of man, and selection in relation to sex*. United Kingdom: D. Appleton.

Desjardins, J. & Fernald, R. (2009). Fish sex: why so diverse? *Current opinion in neurobiology* **19**, 648.

Diedhiou, S., Moritz, T., Bartsch, P. & Kirschbaum, F. (2007). Comparison of Pollimyrus isidori and Pollimyrus adspersus (Mormyridae) based on morphometric, meristic, ontogenetic, and physiological characteristics. *Bulletin of Fish Biology Volume* **9**, 13-25.

Evans, J. P. & Meisner, A. D. (2009). Copulatory Structures: Taxonomic Overview and the Potential for Sexual Selection. In *Reproductive Biology and Phylogeny of Fishes (Agnathans and Bony Fishes): Phylogeny, Reproductive System, Viviparity, Spermatozoa* (Jamieson, B. G., ed.), pp. 138-180. Boca Raton, FL: Science Publishers.

Feulner, P. G., Plath, M., Engelmann, J., Kirschbaum, F. & Tiedemann, R. (2009). Magic trait electric organ discharge (EOD) dual function of electric signals promotes speciation in African weakly electric fish. *Communicative & Integrative Biology* **2**, 329-331.

Finn, R. N. & Kristoffersen, B. A. (2007). Vertebrate vitellogenin gene duplication in relation to the "3R hypothesis": correlation to the pelagic egg and the oceanic radiation of teleosts. *PLoS One* **2**, e169.

Fishelson, L., Delarea, Y. & Gon, O. (2006). Testis structure, spermatogenesis, spermatocytogenesis, and sperm structure in cardinal fish (Apogonidae, Perciformes). *Anatomy and embryology* **211**, 31-46.

Fitzpatrick, J. L., Montgomerie, R., Desjardins, J. K., Stiver, K. A., Kolm, N. & Balshine, S. (2009). Female promiscuity promotes the evolution of faster sperm in cichlid fishes. *Proceedings of the National Academy of Sciences* **106**, 1128-1132.

Fontanele, O. (1948). Contribuição para O Conhecimento da biologia do pirarucu (Arapaima gigas, Cuvier), em cativeiro (Actinopterygii, Osteoglossidae). *Revista Brasileira de Biologia* **8**, 445-459.

Franzén, Å. (1956). On spermiogenesis, morphology of the spermatozoon, and biology of fertilization among invertebrates. *Zool Bidrag, Uppsala* **31**, 355-382.

Franzén, Å. (1982). Ultrastructure of the biflagellate spermatozoon of Tomopteris helgolandica Greef, 1879 (Annelida, Polychaeta). *Gamete research* **6**, 29-37.

Friedmann, H. (1927). Testicular asymmetry and sex ratio in birds. *The Biological Bulletin* **52**, 197-207.

Frey, R. & Goymann, W. (2009). A single functional testis and long deferent duct papillae: the peculiar male reproductive tract of the classically polyandrous, sex-role reversed Black Coucal (Centropus grillii). *Journal of Ornithology* **150**, 827-838.

Froese, R. & Pauly, D. (2019). FishBase. Species list: World Wide Web electronic publication.

Gallant, J. R., Losilla, M., Tomlinson, C. & Warren, W. C. (2017). The genome and adult somatic transcriptome of the mormyrid electric fish Paramormyrops kingsleyae. *Genome biology and evolution* **9**, 3525-3530.

Glasauer, S. M. & Neuhauss, S. C. (2014). Whole-genome duplication in teleost fishes and its evolutionary consequences. *Molecular genetics and genomics* **289**, 1045-1060.

Gonzalez-Voyer, A., Fitzpatrick, J. L. & Kolm, N. (2008). Sexual selection determines parental care patterns in cichlid fishes. *Evolution: International Journal of Organic Evolution* **62**, 2015-2026.

Goymann, W., Moore, I. T., Scheuerlein, A., Hirschenhauser, K., Grafen, A. & Wingfield, J. C. (2004a). Testosterone in tropical birds: effects of environmental and social factors. *The American Naturalist* **164**, 327-334.

Goymann, W. & Wingfield, J. C. (2004). Competing females and caring males. Sex steroids in African black coucals, Centropus grillii. *Animal Behaviour* **68**, 733-740.

Goymann, W., Wittenzellner, A. & Wingfield, J. C. (2004b). Competing females and caring males. Polyandry and sex-role reversal in African black coucals, Centropus grillii. *Ethology* **110**, 807-823.

Grier, H. J., Moody, D. P. & Cowell, B. C. (1990). Internal fertilization and sperm morphology in the brook silverside, Labidesthes sicculus (Cope). *Copeia* **1990**, 221-226.

Grützner, F., Nixon, B. & Jones, R. (2008). Reproductive biology in egg-laying mammals. *Sexual Development* **2**, 115-127.

Guioli, S., Nandi, S., Zhao, D., Burgess-Shannon, J., Lovell-Badge, R. & Clinton, M. (2014). Gonadal asymmetry and sex determination in birds. *Sexual Development* **8**, 227-242.

Guraya, S. S. (2013). *Ovarian follicles in reptiles and birds*: Springer Science & Business Media.

Helfman, G., Collette, B. B., Facey, D. E. & Bowen, B. W. (2009). *The diversity of fishes: biology, evolution, and ecology*: John Wiley & Sons.

Heymer, A. & Harder, W. (1975). Erste Auftreten der electrischen Entladungen bei einem jungen Mormyriden. *Naturwiss* **62**, 489.

Hilton, E. J. & Lavoué, S. (2018). A review of the systematic biology of fossil and living bony-tongue fishes, Osteoglossomorpha (Actinopterygii: Teleostei). *Neotropical Ichthyology* **16**(3), e180031.

Hirohashi, N., Tamura-Nakano, M., Nakaya, F., Iida, T. & Iwata, Y. (2016). Sneaker male squid produce long-lived spermatozoa by modulating their energy metabolism. *Journal of Biological Chemistry* **291**, 19324-19334.

Hjerresen, G. (1937). Pflege und Zucht von Pantodon buchholzi Peters. *Wschr. Aquar.- u. Terrarienkund* **32**, 115-117.

Hopkins, C. (1986). Behavior of Mormyridae. In *Electroreception* (Bullock, T. & Heiligenberg, W., eds.), pp. 527-576. New York, NY: John Wiley & Sons, New York.

Hussain, A., Shakir, H., Ali, S. & Qazi, J. (2015). Growth coefficient and fecundity of Chitala chitala (Osteoglossiformes: Notopteridae) from the river Ravi, Pakistan. *The Journal of Animal & Plant Sciences* **25**, 401.

Iles, R. B. (1960). External sexual differences and their significance in Mormyrus kannume Forskal 1775. *Nature* **188**, 516.

Jamieson, B. G. M. (1989). Complex spermatozoon of the live-bearing half-beak, Hemirhamphodon pogonognathus (Bleeker): Ultrastructural description (Euteleostei, Atherinomorpha, Beloniformes). *Gamete research* **24**, 247-259.

Jamieson, B. G. M. (1991). Fish evolution and systematics: evidence from spermatozoa: with a survey of lophophorate, echinoderm and protochordate sperm and an account of gamete cryopreservation. Cambridge University Press.

Jamieson, B. G. M., Dallai, R. & Afzelius, B. A. (1999). *Insects: their spermatozoa and phylogeny*: Science Publishers, Inc.

Jennions, M. D. & Passmore, N. I. (1993). Sperm competition in frogs: testis size and a ‘sterile male’experiment on Chiromantis xerampelina (Rhacophoridae). *Biological Journal of the Linnean Society* **50**, 211-220.

Karino, K. (2009). Sexual Selection: Signaling and Courtship. In *Reproductive Biology and Phylogeny of Fishes (Agnathans and Bony Fishes): Phylogeny, Reproductive System, Viviparity, Spermatozoa* (Jamieson, B. G., ed.), pp. 181-220. Boca Raton, FL: Science Publishers.

Khallaf, E. & Authman, M. (2012). Interaction of the mormyrid fish Mormyrus kannume (Forsskål, 1775) reproduction and feeding intensity with the environment in a Nile Delta Canal, Egypt. *Egyptian Journal of Aquatic Biology and Fisheries* **16**, 73-94.

Kirschbaum, F. (1987). Reproduction and development of the weakly electric fish, Pollimyrus isidori (Mormyridae, Teleostei) in captivity. *Environmental Biology of Fishes* **20**, 11-31.

Kirschbaum, F. & Schugardt, C. (1995). Vergleichende Daten zur Fortpflanzungsbiologie von zwei Nilhecht-Arten (Mormyridae). In *Fortpflanzungsbiologie der Aquarienfische* (Greven, H. & Riehl, R., eds.), pp. 81-90. Bornheim: Birgit Schmettkamp Verlag.

Kirschbaum, F. & Schugardt, C. (2002). Reproductive strategies and developmental aspects in mormyrid and gymnotiform fishes. *Journal of Physiology-Paris* **96**, 557-566.

Kolm, N. (2009). Parental Care. In *Reproductive Biology and Phylogeny of Fishes (Agnathans and Bony Fishes): Phylogeny, Reproductive System, Viviparity, Spermatozoa* (Jamieson, B. G., ed.), pp. 351-370. Boca Raton, FL: Science Publishers.

Komaru, A. & Konishi, K. (1996). Ultrastructure of biflagellate spermatozoa in the freshwater clam, Corbicula leana (Prime). *Invertebrate Reproduction & Development* **29**, 193-197.

Koya, Y., Munehara, H. & Takano, K. (2002). Sperm storage and motility in the ovary of the marine sculpin Alcichthys alcicornis (Teleostei: Scorpaeniformes), with internal gametic association. *Journal of Experimental Zoology* **292**, 145-155.

Kustra, M. C. & Alonzo, S. H. (2020). Sperm and alternative reproductive tactics: a review of existing theory and empirical data. *Philosophical Transactions of the Royal Society B* **375**, 20200075.

Lahnsteiner, F. (2003). The spermatozoa and eggs of the cardinal fish. *Journal of fish biology* **62**, 115-128.

Lake, J. & Midgley, S. (1970). Australian Osteoglossidae (Teleostei). *Australian Journal of Science* **32**, 442-443.

Lake, P. (1981). Male genital organs. In *Form and function in birds* (AS, K. & J, M., eds.), pp. 1-61. New York: Academic Press.

Landsman, R. E. (1993). Sex differences in external morphology and electric organ discharges in imported Gnathonemus petersii (Mormyriformes). *Animal Behaviour* **46**, 417-429.

Lastein, U. & Van Deurs, B. (1973). The copulatory organ of Pantodon buchholzi Peters (Teleostei). *Acta Zoologica* **54**, 153-160.

Lavoué, S. (2016). Was Gondwanan breakup the cause of the intercontinental distribution of Osteoglossiformes? A time-calibrated phylogenetic test combining molecular, morphological, and paleontological evidence. *Molecular phylogenetics and evolution* **99**, 34-43.

Ligon, J. D. (1997). A single functional testis as a unique proximate mechanism promoting sex-role reversal in coucals. *The Auk* **114**, 800-801.

Liu, Y., Liao, W., Zhou, C., Mi, Z. & Mao, M. (2011). Asymmetry of testes in Guenther's frog, Hylarana guentheri (Anuar: Ranidae). *Asian Herpetol. Res* **2**, 234-239.

Lüling, K.-H. (1964). Zur Biologie und Okologie von Arapaima gigas (Pisces, Osteoglossidae). *Zeitschrift fur Morphologie und Okologie der Tiere* **54**, 436-530.

Luo, D., Sun, J.-J., Lu, X., Liu, L.-Z., Chen, S.-J. & Li, G.-F. (2011). Comparative sperm ultrastructure of three species in Siniperca (Teleostei: Perciformes: Sinipercidae). *Micron* **42**, 884-891.

Lüpold, S. & Pitnick, S. (2018). Sperm form and function: what do we know about the role of sexual selection? *Reproduction* **155**, R229-R243.

Machnik, P. & Kramer, B. (2008). Female choice by electric pulse duration: attractiveness of the males' communication signal assessed by female bulldog fish, Marcusenius pongolensis (Mormyridae, Teleostei). *Journal of Experimental Biology* **211**, 1969-1977.

Mainoya, J. (1981). Observations on the ultrastructure of spermatids in the testis of Chiromantis xerampelina (Anura: Rhacophoridae). *African Journal of Ecology* **19**, 365-368.

Martin, Z. P. & Page, L. M. (2015). Comparative morphology and evolution of genital papillae in a genus of darters (Percidae: Etheostoma). *Copeia* **2015**, 99-124.

Mattei, X. (1970). Spermiogenèse comparée des poissons. In *Comparative spermatology, Proc Ith Int Symp* (Baccetti, B., ed.), pp. 57-71. Rome, Italy: Sièna.

Mattei, X. (1988). The flagellar apparatus of spermatozoa in fish. Ultrastructure and evolution. *Biology of the Cell* **63**, 151-158.

Mattei, X. (1991). Spermatozoon ultrastructure and its systematic implications in fishes. *Canadian Journal of Zoology* **69**, 3038-3055.

Mattei, X., Boisson, C., Mattei, C. & Reizer, C. (1967). Spermatozoïdes aflagellés chez un poisson: Gymnarchus niloticus (Téléostéen, Gymnarchidae). *Comptes rendus hebdomadaires des séances de l'Académie des Sciences, Série D* **265**, 2010-2012.

Mattei, X., Marchand, B. & Quilichini, Y. (2018). A biflagellate spermatozoon in the African bonytongue Heterotis niloticus (Teleostei, Osteoglossidae). *Journal of fish biology* **94**, 335-338.

Mattei, X., Mattei, C., Reizer, C. & Chevalier, J. L. (1972). Ultrastructure des spermatozoïdes aflagellés des mormyres (Poissons Téléostéens). *Journal of Microscopy* **15**, 67-78.

Meisner, A. (2005). Male modifications associated with insemination in teleosts. In *Viviparous fishes* (Uribe, M. & Grier, G., eds.), pp. 167-192. Homestead, Florida: New Life Publications.

Merrick, J. & Green, L. (1982). Pond culture of the spotted barramundi, Scleropages leichardti (Pisces: Osteoglossidae). *Aquaculture* **29**, 171-176.

Merrick, J. R. & Schmida, G. n. E. (1984). *Australian freshwater fishes: biology and management*. South Australia: Griffin Press Ltd.

Millot, T., Anthony, J. & Robineau, D. (1978). *Anatomie de Latimena chalumnae*, Vol 3. Paris: Centre National de la Recherche Scientifique.

Montgomerie, R. & Fitzpatrick, J. L. (2009). Testes, Sperm, and Sperm Competition. In *Reproductive Biology and Phylogeny of Fishes (Agnathans and Bony Fishes): Phylogeny, Reproductive System, Viviparity, Spermatozoa* (Jamieson, B. G., ed.), pp. 1-53. Boca Raton, FL: CRC Press.

Mookerjee, H. & Mazumdar, S. (1946). On the life history of Notopterus notopterus (Pallas). *Journal of the Department of Sciences, Calcutta University* **2**, 88-100.

Moreau, J. (1974). Premiéres observations écologiques sur la reproduction d'Heterotis niloticus (Osteoglossidae). *Ann. Hydrobiol* **5**, 1-13.

Moreau, J. (1982). Exposé synoptique des données biologiques sur Heterotis niloticus (Cuvier, 1829). *Synopsis FAO sur les pêches* **131**, 1-45.

Morisawa, M. & Suzuki, K. (1980). Osmolality and potassium ion: their roles in initiation of sperm motility in teleosts. *science* **210**, 1145-1147.

Morrow, E. H. (2004). How the sperm lost its tail: the evolution of aflagellate sperm. *Biological Reviews* **79**, 795-814.

Nagel, R., Kirschbaum, F., Engelmann, J., Hofmann, V., Pawelzik, F. & Tiedemann, R. (2018). Male-mediated species recognition among African weakly electric fishes. *Royal Society open science* **5**, 170443.

Nawar, G. (1959). Observations on breeding of six members of the Nile Mormyridae. *Journal of Natural History* **2**, 493-504.

Neff, B. D. (2003). Decisions about parental care in response to perceived paternity. *Nature* **422**, 716-719.

Neff, B. D., Fu, P. & Gross, M. R. (2003). Sperm investment and alternative mating tactics in bluegill sunfish (Lepomis macrochirus). *Behavioral Ecology* **14**, 634-641.

Nelson, J. S., Grande, T. C. & Wilson, M. V. (2016). *Fishes of the World*: John Wiley & Sons.
Nielsen, J. G. (1984). Two new, abyssal Barathronus spp. from the North Atlantic (Pisces: Aphyonidae). *Copeia*, 579-584.

Núñez, J., Chu-Koo, F., Berland, M., Arévalo, L., Ribeyro, O., Duponchelle, F. & Renno, J. F. (2011). Reproductive success and fry production of the paiche or pirarucu, Arapaima gigas (Schinz), in the region of Iquitos, Perú. *Aquaculture Research* **42**, 815-822.

Nyonje, B. M. (2006). Experimental studies on cyclical reproduction of tropical African freshwater fishes. *Humboldt Universität zu Berlin*.

Nysten, M. (1962). Étude anatomique des rapports de la vessie aérienne avec l'axe vertébral chez Pantodon buchholzi Peters. *Annales du Musee Royal de l Afrique Centrale Serie 8: Sciences Zoologiques*, **108**, 185-225.

Opadokun, I. & Ajani, E. (2015). Some aspects of the reproductive biology of Gymnarchus niloticus Cuvier, 1829 (Knifefish) in Lekki Lagoon, Nigeria. *International Journal of Fisheries and Aquatic Studies* **2**, 166-170.

Orido, Y. (1988). Ultrastructure of spermatozoa of the lung fluke, Paragonimus ohirai (Trematoda: Troglotrematidae), in the seminal receptacle. *Journal of Morphology* **196**, 333-343.

Parker, G. (1979). Sexual selection and sexual conflict. In *Sexual selection and reproductive competition in insects* (Blum, M. S. & Blum, N. A., eds.), pp. 123-166. New York, NY: Academic Press, Inc.

Parker, G. A. (1990). Sperm competition games: raffles and roles. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **242**, 120-126.

Parker, G. A. (2014). The sexual cascade and the rise of pre-ejaculatory (Darwinian) sexual selection, sex roles, and sexual conflict. *Cold Spring Harbor perspectives in biology* **6**, a017509.

Parker, G. A. & Pizzari, T. (2010). Sperm competition and ejaculate economics. *Biological Reviews* **85**, 897-934.

Peterson, C. W. & Warner, R. R. (1998). Sperm Competition in Fishes. In *Sperm Competition and Sexual Selection* (Birkhead, T. & Møller, A., eds.). San Diego, CA: Academic Press.

Pezzanite, B. & Moller, P. (1998). A sexually dimorphic basal anal-fin ray expansion in the weakly discharging electric fish Gnathonemus petersii. *Journal of fish biology* **53**, 638-644.
van Pinxteren, M. C. A. (1974). Gelungene Zucht mit einem Messerfisch, Notopterus spec. *Aquarien und Terrarien Zeitschrift* **37**, 364-369.

Pitnick, S. (1996). Investment in testes and the cost of making long sperm in Drosophila. *The American Naturalist* **148**, 57-80.

Pitnick, S., Hosken, D. J. & Birkhead, T. R. (2009). Sperm morphological diversity. In *Sperm biology, An Evolutionary Perspective* (Pitnick, S., Hosken, D. J. & Birkhead, T. R., eds.), pp. 69-149. Cambridge, MA: Elsevier.

Pusey, B. & Stewart, T. (1989). Internal fertilization in Lepidogalaxias salamandroides Mees (Pisces: Lepidogalaxiidae). *Zoological Journal of the Linnean Society* **97**, 69-79.

Queiroz, H. L. d. (2008). Investimento parental e reprodução do Aruanã branco, Osteoglossum bicirrhosum, na Reserva Mamirauá. In *Biologia, Conservação e Manejo dos Aruanãs na Amazônia Brasileira. Téfé, Brasil, Inst. Desemvolv. Sustentavel de Mamirauá*, pp. 119-132. Téfé, Brasil: Inst. Desemvolv. Sustentavel de Mamirauá.

Rabosky, D. L., Santini, F., Eastman, J., Smith, S. A., Sidlauskas, B., Chang, J. & Alfaro, M. E. (2013). Rates of speciation and morphological evolution are correlated across the largest vertebrate radiation. *Nature communications* **4**, 1-8.

Rand, A. (1933). Testicular asymmetry in the Madagascar coucal. *The Auk* **50**, 219-220.
Ravaglia, M. A. & Maggese, M. C. (2002). Oogenesis in the swamp eel Synbranchus marmoratus (Bloch, 1795)(Teleostei; synbranchidae). Ovarian anatomy, stages of oocyte development and micropyle structure. *Biocell* **26**, 325.

Salazar, V. L., Krahe, R. & Lewis, J. E. (2013). The energetics of electric organ discharge generation in gymnotiform weakly electric fish. *Journal of Experimental Biology* **216**, 2459-2468.

Schugardt, C. & Kirschbaum, F. (1998). Sozial-und Fortpflanzungsverhalten von Mormyriden (Nilhechten). *Verhalten der Aquarienfische. Bornheim, Germany: Brigit Schmettkamp. p,* 87-98.

Schugardt, C. & Kirschbaum, F. (2004). Control of Gonadal Maturation and Regression by Experimental Variation of Environmental Factors in the Mormyrid Fish, Mormyrus rume proboscirostris. *Environmental Biology of Fishes* **70**, 227-233.

Scott, D. B. C. (1973). The reproductive cycle of Mormyrus kannume Forsk. (Osteoglossomorpha, Mormyriformes) in Lake Victoria Uganda. *Journal of fish biology* **6**, 447-454.

Scott, D. B. C. & Fuller, J. (1976). The reproductive biology of Scleropages formosus (Müller & Schlegel)(Osteoglossomorpha, Osteoglossidae) in Malaya, and the morphology of its pituitary gland. *Journal of fish biology* **8**, 45-53.

Scrimshaw, N. S. (1945). Embryonic development in poeciliid fishes. *The Biological Bulletin* **88**, 233-246.

Siraad, H. A. (1999). Kweek van Xenomystus nigri, de Afrikaanese mesvis. *Het Aquarium* **69**, 90-92.

Smith, C. & Wootton, R. J. (2016). The remarkable reproductive diversity of teleost fishes. *Fish and Fisheries* **17**, 1208-1215.

Smith, H. M. (1933). Contributions to the ichthyology of Siam. VII. The Featherback fish Notopterus chitala in Siam with notes on its egg-laying and young. *Journal of the Siam Society of Natural History*, 245-258.

Southwell, T. & Prashad, B. (1919). Notes from the Bengal Fisheries Laboratory, no. 6: embryological and developmental studies of Indian fishes. *Records of the Indian Museum* **16**, 215-240.

Snook, R. R. (2005). Sperm in competition: not playing by the numbers. *Trends in Ecology & Evolution* **20**, 46-53.

Spadella, M. A., Oliveira, C., Ortega, H., Quagio-Grassiotto, I. & Burns, J. R. (2012). Male and female reproductive morphology in the inseminating genus Astroblepus (Ostariophysi: Siluriformes: Astroblepidae). *Zoologischer Anzeiger-A Journal of Comparative Zoology* **251**, 38-48.

Suarez, S. S. (1975). The reproductive biology of Ogilbia cayorum, a viviparous brotulid fish. *Bulletin of Marine Science* **25**, 143-173.

Svensson, G. S. (1933). Fresh water fishes from the Gambia river (British West Africa): results of the Swedish expedition 1931. **12**, 1-102.

Taborsky, M. (1998). Sperm competition in fish:bourgeois' males and parasitic spawning. *Trends in Ecology & Evolution* **13**, 222-227.

Turner, E. & Montgomerie, R. (2002). Ovarian fluid enhances sperm movement in Arctic charr. *Journal of fish biology* **60**, 1570-1579.

Uribe, M., Grier, H. J. & Parenti, L. R. (2012). Ovarian structure and oogenesis of the oviparous goodeids Crenichthys baileyi (Gilbert, 1893) and Empetrichthys latos Miller, 1948 (Teleostei, Cyprinodontiformes). *Journal of Morphology* **273**, 371-387.

Val, A. L. & de Salvo Souza, R. H. (1990). Pirarucu-O gigante vermelho das águas Amazônicas. *Ciéncia Hoje* **11**, 10-12.

Van Deurs, B. (1975). The sperm cells of Pantodon (Teleostei) with a note on residual body formation. In *The functional anatomy of the spermatozoon* (Afzelius, B. A., ed.), pp. 311-318. Oxford, UK: Pergamon.

Van Deurs, B. & Lastein, U. (1973). Ultrastructure of the spermatozoa of the teleost Pantodon buchholzi Peters, with particular reference to the midpiece. *Journal of ultrastructure research* **42**, 517-533.

Verba, J. T., Rabello Neto, J. G., Zuanon, J. & Farias, I. (2014). Evidence of multiple paternity and cooperative parental care in the so called monogamous silver arowana Osteoglossum bicirrhosum (Osteoglossiformes: Osteoglossidae). *Neotropical Ichthyology* **12**, 145-151.

Vialle, R. A., de Souza, J. E. S., Lopes, K. d. P., Teixeira, D. G., Alves Sobrinho, P. d. A., Ribeiro-dos-Santos, A. M., Furtado, C., Sakamoto, T., Oliveira Silva, F. A. & Herculano Corrêa de Oliveira, E. (2018). Whole genome sequencing of the pirarucu (Arapaima gigas) supports independent emergence of major teleost clades. *Genome biology and evolution* **10**, 2366-2379.

Voigt, C. & Goymann, W. (2007). Sex-role reversal is reflected in the brain of African black coucals (Centropus grillii). *Developmental neurobiology* **67**, 1560-1573.

Wakamatsu, M., Yamagata, T., Namikawa, T. & Mizutani, M. (2000). Hereditary persistent right oviduct in the chicken PNP/DO line. *Poultry science* **79**, 1075-1081.

Walter, H. (1979). *Eleonora's falcon: adaptations to prey and habitat in a social raptor*: Chicago: University of Chicago Press.

Weitkamp, H. (2005). Untersuchungen zur Reproduktionsbiologie von Notopterus notopterus (Pallas, 1769). In *Arbeit im Studiengang Agrarwissenschaften*: Humboldt-Universität zu Berlin.

Werneyer, M. & Kramer, B. (2006). Ontogenetic development of electric-organ discharges in a mormyrid fish, the bulldog Marcusenius macrolepidotus (South African form). *Journal of fish biology* **69**, 1190-1201.

West-Eberhard, M. J. (1983). Sexual selection, social competition, and speciation. *The Quarterly Review of Biology* **58**, 155-183.

Wong, R. Y. & Hopkins, C. D. (2007). Electrical and behavioral courtship displays in the mormyrid fish Brienomyrus brachyistius. *Journal of Experimental Biology* **210**, 2244-2252.

Wootton, R. J. & Smith, C. (2014). *Reproductive biology of teleost fishes*: John Wiley & Sons.

Wourms, J. P. (1977). Reproduction and development in chondrichthyan fishes. *American Zoologist* **17**, 379-410.

Yanwirsal, H. (2013). Reproductive styles of Osteoglossomorpha with emphasis on Notopterus notopterus and Osteoglossum bicirrhosum. (Doctoral thesis, Humboldt University of Berlin, Germany). Retrieved from: edoc.hu-berlin.de/handle/18452/17413.

Yanwirsal, H., Bartsch, P. & Kirschbaum, F. (2017). Reproduction and development of the asian bronze featherback Notopterus notopterus (Pallas, 1769)(Osteoglossiformes, Notopteridae) in captivity. *Zoosystematics and Evolution* **93**, 299-324.

Yao, Z., Emerson, C. & Crim, L. (1995). Ultrastructure of the spermatozoa and eggs of the ocean pout (Macrozoarces americanus L.), an internally fertilizing marine fish. *Molecular reproduction and development* **42**, 58-64.

Young, J., Blaber, S. & Rose, R. (1987). Reproductive biology of three species of midwater fishes associated with the continental slope of eastern Tasmania, Australia. *Marine Biology* **95**, 323-332.

Yu, Z. H. (1998). Asymmetrical testicular weights in mammals, birds, reptiles and amphibia. *International journal of andrology* **21**, 53-55.

Zhang, G., Li, C., Li, Q., Li, B., Larkin, D. M., Lee, C., Storz, J. F., Antunes, A., Greenwold, M. J. & Meredith, R. W. (2014). Comparative genomics reveals insights into avian genome evolution and adaptation. *Science* **346**, 1311-1320.

Zhou, C. Q., Mao, M., Liao, W. B., Mi, Z. P. & Liu, Y. H. (2011). Testis asymmetry in the dark-spotted frog Rana nigromaculata. *The Herpetological Journal* **21**, 181-185.

# CONCLUSION

This work contributed new knowledge about the predator-prey interactions of grasshopper mice and stink beetles, and new insight about the relationship between sperm competition, sexual selection, and the reproductive biology of Osteoglossiformes fishes. Befitting Krogh's principle, grasshopper mice and osteoglossiforms exhibit exceptional traits well-suited for studying particular problems in biology.

Grasshopper mice's specialized predatory behavior is highly tractable for studies of sensory biology and the genetic basis of behavior, while osteoglossiforms' reproductive diversity can inform investigations of form and function and speciation. Thus, like standard models, research on these animals' morphology and behavior goes beyond species-specific results to shed light on broader biological processes. Moreover, grasshopper mice's and osteoglossiforms' relatedness to their standard model counterparts, lab mice and zebrafish respectively, highlights the value of using these non-model organisms to evaluate biological questions at the same organizational level across species. By comparing the exceptional traits of grasshopper mice and osteoglossiforms within their own families and with traditional lab models, this dissertation demonstrates that these non-model organisms provide novel perspectives to studies of ecology, evolution, and behavior, as well as to broader applications of research that links genes to behavior.

## 1. Grasshopper Mice

In Chapter 1, I showed that grasshopper mice have specialized behaviors that enable them to be more efficient predators and consume different prey than related desert rodents. Compared to deer mice and pocket mice, only grasshopper mice attacked and consumed highly caloric stink beetles in spite of being sprayed in the eyes, nose, and mouth with high concentrations of the beetles' benzoquinone defenses. As depicted in Figure 1.1 in Chapter 1, grasshopper mice attacked beetles more than they attacked crickets, which lack chemical defenses. Moreover, grasshopper mice oriented their attacks towards the beetles' heads more than their abdomens, although attacking the head did not appear to reduce their handling time of prey. Beetles represented challenging prey for grasshopper mice: larger beetles took longer to subdue than smaller beetles and crickets took less time to incapacitate overall. Figure 1.2 in this chapter shows that rodents performed different amounts of total defensive behaviors in response to either crickets or beetles, but grasshopper mice were the only species to exhibit face shoveling behavior upon encountering beetles. Nonetheless, grasshopper mice were much quicker at killing crickets than deer mice. This study therefore provides support for a connection between grasshopper mice's adaptive predatory traits and their uniquely carnivorous dietary niche among rodents within a resource-poor desert. Other rodents' aversion to the stink beetles' chemical spray suggests that grasshopper mice may have an underlying physiological or genetic mechanism that protects them from the spray's noxious effects.

Grasshopper mice's exceptional hunting strategies, especially when compared to omnivorous deer mice, allow them to serve as convenient models for testing foraging theory concepts using a predator with multiple prey types as well as the limitations and plasticity of aversive conditioning. Foraging success may largely be defined by their ability to speedily evaluate sensory cues and assess the risk of consuming certain prey (Rowe and Rowe, 2006) combined with their quick capture and consumption of food to turn an energetic profit (Slobodchikoff *et al.*, 1987). Both juveniles and adults appear capable of learning to improve their hunting strategies through experience (Cyr, 1972; Slobodchikoff *et al.*, 1987). However, it remains unclear the extent to which they employ prey-specific strategies compared to generalist strategies, and there is conflicting evidence regarding which sensory cues or experiences are required for them to undergo aversive conditioning (Langley, 1981; Whitman *et al.*, 1986). Resolving these questions in grasshopper mice could potentially reveal new information about learning pathways and how they are shaped by the environmental and selective forces that determine a species' dietary niche.

In Chapter 2, I used a two-bottle choice test to demonstrate that grasshopper mice are as gustatorily sensitive as omnivorous house mice to the benzoquinone found in stink beetles' spray. As shown in Figure 2.1 from this chapter, both grasshopper mice and house mice preferred water to benzoquinone; their aversion to benzoquinone was present at concentrations ten times lower than those that grasshopper mice encounter when they consume stink beetles. I also demonstrated in Figure 2.2 that compared to related rodent species, grasshopper mice have mutations in their TRPA1 receptors, a chemical sensor activated by benzoquinone, that may mediate differences in their chemosensitivity and predatory behavior. Differential expression of TRPA1 and the relationship between hunger and taste sensitivity are two factors discussed in this chapter, among other possible explanations, that need to be tested to understand why grasshopper mice consume noxious chemicals. Taken together, this work underscores that the physiological process enabling grasshopper mice predatory behavior is likely due to complex interactions between the structure and expression of genes activated by benzoquinone combined with gustatory, olfactory, and chemosensitive mechanisms.

Grasshopper mice have the potential be valuable biomedical models. Their ability to consume noxious and toxic prey without adverse effects makes them suited for expanding current research on the relationship between palatability and postingestive effects on diet and health; this research typically relies on standard rodent models. Moreover, elucidating the effects of mutations in their TRPA1 channels, especially the cysteine variants identified in Chapter 2, may inform understanding of the role this generally conserved channel plays in chemosensation and basic function. TRPA1 has been implicated in inflammatory and neuropathic pain throughout the body, including skin irritation, itch, dental sensitivity, migraine, arthritis, and respiratory disorders (Benemei *et al.*, 2013; Caceres *et al.*, 2009; Fernandes *et al.*, 2011; Haas *et al.*, 2011; Mukhopadhyay *et al.*, 2011; Sekiguchi *et al.*, 2009; Wilson *et al.*, 2013). It is also connected to acquired diseases, such as cardiovascular, gastrointestinal, and urogenital disease, which has led to numerous reviews seeking to integrate the channel's function with knowledge of its structure, sequence, and activation pathways (e.g., Mickle *et al.*, 2015; Nilius *et al.*, 2012; Paulsen *et al.*, 2015; Talavera *et al.*, 2020; Viana, 2016). As such, TRPA1 has become widely recognized as a promising therapeutic and analgesic target over the last decade (Andrade *et al.*, 2012; Banzawa *et al.*, 2014; Chen and Hackos, 2015; Dai, 2016; De Logu *et al.*, 2019; Fresno *et al.*, 2014; Garrison and Stucky, 2011; Mukhopadhyay *et al.*, 2016; Radresa *et al.*, 2013). Work on grasshopper mouse TRPA1 contributes to the growing research that has described the adaptive value and biomedical applicability of natural variation in TRPA1, both in traditional laboratory species, like frogs (Nakatsuka *et al.*, 2013) and flatworms (Arenas *et al.*, 2017), and in non-model species, such as snakes (Gracheva *et al.*, 2010) and mole-rats (Eigenbrod *et al.*, 2019; Smith *et al.*, 2020).

Mutations in grasshopper mice's TRPA1 sequences can also inform evolutionary questions that use this conserved ion channel as a bioinformatics tool. TRPA1 channels have undergone intense evolutionary selection as chemoreceptors in organisms of very different lineages (Gracheva *et al.*, 2010). Subsequently, TRPA1 channels provide new genetic and physiological markers to identify evolutionary relationships across the animal kingdom (Gracheva *et al.*, 2010).

Grasshopper mice are useful non-model models in part due to extensive research conducted on related rodents. Deer mice, close relatives of grasshopper mice, have become increasingly popular as emerging models for linking genes to behavior (Bedford and Hoekstra, 2015; Bendesky *et al.*, 2017; Weber *et al.*, 2013). New research on grasshopper mice can take advantage of infrastructure built for traditional house mice models and join both house mice and deer mice in a comparative framework for addressing the genetic underpinnings of behavior within evolutionary and biomedical research.

## 2. Osteoglossiformes

In Chapter 3, I evaluated the influence of sperm competition and sexual selection in shaping the diverse reproductive morphology and behavior of osteoglossiforms, an ancient group of teleost fishes. Many of osteoglossiforms' reproductive traits indicate that they experience low levels of sperm competition. Most species have singular gonads; two families, mormyrids and gymnarchids, are the only vertebrates known to have aflagellate sperm. Most families also exhibit modified anatomy often associated with internal fertilization, even though they perform external fertilization; potentially, osteoglossiforms may exist at a midpoint in the evolutionary transition from external to internal fertilization. Their ancestral position among teleosts suggests that understanding the selective forces that shaped osteoglossiform diversification may help improve understanding of the processes that drove early teleosts' adaptive radiation.

Osteoglossiforms may be a useful model for research seeking to test the relationship between reproductive traits and speciation. In combination with ecological selection, sexual selection can generate reproductive isolation (Kraaijeveld *et al.*, 2011; Panhuis *et al.*, 2001; Ritchie, 2007; West-Eberhard, 1983), potentially through diversification of genes involved in sexual reproduction (Carson, 1997; Ferris *et al.*, 1997; Lande, 1981; Singh and Kulathinal, 2000; Swanson and Vacquier, 2002). Investigating the genetic bases of osteoglossiforms' reproductive traits, including the genes involved in spermatogenesis, sperm-egg interaction, and sperm morphology, could resolve phylogenetic disputes and enigmatic species differences among osteoglossiform families (Hilton and Lavoué, 2018). For instance, research on closely related species of Indo-Pacific sea urchins found that these species may have diverged due to species-specific differences in gamete recognition proteins that became fixed at the time of speciation (Metz and Palumbi, 1996). In some *Drosophila* species, testis and male seminal fluid proteins have lineage-specific bursts of accelerated evolution and positive selection (Haerty *et al.*, 2007) that may be involved in speciation due to their role in post-mating effects, such as reducing female receptivity to remating (Ravi Ram and Wolfner, 2007). Subsequently, any work using osteoglossiforms as a model system to examine the selective pressures and genetic changes that contributed to teleost speciation would benefit from more information on osteoglossiform egg micropylar structure, female gametic proteins, and sexual conflict.

Research on adaptive radiation and reproductive behavior has focused on one particular osteoglossiform family, Mormyridae, by investigating the evolution of these fishes' diverse electrical signals in contributing to reproductive isolation. Sexual selection and sensory evolution have been considered to drive speciation in *Paramormyrops spp.*, which demonstrate rapid signal evolution (Arnegard *et al.*, 2010; Carlson *et al.*, 2011). Due to available genomics resources for mormyrids and extensive work on their physiology, electrosensory systems, and mechanisms of electric signal generation and reception, mormyrids have been called an emerging model system for identifying cellular mechanisms of behavior (Carlson and Gallant, 2013).

The diversity of osteoglossiforms' reproductive behavior also enables this group to serve as a model for understanding the evolution of fertilization strategies and parental care. Many species exhibit different combinations of features attributed to both external and internal fertilization, which makes them useful for empirically testing the contribution of sexual selection and environmental constraints to these traits. In addition, osteoglossiforms appear to exhibit every type of parental care demonstrated by other teleosts. Multiple species perform paternal, maternal, and biparental care, while others lack parental care entirely. With the exception of mormyrids, there appears to be a correlation between high sperm competition and parental care in osteoglossiforms. Osteoglossiforms may therefore be a useful comparative model system, like Cichlidae (Gonzalez-Voyer *et al.*, 2008), for investigating the role of sexual selection in the evolution of parental care from the ancestral state of no care.

Osteoglossiforms may serve as a model for body development due to one of their most distinctive and enigmatic features – singular gonads. All osteoglossiforms studied to date have unpaired gonads with the exception of one species, *Pantodon buccholzi*, whose taxonomic position has been under debate (Lavoué, 2016). Revealing the genetic basis of this trait may reveal new information about the origins and mechanisms of body asymmetry, especially since singular gonads are found in ancestral species like the coelacanth (Millot *et al.*, 1978). Investigating potential orthologues of these genes in fish and bird species with unpaired gonads may be the first step towards developing functional hypotheses related to the adaptive value of singular gonads, such as whether they evolved as a means of promoting egg survival (Guioli *et al.*, 2014).

The ability to link genotype to phenotype in osteoglossiforms is especially important for conservation genetics and management due to the evolutionary effects of fishing and adaptation to environmental change (Naish and Hard, 2008). Spread across five continents, many osteoglossiforms are of commercial interest and threatened by native habitat loss (Castello *et al.*, 2015). The Asian arowana (*Scleropages formosus*) is the most expensive fish in the exotic aquarium pet trade due to its vivid color morphs and there has been extensive interest in identifying the genes responsible for its pigmentation (Austin *et al.*, 2015; Bian *et al.*, 2016; Fernando *et al.*, 1997; Manoharan *et al.*, 2011; Shen *et al.*, 2014; Yue *et al.*, 2020; Yue *et al.*, 2006b). Further development of genetic markers for its different colored phenotypes would be helpful for breeding and maintaining genetic diversity of this species (Rahman *et al.*, 2010; Yue *et al.*, 2002; Yue *et al.*, 2006a; Yue *et al.*, 2004). These efforts, combined with a more detailed understanding of osteoglossiform courtship, parental care behaviors, and how to induce reproductive behavior in captivity, would be invaluable in saving these species from extinction and enabling their reintroduction to the wild (Fernando *et al.*, 1997; Yue *et al.*, 2020)

## Final Remarks

In an editorial piece entitled "The Institute for the Study of Non-Model Organisms and other fantasies," William Sullivan imagines an entire research center designated for learning about the "countless examples of organisms with fascinating biology that have been largely overlooked by the molecular and cell biologists" (Sullivan, 2015). Osteoglossiforms and grasshopper mice would clearly find a home in this imaginary institution. Although the definition of "model organisms" remains up for debate, the growing promise of nontraditional model species suggests that we may require a larger consensus regarding whether to incorporate non-laboratory species under this umbrella term, or establish a new term for these exemplary organisms with specialized traits. Species-agnostic genomics tools and infrastructure has changed the future of non-model species so that they are becoming more tractable systems for use in existent laboratories. Equipment has also become increasingly smaller, portable, and durable, meaning that research previously only practical in the lab can now be conducted in the field and on a greater variety of organisms. Depending on the biological question of interest, the choice of study species for addressing that question is not necessarily restricted to organisms that can be raised or kept long-term in captivity. Rather, a comparative biology approach may allow for the expansion of Krogh's principle, such that specific model systems that include a traditional model species and related species, rather than a single organism, may become the most convenient tool for examining a biological question from genes to behavior. Refinement of techniques practiced on traditional models suggests that realizing the potential of non-model species may no longer be an imaginative fantasy, but a feasible and welcome integration of new perspectives in the scientific community.

# REFERENCES

# REFERENCES

Andrade, E., Meotti, F. & Calixto, J. (2012). TRPA1 antagonists as potential analgesic drugs. *Pharmacology & therapeutics* **133**, 189-204.

Arenas, O. M., Zaharieva, E. E., Para, A., Vásquez-Doorman, C., Petersen, C. P. & Gallio, M. (2017). Activation of planarian TRPA1 by reactive oxygen species reveals a conserved mechanism for animal nociception. *Nature neuroscience* **20**, 1686-1693.

Arnegard, M. E., McIntyre, P. B., Harmon, L. J., Zelditch, M. L., Crampton, W. G., Davis, J. K., Sullivan, J. P., Lavoué, S. & Hopkins, C. D. (2010). Sexual signal evolution outpaces ecological divergence during electric fish species radiation. *The American Naturalist* **176**, 335-356.

Austin, C. M., Tan, M. H., Croft, L. J., Hammer, M. P. & Gan, H. M. (2015). Whole genome sequencing of the Asian arowana (Scleropages formosus) provides insights into the evolution of ray-finned fishes. *Genome biology and evolution* **7**, 2885-2895.

Banzawa, N., Saito, S., Imagawa, T., Kashio, M., Takahashi, K., Tominaga, M. & Ohta, T. (2014). Molecular basis determining inhibition/activation of nociceptive receptor TRPA1 protein. *Journal of Biological Chemistry* **289**, 31927-31939.

Baraldi, P. G., Preti, D., Materazzi, S. & Geppetti, P. (2010). Transient receptor potential ankyrin 1 (TRPA1) channel as emerging target for novel analgesics and anti-inflammatory agents. *Journal of medicinal chemistry* **53**, 5085-5107.

Bedford, N. L. & Hoekstra, H. E. (2015). The natural history of model organisms: Peromyscus mice as a model for studying natural variation. *Elife* **4**, e06813.

Bendesky, A., Kwon, Y.-M., Lassance, J.-M., Lewarch, C. L., Yao, S., Peterson, B. K., He, M. X., Dulac, C. & Hoekstra, H. E. (2017). The genetic basis of parental care evolution in monogamous mice. *Nature* **544**, 434-439.

Benemei, S., De Cesaris, F., Fusi, C., Rossi, E., Lupi, C. & Geppetti, P. (2013). TRPA1 and other TRP channels in migraine. *The journal of headache and pain* **14**, 1-8.

Bian, C., Hu, Y., Ravi, V., Kuznetsova, I. S., Shen, X., Mu, X., Sun, Y., You, X., Li, J. & Li, X. (2016). The Asian arowana (Scleropages formosus) genome provides new insights into the evolution of an early lineage of teleosts. *Scientific reports* **6**, 1-17.

Caceres, A. I., Brackmann, M., Elia, M. D., Bessac, B. F., del Camino, D., D'Amours, M., Witek, J. S., Fanger, C. M., Chong, J. A. & Hayward, N. J. (2009). A sensory neuronal ion channel essential for airway inflammation and hyperreactivity in asthma. *Proceedings of the National Academy of Sciences* **106**, 9099-9104.

Carlson, B. A. & Gallant, J. R. (2013). From sequence to spike to spark: evo-devo-neuroethology of electric communication in mormyrid fishes. *Journal of neurogenetics* **27**, 106-129.

Carlson, B. A., Hasan, S. M., Hollmann, M., Miller, D. B., Harmon, L. J. & Arnegard, M. E. (2011). Brain evolution triggers increased diversification of electric fishes. *science* **332**, 583-586.
Carson, H. (1997). Sexual selection: a driver of genetic change in Hawaiian Drosophila. *Journal of Heredity* **88**, 343-352.

Castello, L., Arantes, C. C., Mcgrath, D. G., Stewart, D. J. & Sousa, F. S. D. (2015). Understanding fishing-induced extinctions in the Amazon. *Aquatic Conservation: Marine and Freshwater Ecosystems* **25**, 587-598.

Chen, J. & Hackos, D. H. (2015). TRPA1 as a drug target—promise and challenges. *Naunyn-Schmiedeberg's archives of pharmacology* **388**, 451-463.

Cruz-Orengo, L., Dhaka, A., Heuermann, R. J., Young, T. J., Montana, M. C., Cavanaugh, E. J., Kim, D. & Story, G. M. (2008). Cutaneous nociception evoked by 15-delta PGJ2 via activation of ion channel TRPA1. *Molecular pain* **4**, 1744-8069-1744-1730.

Cyr, M. A. (1972). Predatory Behavior of the Grasshopper Mouse (Onychomys). In *Zoology*: University of California, Los Angeles.

Dai, Y. (2016). TRPs and pain. In *Seminars in Immunopathology*, pp. 277-291: Springer.

De Logu, F., Puma, S. L., Landini, L., Tuccinardi, T., Poli, G., Preti, D., De Siena, G., Patacchini, R., Tsagareli, M. G. & Geppetti, P. (2019). The acyl-glucuronide metabolite of ibuprofen has analgesic and anti-inflammatory effects via the TRPA1 channel. *Pharmacological research* **142**, 127-139.

Eigenbrod, O., Debus, K. Y., Reznick, J., Bennett, N. C., Sánchez-Carranza, O., Omerbašić, D., Hart, D. W., Barker, A. J., Zhong, W. & Lutermann, H. (2019). Rapid molecular evolution of pain insensitivity in multiple African rodents. *science* **364**, 852-859.

Fanger, C. M., Camino, D. d. & Moran, M. M. (2010). TRPA1 as an analgesic target. *The Open Drug Discovery Journal* **2**.

Fernandes, E. S., Russell, F. A., Spina, D., McDougall, J. J., Graepel, R., Gentry, C., Staniland, A. A., Mountford, D. M., Keeble, J. E. & Malcangio, M. (2011). A distinct role for transient receptor potential ankyrin 1, in addition to transient receptor potential vanilloid 1, in tumor necrosis factor α–induced inflammatory hyperalgesia and Freund's complete adjuvant–induced monarthritis. *Arthritis & Rheumatism* **63**, 819-829.

Fernando, A., Lim, L., Jeyaseelan, K., Teng, S., Liang, M. & Yeo, C. (1997). DNA fingerprinting: application to conservation of the CITES-listed dragon fish, Scleropages formosus (Osteoglossidae). *Aquarium Sciences and Conservation* **1**, 91-104.

Ferris, P. J., Pavlovic, C., Fabry, S. & Goodenough, U. W. (1997). Rapid evolution of sex-related genes in Chlamydomonas. *Proceedings of the National Academy of Sciences* **94**, 8634-8639.
Fresno, N., Pérez-Fernández, R., Goicoechea, C., Alkorta, I., Fernández-Carvajal, A., De la Torre-Martinez, R., Quirce, S., Ferrer-Montiel, A., Martín, M. I. & Goya, P. (2014). Adamantyl analogues of paracetamol as potent analgesic drugs via inhibition of TRPA1. *PLoS One* **9**, e113841.

Garrison, S. & Stucky, C. (2011). The dynamic TRPA1 channel: a suitable pharmacological pain target? *Current pharmaceutical biotechnology* **12**, 1689-1697.

Gonzalez-Voyer, A., Fitzpatrick, J. L. & Kolm, N. (2008). Sexual selection determines parental care patterns in cichlid fishes. *Evolution: International Journal of Organic Evolution* **62**, 2015-2026.

Gracheva, E. O., Ingolia, N. T., Kelly, Y. M., Cordero-Morales, J. F., Hollopeter, G., Chesler, A. T., Sánchez, E. E., Perez, J. C., Weissman, J. S. & Julius, D. (2010). Molecular basis of infrared detection by snakes. *Nature* **464**, 1006-1011.

Guioli, S., Nandi, S., Zhao, D., Burgess-Shannon, J., Lovell-Badge, R. & Clinton, M. (2014). Gonadal asymmetry and sex determination in birds. *Sexual Development* **8**, 227-242.

Haas, E. T., Rowland, K. & Gautam, M. (2011). Tooth injury increases expression of the cold sensitive TRP channel TRPA1 in trigeminal neurons. *Archives of oral biology* **56**, 1604-1609.

Haerty, W., Jagadeeshan, S., Kulathinal, R. J., Wong, A., Ravi Ram, K., Sirot, L. K., Levesque, L., Artieri, C. G., Wolfner, M. F. & Civetta, A. (2007). Evolution in the fast lane: rapidly evolving sex-related genes in Drosophila. *Genetics* **177**, 1321-1335.

Hilton, E. J. & Lavoué, S. (2018). A review of the systematic biology of fossil and living bony-tongue fishes, Osteoglossomorpha (Actinopterygii: Teleostei). *Neotropical Ichthyology* **16**.

Kraaijeveld, K., Kraaijeveld-Smit, F. J. & Maan, M. E. (2011). Sexual selection and speciation: the comparative evidence revisited. *Biological Reviews* **86**, 367-377.

Lande, R. (1981). Models of speciation by sexual selection on polygenic traits. *Proceedings of the National Academy of Sciences* **78**, 3721-3725.

Langley, W. (1981). Failure of food-aversion conditioning to suppress predatory attack of the grasshopper mouse, Onychomys leucogaster. *Behavioral and Neural Biology* **33**, 317-333.

Lavoué, S. (2016). Was Gondwanan breakup the cause of the intercontinental distribution of Osteoglossiformes? A time-calibrated phylogenetic test combining molecular, morphological, and paleontological evidence. *Molecular phylogenetics and evolution* **99**, 34-43.

Manoharan, B., Sulaimen, Z., Omar, F., Othman, R., Mohamed, S. & Bhassu, S. (2011). Isolation and characterization of novel microsatellite markers in commercial selected golden

Malaysian arowana fish, Scleropages formosus (Osteoglossidae). *Genetics and Molecular Research* **10**, 712-716.

Metz, E. C. & Palumbi, S. R. (1996). Positive selection and sequence rearrangements generate extensive polymorphism in the gamete recognition protein bindin. *Molecular biology and evolution* **13**, 397-406.

Mickle, A. D., Shepherd, A. J. & Mohapatra, D. P. (2015). Sensory TRP channels: the key transducers of nociception and pain. *Progress in molecular biology and translational science* **131**, 73-118.

Millot, T., Anthony, J. & Robineau, D. (1978). Anatomie de Latimena chalumnae. *Editions du Centre National de la Recherche Scientifique, Paris* **3**.

Mukhopadhyay, I., Gomes, P., Aranake, S., Shetty, M., Karnik, P., Damle, M., Kuruganti, S., Thorat, S. & Khairatkar-Joshi, N. (2011). Expression of functional TRPA1 receptor on human lung fibroblast and epithelial cells. *Journal of Receptors and Signal Transduction* **31**, 350-358.

Mukhopadhyay, I., Kulkarni, A. & Khairatkar-Joshi, N. (2016). Blocking TRPA1 in respiratory disorders: does it hold a promise? *Pharmaceuticals* **9**, 70.

Naish, K. A. & Hard, J. J. (2008). Bridging the gap between the genotype and the phenotype: linking genetic variation, selection and adaptation in fishes. *Fish and Fisheries* **9**, 396-422.

Nakatsuka, K., Gupta, R., Saito, S., Banzawa, N., Takahashi, K., Tominaga, M. & Ohta, T. (2013). Identification of molecular determinants for a potent mammalian TRPA1 antagonist by utilizing species differences. *Journal of Molecular Neuroscience* **51**, 754-762.

Nilius, B., Appendino, G. & Owsianik, G. (2012). The transient receptor potential channel TRPA1: from gene to pathophysiology. *Pflügers Archiv-European Journal of Physiology* **464**, 425-458.

Panhuis, T. M., Butlin, R., Zuk, M. & Tregenza, T. (2001). Sexual selection and speciation. *Trends in Ecology & Evolution* **16**, 364-371.

Paulsen, C. E., Armache, J.-P., Gao, Y., Cheng, Y. & Julius, D. (2015). Structure of the TRPA1 ion channel suggests regulatory mechanisms. *Nature* **520**, 511-517.

Radresa, O., Dahllof, H., Nyman, E., Nolting, A., Albert, J. S. & Raboisson, P. (2013). Roles of TRPA1 in pain pathophysiology and implications for the development of a new class of analgesic drugs. *The Open Pain Journal* **6**.

Rahman, S., Zakaria-Ismail, M., Tang, P. Y., Rizman-Idid, M. & Muniandy, S. (2010). Genetic variability and estimation of effective population sizes of the natural populations of green arowana, Scleropages formosus in Peninsular Malaysia. *Advances in Environmental Biology* **4**, 14-23.

Ravi Ram, K. & Wolfner, M. F. (2007). Seminal influences: Drosophila Acps and the molecular interplay between males and females during reproduction. *Integrative and comparative biology* **47**, 427-445.

Ritchie, M. G. (2007). Sexual selection and speciation. *Annu. Rev. Ecol. Evol. Syst.* **38**, 79-102.
Rowe, A. H. & Rowe, M. P. (2006). Risk assessment by grasshopper mice (Onychomys spp.) feeding on neurotoxic prey (Centruroides spp.). *Animal Behaviour* **71**, 725-734.

Sekiguchi, M., Futei, Y., Fujii, Y., Iwasaki, T., Nishikawa, T. & Amagai, M. (2009). Non-neuronal expression of transient receptor potential type A1 (TRPA1) in human skin. *Journal of Investigative Dermatology* **129**, 2312-2315.

Shen, X., Kwan, H., Thevasagayam, N., Prakki, S., Kuznetsova, I., Ngoh, S., Lim, Z., Feng, F., Chang, A. & Orbán, L. (2014). The first transcriptome and genetic linkage map for Asian arowana. *Molecular ecology resources* **14**, 622-635.

Singh, R. S. & Kulathinal, R. J. (2000). Sex gene pool evolution and speciation: a new paradigm. *Genes & genetic systems* **75**, 119-130.

Slobodchikoff, C., Vaughan, T. A. & Warner, R. M. (1987). How prey defenses affect a predator's net energetic profit. *Journal of Mammalogy* **68**, 668-671.

Smith, E. S. J., Park, T. J. & Lewin, G. R. (2020). Independent evolution of pain insensitivity in African mole-rats: origins and mechanisms. *Journal of Comparative Physiology A* **206**, 313-325.

Stucky, C. L., Dubin, A. E., Jeske, N. A., Malin, S. A., McKemy, D. D. & Story, G. M. (2009). Roles of transient receptor potential channels in pain. *Brain research reviews* **60**, 2-23.

Sullivan, W. (2015). The Institute for the Study of Non–Model Organisms and other fantasies. *Molecular biology of the cell* **26**, 387-389.

Swanson, W. J. & Vacquier, V. D. (2002). The rapid evolution of reproductive proteins. *Nature Reviews Genetics* **3**, 137-144.

Talavera, K., Startek, J. B., Alvarez-Collazo, J., Boonen, B., Alpizar, Y. A., Sanchez, A., Naert, R. & Nilius, B. (2020). Mammalian transient receptor potential TRPA1 channels: from structure to disease. *Physiological reviews* **100**, 725-803.

Taylor-Clark, T. E., Nassenstein, C., McAlexander, M. A. & Undem, B. J. (2009). TRPA1: a potential target for anti-tussive therapy. *Pulmonary pharmacology & therapeutics* **22**, 71-74.

Viana, F. (2016). TRPA1 channels: molecular sentinels of cellular stress and tissue damage. *The Journal of physiology* **594**, 4151-4169.

Weber, J. N., Peterson, B. K. & Hoekstra, H. E. (2013). Discrete genetic modules are responsible for complex burrow evolution in Peromyscus mice. *Nature* **493**, 402-405.

West-Eberhard, M. J. (1983). Sexual selection, social competition, and speciation. *The Quarterly Review of Biology* **58**, 155-183.

Whitman, D. W., Blum, M. S. & Jones, C. G. (1986). Olfactorily mediated attack suppression in the southern grasshopper mouse toward an unpalatable prey. *Behavioural processes* **13**, 77-83.

Wilson, S. R., Nelson, A. M., Batia, L., Morita, T., Estandian, D., Owens, D. M., Lumpkin, E. A. & Bautista, D. M. (2013). The ion channel TRPA1 is required for chronic itch. *Journal of Neuroscience* **33**, 9283-9294.

Yue, G., Li, Y., Chen, F., Cho, S., Lim, L. C. & Orban, L. (2002). Comparison of three DNA marker systems for assessing genetic diversity in Asian arowana (Scleropages formosus). *Electrophoresis* **23**, 1025-1032.

Yue, G., Zhu, Z., Lin, G., Lo, L. & Feng, F. (2006a). Novel polymorphic microsatellites for studying genetic diversity of red Asian arowanas. *Conservation Genetics* **7**, 627-629.

Yue, G. H., Chang, A., Yuzer, A. & Suwanto, A. (2020). Current Knowledge on the Biology and Aquaculture of the Endangered Asian Arowana. *Reviews in Fisheries Science & Aquaculture* **28**, 193-210.

Yue, G. H., Li, Y., Lim, L. C. & Orban, L. (2004). Monitoring the genetic diversity of three Asian arowana (Scleropages formosus) captive stocks using AFLP and microsatellites. *Aquaculture* **237**, 89-102.

Yue, G. H., Liew, W. C. & Orban, L. (2006b). The complete mitochondrial genome of a basal teleost, the Asian arowana (Scleropages formosus, Osteoglossidae). *BMC genomics* **7**, 242.