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The Adaptive Evolution of Herbivory in Freshwater Systems

Jessica Lynn Sanchez Montelongo
Florida International University, jsanc318@fiu.edu

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

THE ADAPTIVE EVOLUTION OF HERBIVORY IN FRESHWATER SYSTEMS

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by

Jessica L. Sanchez Montelongo

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To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Jessica L. Sanchez Montelongo, and entitled The Adaptive Evolution of Herbivory in Freshwater Systems, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

_____
John Berry

_____
Heather Bracken-Grissom

_____
Deron Burkepile

_____
Evelyn Gaiser

_____
Joel Trexler, Major Professor

Date of Defense: May 29, 2018

The dissertation of Jessica L. Sanchez Montelongo is approved.

_____
Dean Michael R. Heithaus
College of Arts, Sciences and Education

_____
Andrés G. Gil
Vice President for Research and Economic Development
and Dean of the University Graduate School

Florida International University, 2018

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ABSTRACT OF THE DISSERTATION

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Jessica L. Sanchez Montelongo

Florida International University, 2018

Miami, Florida

Professor Joel Trexler, Major Professor

Herbivory is thought to be nutritionally inefficient relative to carnivory and omnivory. But, herbivory evolved from carnivory in many lineages, suggesting that there are advantages to eating plants. To understand the adaptive significance of the transition from carnivory to herbivory, I proposed five hypotheses for the adaptive evolution of herbivory and reviewed the current freshwater literature to identify conditions where eating plants might be adaptive over eating animals. I tested three of these ideas (Suboptimal Habitat, Heterotroph Facilitation, and Lipid Allocation) using the herbivorous Sailfin Molly (*Poecilia latipinna)* and identified each as a potential mechanism for the evolution of herbivory.

To understand the origins of herbivory in Sailfin Mollies, I reconstructed ancestral habitats and diets across a phylogeny of the genus *Poecilia* and then used phylogenetically independent contrasts to identify patterns of diet evolution. I found that the degree of herbivory increases with increasing salinity affiliation, suggesting that in this genus, herbivory evolved as an adaptation for invading less productive saline habitats from freshwaters. This result is consistent with the Suboptimal Habitat hypothesis, which states that herbivory allows organisms to invade and persist in 'suboptimal' habitats. To

understand how herbivory is maintained in extant populations, I raised juvenile Sailfin Mollies in mesocosms and enclosure cages placed in the Everglades to document that dietary autotrophic lipids play a role in early life history by supporting rapid growth (Lipid Allocation). However, dietary bacterial fatty acids promoted fish survival, consistent with the Heterotroph Facilitation hypothesis, which states that indirect detritivory supplements the herbivorous diet. Finally, I quantified periphyton quality/availability and consumer density across the Everglades landscape to examine the correlates of trophic dynamics in nature. Results revealed that herbivores can persist in diverse habitats and survive on varying resources when habitats are unfavorable, supporting the Suboptimal Habitat hypothesis.

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CHAPTER 1

INTRODUCTION

Herbivores are key consumers in ecosystems because they harvest energy from plants, which is thereby transferred up the food web when other animals eat herbivores. Without this important diet strategy, energy would not reach higher order consumers (e.g., large game fish, humans, etc.) and their populations could not be sustained. However, from a nutritional perspective, carnivory (eating animals) and omnivory (eating both plants and animals) are "better" diets than herbivory (Sanchez and Trexler 2016). Omnivores and carnivores consume animal prey that are high in nutritional value (Mattson, 1980; Sterner & Hessen, 1994; Choat & Clements, 1998; Karban & Agrawal, 2002), and omnivores have the additional advantage of supplementing their diets with abundant and easy to obtain plant items (Coll & Guershon 2002; Diehl, 2003). Obtaining comparable energy from an exclusively herbivorous diet is difficult because plants are nutritionally variable and usually employ structural and/or biochemical mechanisms to deter herbivores (Mattson, 1980; Porter & McDonough, 1984; Horn 1989, Chivers & Langer, 1994; Sterner & Hessen, 1994; Choat & Clements, 1998; and others). Herbivores may also be limited by time and/or space by predators and competitors, by the ability to produce digestive or detoxifying enzymes (see Karban & Agrawal, 2002), or the amount of time it takes for food to pass through the gut (Horn, 1989; Bruggeman et al. 1994; Bellwood, 1995; Choat & Clements 1998). The unfavorable characteristics of herbivore diets affect consumer life histories (i.e., reproduction, growth, survival) and raise the question of why herbivory is common in nature. However, many herbivores have evolved from carnivorous ancestors (see Sanchez and Trexler 2016)., suggesting that there are adaptive advantages to this seemingly inferior diet strategy.

Current research has thoroughly delineated the ecological context of the herbivorous diet (i.e., food selection, assimilation, nutrient regulation, etc.), but has yet to determine the conditions that favor herbivory over eating animals. The paucity of knowledge concerning the adaptive evolution of herbivory is a missing piece to an overall theory of the origins of diet. To understand the adaptive significance of the herbivorous diet, I posed five hypotheses on the evolution of herbivory from carnivory: 1*) Intake-Efficiency* - herbivores use part of their food source as habitat, thus minimizing the energy/time spent searching for food and avoiding predators; 2) *Suboptimal Habitat* - herbivory allows organisms to invade and establish populations in habitats that have high primary production but low abundance of animal prey; 3) *Heterotroph Facilitation* - herbivory is adaptive because herbivores consume microbes associated with producers; 4) *Lipid Allocation* - herbivory is adaptive because producers are rich in fatty acids, which fuel reproduction and storage; and 5) *Disease Avoidance* - herbivory minimizes animal-facilitated disease transmission. In Chapter 2, I reviewed the current literature and used evidence from these works as support for these five adaptive hypotheses in order to establish a framework to test them.

The genus *Poecilia* is an excellent model system for studying the evolution of herbivory because *Poecilia* species exhibit a variety of diet preferences, with herbivory concentrated in the subgenus *Mollienesia*. Furthermore, this group has evolved the ability to disperse across marine water barriers, and extant species inhabit both fresh and euryhaline habitat types (Meffe and Snelson 1989).

Although marine systems cover 99% of the Earth's surface, these habitats are less productive per unit area than freshwater aquatic habitats (e.g., Colinvaux 1980; May and Godfrey 1994; Vermeij and Grosberg 2010) and could therefore be considered 'suboptimal' under the Suboptimal Habitat Hypothesis. As such, transitions from freshwater to less productive marine waters may have prompted the evolution of the herbivorous strategy in the genus *Poecilia*, particularly in the subgenus *Mollienesia*. In Chapter 3, I evaluated the Suboptimal Habitat Hypothesis by reconstructing ancestral states of habitat and diet across a phylogeny of the genus *Poecilia* to identify patterns of diet evolution and habitat transition. I then used phylogenetically independent contrasts to identify patterns of diet evolution in response to habitat transition.

In Chapters 4 and 5, I tested the Heterotroph Facilitation and Lipid Allocation Hypotheses using the Sailfin Molly (*Poecilia latipinna*), an exclusively herbivorous member of the subgenus *Mollienesia*. The herbivorous Sailfin Molly is native to the Florida Everglades, although there is evidence that herbivory is not an efficient diet in this area. Several studies have suggested that periphyton (the primary basal resource in the Everglades) is a poor-quality food source for herbivores (e.g., Geddes and Trexler 2003).

As a result, the system supports a low diversity and abundance of higher order consumers relative to primary producers (Turner et al. 1999; Geddes and Trexler 2003).

To test my adaptive hypotheses, I used enclosure cages stocked with juvenile Sailfin Mollies placed in the field (Chapter 4) and lab (Chapter 5). I used shading and phosphorus addition to manipulate the heterotrophic and autotrophic composition of colonizing epiphyton (food for Sailfin Mollies), and then examined the effects of this

varying food quality on Sailfin Molly life history to determine the explanatory power of these alternative adaptive hypotheses in nature.

Although my posed hypotheses were developed to describe the evolution of herbivory, they may also be incorporated into current ecological theory to describe how communities are assembled based on the role of consumers in a food web. Ecological niche-based models predict that species' abilities to establish in a locality are determined by their traits (Chase and Leibold 2003), whereas dispersal-based models predict that community assembly is driven by stochastic colonization, independent from species traits (Hubbell 2001; Chase and Leibold 2003; Chase 2007). Some studies have shown that dispersal-based models yield similar results to relatively complicated niche-based models (e.g., Condit et al. 2000; Bell 2001; Hubbell 2001, Volkov et al. 2003), suggesting that we can predict community assembly without considering the species traits. But, in nature, resources vary across the landscape, resulting in natural variation in consumer life history that drives species relative abundances and distributions (Kareiva 1990; Tilman 1994; Polis et al. 1997; Power and Dietrich 2002; McIntosh et al. 2004; Torres-Ruiz et al. 2007; Doi 2009; Guo et al. 2016). Therefore, relying on models that ignore the role of species traits in shaping communities limits our ability to understand the evolutionary consequences of ecological processes. In Chapter 6, I determined if niche- or dispersal-based predictions best described consumer dynamics in the Florida Everglades based on the nutritional landscape and interpreted these results in the contexts of the Heterotroph Facilitation and Suboptimal Habitat Hypotheses. By identifying an evolutionary mechanism that promotes herbivory, I was able to more fully describe the complex role of these consumers in functional food webs.

Herbivory has been the focus of many ecological studies spanning many sub-disciplines, but there is a significant gap in knowledge pertaining to the adaptive evolution of herbivory in nature. I began this research to explore the conditions that would favor the evolution of an herbivorous diet from a carnivorous or omnivorous one. These studies represent a starting point that may lead to more comprehensive studies of diet evolution.

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CHAPTER 2

THE ADPATIVE EVOLUTION OF HERBIVORY IN FRESHWATER SYSTEMS

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Abstract

Herbivory is thought to be nutritionally inefficient relative to carnivory and omnivory. But, herbivory evolved from carnivory in many terrestrial and aquatic lineages, suggesting that there are advantages of eating plants. Herbivory has been well-studied in both terrestrial and aquatic systems and there is abundant information on feedbacks between herbivores and plants, coevolution of plant and herbivore defenses, mechanisms for mediating nutrient limitation, effects of nutrient limitation on herbivore life history and more recently, the origins of the herbivorous diet. Researchers have sufficiently defined the ecological context and evolutionary origins of the herbivorous diet, and these main areas of research have laid the groundwork for studying herbivory as an adaptation. However, I have yet to synthesize this information in a way that allows us to establish a framework of testable adaptive hypotheses.

To understand the adaptive significance of this diet transition, I review the current literature and use evidence from these works as support for five hypotheses on the evolution of herbivory from carnivory: 1*) Intake-efficiency* - herbivores use part of their food source as habitat, thus minimizing the energy/time spent searching for food and avoiding predators; 2) *Suboptimal habitat* - herbivory allows organisms to invade and establish populations in habitats that have high primary production but low abundance of animal prey; 3) *Heterotroph facilitation* - herbivory is adaptive because herbivores consume microbes associated with producers; 4) *Lipid allocation* - herbivory is adaptive because producers are rich in fatty acids, which fuel reproduction and storage; and 5) *Disease avoidance* - herbivory minimizes animal-facilitated disease transmission.

Due to the extensive literature, I have limited this review to discussing herbivory in freshwater systems. To my knowledge, no prior work has compiled a comprehensive list of conditions that favor an herbivorous diet in nature. With backgrounds in both theoretical and experimental ecology, the incorporation of these hypotheses to the current literature will provide information about diet evolution, where it is currently lacking.

Key-words: Adaptive evolution, diet evolution, freshwater herbivory, herbivory

Introduction

Herbivory is thought to be an inefficient feeding strategy relative to omnivory and carnivory (Sterner and Elser 2002, Laspoumaderes et al. 2010). From an energetic perspective, herbivores are important consumers because they process primary production for use at higher trophic levels. However, at the individual level, the adaptive significance of herbivory is unclear. Omnivory is adaptive because food abundance is usually highest at lower trophic levels, whereas food quality (relative measure of energy content; defined below) increases with trophic position (e.g. Hastings and Conrad 1979, Hairston and Hairston 1993, Elser et al. 2000; Coll and Guershon 2002, Eubanks et al. 2003, Diehl 2003). Omnivores benefit by supplementing energetically costly prey with easy to obtain, but nutritionally variable, food items (Diehl 2003). Similarly, a carnivorous diet may be adaptive because prey items are of high quality and readily digested and assimilated (Stevens and Hume 2004, Choat and Clements 1998; Raubenheimer et al. 2005). Despite the vast herbivory literature on both terrestrial and aquatic systems, comparable hypotheses of herbivory are lacking.

There are few similarities of herbivory patterns between terrestrial and aquatic systems, and as a result, these literatures have developed independently. However, the majority of herbivory work in both systems focuses on these five ideas:

Feedback between herbivores and primary producers

Herbivores can control nutrient storage and recycling through their consumption rate of primary production (Cebrian and Lartigue 2004). In turn, herbivore consumption rates can be affected by nutrient composition of the producers (Sterner et al. 1997; Cebrian et al. 1998; Griffin et al 1998; Cebrian and Lartigue 2004). These top-down and bottom-up processes drive both producer and consumer population dynamics in terrestrial and aquatic systems, although the relative strength of these forces is different between systems (see Burkepile 2013). There is a large literature (e.g. Hairston et al. 1960, Murdoch 1966, Ehrlich and Birch 1967, Slobodkin et al. 1967, Wiegert and Owen 1971, Fretwell 1977, Oksanen 1988 and others) and numerous reviews (see Power 1992, Strong 1992) on feedback mechanisms as they are one of the fundamental ideas in herbivory research.

Coevolution of plant and herbivore defenses

Increased plant mortality by grazers may lead to changes in the life history and population dynamics of producers. For example, many plants can produce harmful secondary metabolites in response to herbivory (e.g. Pare and Tumlinson 1999, Howe and Jander 2008, etc.), but this is energetically costly (Crawley 1983) and limits energy available for other life processes (e.g. Herms and Mattson 1992).

In turn, herbivores expend energy in response to these defenses in order to obtain nutritional value from producers (e.g. detoxification pathways, Wiegand and Pflugmacher 2005, Jiang et al. 2012, Zhang et al. 2012) or to defend themselves against predators (e.g. sequestering plant metabolites, Duffy and Hay 1994, Stachowicz and Hay 1999, Nishida 2002), also diverting energy from other processes. Co-evolution of these and other plant and animal defenses (e.g. altered plant morphology/phenology/nutrient composition versus altered animal morphology/behavior/ digestive physiology) has been shown to influence population dynamics of both producers and herbivores.

How herbivores mediate the effects of nutrient limitation

When consumers are confined to relatively poor quality diets, they may compensate by increasing the amount of food they consume (e.g. Sinclair et al. 1982, Targett and Targett 1990, Simpson and Simpson 1990, Pennings et al. 1993, Stachowicz and Hay 1996, Cruz-Rivera and Hay 2000b, Van der Wal et al. 2000, Fink and von Elert 2006), allowing them to obtain sufficient nutrients and potentially offset the negative fitness consequences of a low quality diet (Vanni and Lampert 1992, Cruz-Rivera and Hay 2000b, Fink and von Elert 2006). Diet selectivity has also been proposed as a mechanism to permit subsistence on the relatively poor quality herbivorous diet (outlined in Karasov and Martinez del Rio 2007; e. g., Grasshoppers, Behmer and Joern 1993; amphipods, Cruz-Rivera and Hay 2000b). Alternatively, organisms may differentially assimilate or excrete nutrients, allowing them to attain suitable quantities of limiting nutrients (Behmer 2009).

Herbivores may also supplement their diets with food items of higher quality (e.g. other basal resources and/or animal prey), in order to sustain their imbalanced diet of primary food items (the "diet mixing hypothesis"; Simmonds et al. 1992, Bernays et al. 1994, Simpson and Rauenheimer 1996, Singer et al. 2002). Similarly, herbivores consuming chemically defended diets may consume other items of various qualities in order to "dilute" the toxin to benign concentrations ("toxin dilution hypothesis"; Freeland and Janzen 1974, Freeland and Saladin 1989). Herbivores may also consume less digestible items such as cellulose in order to increase the rate of food passage, thereby minimizing exposure of toxins in the diet (Berg et al. 2012). These hypotheses of nutrient acquisition by herbivores and resulting life history trade-offs (e.g. Duffy and Paul 1992, Raubenheimer and Simpson 1997, Cruz-Rivera and Hay 2000a-b, 2003, Ojala et al. 2005, Clements et al. 2009) have been a productive area of herbivory research.

Effects of nutrient limitation on herbivore life history

Basal resources are variable in their nutrient content as compared to animal prey (Sterner and Elser 2002), which limits energy allocation to individual growth and reproduction of primary consumers (Mattson 1980, Lika and Kooijman 2003). A multitude of studies on herbivores from terrestrial, marine and freshwater systems show that diet quality is linked to tradeoffs among life history traits (e.g. Rushton and Hassall 1983, Sterner 1993, Caceres et al.1994, Hietala et al. 1995, Lampert and Trubetskova 1996, Kilham et al. 1997, Schmidt and Jonasdottir 1997, Cruz-Rivera and Hay 2000b, Shin et al. 2003, Ojala et al. 2005, Trubetskova and Haney 2006, Guo and Xie 2011, Mitchell et al. 2012, Ortiz-Rodriguez et al. 2012).

Growth of an organism affects overall fitness via changes in survival and reproduction (Hairston et al. 2001), and reproductive output can have implications for population regulation (Stearns 1992).

Comparative analyses of related species with varying diet strategies

There are some diet characters that distinguish herbivores and carnivores. For example, post-foraging food processing (i.e. digestion, assimilation, etc.) by omnivorous or carnivorous animals may be more efficient than that of herbivores (Mattson 1980, Sterner and Hessen 1994, Choat and Clements 1998, Sterner and Elser 2002), and herbivores have evolved gut morphologies that may increase food assimilation (Kramer and Bryant 1995) as a result of this processing deficit (e.g. German et al. 2010). Furthermore, "dull" teeth (e.g. German et al. 2010) or specialized feeding apparatuses (e.g. intramandibular bending; Gibb et al. 2008) may be typical of benthic herbivores. Many terrestrial studies have included digestive physiology as a characteristic of diet and recent aquatic studies have begun to do so as well (see Choat and Clements 1998). Recent comparative studies have used these and other characters to document the evolution of herbivory from carnivorous ancestors (e.g. lizards: Van Damme 1999, Espinoza et al. 2004, mollusks: deMaintenon 1999, heteropteran insects: Eubanks et al. 2003, caddisflies: Pauls et al. 2008, fishes: Bellwood 2003, Bellwood et al. 2014), bringing us closer to understanding the adaptive significance of herbivory. These evolutionary studies are the bases for future work examining diets from an adaptive perspective.

Researchers have done a good job in delineating the ecological context and evolutionary origins of the herbivorous diet, and these main areas of research have laid the groundwork for studying herbivory as an adaptation. However, we have yet to synthesize this information in a way that allows us to establish a framework of testable adaptive hypotheses, which is a missing piece in the overall theory of diet evolution. For herbivorous lineages, at some point in time the cumulative benefits of switching from carnivory to herbivory were greater than both the costs of doing so, and the benefits of maintaining carnivory. However, carnivory remains a strategy in nature, suggesting that there are costs associated with herbivory. Similarly, the evolution of omnivory from herbivory seems beneficial, but both strategies are maintained in nature, further suggesting that there are adaptive advantages to herbivory. To understand the adaptive significance of this diet transition, I review the current literature and use evidence from these works as support for my ideas on the evolution of herbivory from carnivory (Table 1). Due to the abundant literature on this topic, I have limited this review to discussing herbivory in freshwater systems.

I propose five hypotheses that evaluate the adaptive evolution of herbivorous diets in freshwater systems (Table 2.1). I assume that in order for herbivory to evolve from a carnivorous ancestor: 1) there must be adequate genetic variation for herbivorous strategies to evolve; and 2) the ecological relationships revealed by contemporary research are similar to those that were present in the past. Here "herbivory" is defined as the consumption of algae and/or phytoplankton, and less commonly, the consumption of aquatic vascular macrophytes (reviewed by Newman 1991).

Furthermore, "herbivore" refers to an organism that mainly eats primary producers but may indirectly consume detritus. A "carnivore" is defined as an organism that eats animals and an "omnivore" refers to an organism that eats both plants and animals. Arguments regarding sub-classifications of these diet strategies (e.g. obligate v. facultative herbivore) or other specialized feeding strategies (e.g. wood-eating, frugivory, etc.) are not discussed here. In freshwater systems, grazers include organisms that graze algae (Feminella and Hawkins 1995, Newman and Rotjan 2013); therefore, in this paper, "grazer" and "herbivore" are used interchangeably. The term "food quality" is used to describe the nutritional worth of a diet item to a consumer. Worth of a food item may be defined by macronutrient (e.g. nutritional ecology) or elemental (e.g. stoichiometry) composition, where food items are rich in protein or phosphorus, respectively. Alternatively, food quality may be defined as the ratio of food energy content to that assimilated by consumers. For both definitions, "food quality" is a relative term and can only be interpreted relative to other diets (e.g. a diet item can be both high and low quality depending on the comparison diet). The hypotheses presented here were developed to reflect the life cycles of freshwater organisms and may or may not be applicable to organisms that do not spend their entire lives in freshwater (e.g. diadromous fishes or terrestrial insects with aquatic larvae). Although the concepts behind these hypotheses are not novel, to my knowledge, no compilation of hypotheses exist. In the following sections, I discuss the five proposed adaptive hypotheses: 1) intake-efficiency; 2) suboptimal habitat; 3) heterotroph facilitation; 4) lipid allocation; 5) and disease avoidance.

I. Intake-efficiency hypothesis

The intake-efficiency hypothesis is based on the predictions of simple optimal foraging models, which have proven robust for herbivores (Sih and Christensen 2001). This hypothesis states that selection favors herbivory over animal-containing diets because herbivorous organisms maximize energy intake by minimizing the energy and time spent searching for and subduing prey. Further, aquatic herbivores may use their food source as habitat (Brönmark and Vermaat 1998), or seek refuge in aquatic vegetation associated with their preferred food source (e.g. submerged vegetation and epiphytic algae, respectively; Alvarez and Peckarsky 2013), thereby decreasing energy expenditures related to locomotion (Cummins 1973) and/or predator avoidance. Therefore, the net energy gained from an herbivorous diet may be greater than a diet comprised of metazoan prey.

Herbivores are constantly grazing in order to meet energetic needs (e.g. Simpson and Simpson 1990, Cruz-Rivera and Hay 2000b), whereas energetic, physiological and encounter rate constraints prevent animal-consuming taxa from continuously foraging (Arrington et al. 2002; Karasov and Martinez del Rio 2007). As a result of these different foraging behaviors, herbivores continuously have plant material in their gut and omnivores/carnivores process their food in "batches" (discussed in Karasov and Martinez del Rio 2007). Batch processing may be followed by periods of hunger; therefore, herbivores are probably more continuously satiated relative to omnivores/carnivores. According to optimal foraging theory, satiated animals expend less energy foraging and more energy doing other activities such as mating (Krebs et al. 1983).

Therefore, herbivores may gain an adaptive advantage by shifting their energetic focus from foraging to reproducing.

II. Suboptimal habitat hypothesis

The suboptimal habitat hypothesis states that herbivory may be adaptive by allowing organisms to invade suboptimal habitats. Here, the term "suboptimal habitat" is relative to habitats that support high abundance and diversity of secondary consumers. Food web interactions often occur over spatially heterogeneous landscapes (Oksanen et al. 1995), or "patches" of varying resource quality and quantity. Therefore, an optimal habitat might be a suboptimal habitat at another point in space or time. In freshwater systems, it is generally thought that habitat patches are strongly influenced by abiotic factors such as nutrient availability and/or disturbance frequency (Pringle et al. 1988). Higher trophic levels dominate communities when habitat productivity is increased (e.g. Marks et al. 2000, Deegan et al. 2002, Beveridge et al. 2010) or when disturbance occurs at low to intermediate frequencies (Marks et al. 2000). However, consuming a plant-dominated diet is favored in habitats where animal prey are scarce and plant abundance is high (Chubaty et al. 2014), such as those with frequent disturbance. Furthermore, the palatability of plants is thought to play a key role in structuring herbivore populations (Elger et al. 2004). The most palatable benthic and phytoplankton species are associated with early stages of succession, because fast-growing plants invest less energy in structural and toxic elements (e.g. Porter 1977, Elger et al. 2004).

Elger et al. (2002) investigated the effects of disturbance and nutrient availability on freshwater plant palatability for herbivorous snails (*Lymnaea stagnalis*) and found that increased disturbance frequency, but not nutrient availability, positively influenced food availability for herbivores (Elger et al. 2002), providing evidence for an herbivore advantage in disturbed habitats (e.g. suboptimal habitats).

Classic optimal foraging theory (i.e. optimal diet) predicts that if a resource is abundant, specializing on that resource is preferred (see Chubaty et al. 2014). These predictions are supported by early food preference studies, which suggest that herbivores evolved in response to food availability rather than food value (e.g. Paine and Vadas 1969). Using an evolutionary simulation model, Chubaty et al. (2014) examined how quality and availability of plant and animal prey shapes the evolution of diet. Results indicate that relative availability of resources can predict an individual's trophic level (Chubaty et al. 2014). More specifically, an increased abundance of plants increases herbivore abundance relative to carnivorous animals (Chubaty et al. 2014) demonstrating that herbivory may be adaptive when plants are abundant and prey are not (e.g. suboptimal habitats).

Seasonality can also influence habitat quality and resource availability. Organisms are limited to resources that are immediately available. Constant and seasonally varying food supplies are known to influence life histories of many aquatic consumers by altering individual growth and reproduction (output, patterns, mode, etc.). The effects of seasonal food limitation have been well studied in *Daphnia* (e.g. Chapman and Burns 1994) and other cladocerans (e.g. DeMott and Kerfoot 1982, Boersma and Vijverberg 1996).

More specifically, constant food supplies are known to increase growth and brood size of cladocerans. However, food supplies vary in nature and herbivores may gain an advantage by consuming different species or by switching between green, detrital and/or animal diets seasonally, thereby reducing the effects of specializing on a single food type (e.g. Kitting 1980, Sanders et al. 1996, DeMott 1998, Cruz-Rivera and Hay 2000a). Herbivory may allow organisms to minimize interspecific competition (via decreased niche overlap) by invading and establishing populations in suboptimal habitats. For example, the globally invasive golden apple snail (*Pomacea canaliculata*) specializes on freshwater macrophytes and has established successful populations in areas that are uncolonized by other phylogenetically similar species. Further, invading a suboptimal habitat may allow herbivores to escape predation. Trade-offs between foraging and predator avoidance in aquatic consumers are well documented (reviewed by Milinski 1985). Camacho and Thacker (2013) showed that freshwater amphipods exposed to fish predators sought refuge in toxic cyanobacterial mats. Further, amphipods exposed to predators showed higher survivorship on toxic mats as compared to non-toxic mats. These results suggest that herbivores at risk from predators benefit by seeking refuge in suboptimal habitats. If herbivores benefit from invading suboptimal habitats by avoiding predation, equally performing herbivores could be aggregated in both high and low-quality patches as predicted by an "ideal free distribution" (Fretwell and Lucas 1970). Therefore, the ability to colonize and persist equally in both inferior and relatively superior habitats can promote survival of herbivores by exploiting niche opportunities that are unavailable to carnivorous species.

III. Heterotroph facilitation hypothesis

The heterotroph-facilitation hypothesis states that herbivory is adaptive because herbivores indirectly consume heterotrophic microbes (bacteria, fungi and/or protozoa) that are associated with primary producer communities. It has been shown that aquatic herbivores supplement their diets with essential nutrients originating from heterotrophic bacteria (Bowen 1984, Smoot and Findlay 2010, Belicka et al. 2012) and a strong positive correlation between primary production and bacteria has been documented in several aquatic systems (Cole 1982). In limnetic waters, heterotrophic microbes largely contribute to planktonic biomass and are under strong grazing pressure by zooplankton (Arndt 1993). Benthic algae in close association with heterotrophic microbes come in several forms (collectively called "periphyton") and are the primary food source for herbivores in benthic systems (Wetzel 2001).

Relative to algae, heterotrophic bacteria are superior competitors for phosphorus (P), incorporating the nutrient into their cell walls (Martin-Creuzburg et al. 2011); therefore, these microbes are a rich source of the limiting nutrient for herbivores (Martin-Creuzburg et al. 2011). Although P is important for metazoan growth (Sterner and Elser 2002), diets composed only of heterotrophs are of poor quality for *Daphnia magna* suggesting that herbivores may rely on other dietary items for essential biochemicals like sterols (e.g. invertebrates) or fatty acids (Martin-Creuzburg et al. 2011). For example, growth rates of *Daphnia magna* increased when fed heterotrophic bacteria supplemented with sterols (important for molting) relative to growth of those fed only bacteria (Martin-Creuzburg et al. 2011).

Related studies found that *Daphnia* require a diet composed of at least 50% green algae to compensate for a sterol deficiency (Martin-Creuzburg et al. 2005). In a vertebrate example, the sailfin molly (*Poecilia latipinna*) was shown to assimilate both algal material and fatty acids derived from heterotrophic bacteria (Belicka et al. 2012). Consumption of heterotrophs along with consumption of autotrophs may allow herbivores to obtain adequate amounts of both P and fatty acids for growth and other life processes, respectively.

IV. Lipid allocation hypothesis

The lipid allocation hypothesis states that herbivory is adaptive because higher consumption of algae with high lipid concentrations may increase fitness. Algae are primary producers of essential lipids that cannot be synthesized by metazoans, but are necessary for their survival (Ahlgren et al. 1990, Sargent et al. 1995, Sharathchandra and Rajashekhar 2009, Guo et al. 2016). Although animal-prey are rich in lipids relative to algae, wild-caught herbivorous fishes have higher lipase activities in the gut than carnivores, suggesting that lipids are of major importance to herbivores (Nayak et al. 2003, German et al. 2004, Drewe et al. 2004).

Fatty acids can be incorporated into lipid bilayers of metazoan cells (phospholipids; Karasov and Martinez del rio 2007), can serve as precursors for important animal hormones (Brett and Muller-Navarra 1997), and can be stored as energy (Wiegand 1996) in aquatic consumers. Excess carbon that does not originate from fatty acids can also be stored as lipid reserves in primary consumers (e.g. *Daphnia*: Sterner and Hessen 1994, Gulati and DeMott 1997), emphasizing the importance of lipid storage.

In aquatic organisms, a primary role of lipids is energy storage for reproductive purposes, as they are the main components of ova (Brooks et al. 1997). During reproductive periods, lipid compounds are mobilized to the gonads in fish (Wiegand 1996, Guler et al. 2007, Wang et al. 2013) and increased dietary lipids (from 12%-18%) result in increased fecundity (e.g. Durray et al. 1994). Lipid ingestion from algal sources has also been shown to positively correlate with reproductive success in several aquatic organisms (*Daphnia*, copepods, fishes), and with clutch size in particular (e.g. Goulden et al. 1982, Tessier et al. 1983, Schmidt and Jonasdottier 1997, Weers and Gulati 1997, Martin-Cruezburg et al. 2008, Guo and Xie 2011). In addition, organisms consuming diets rich in phospholipids allocate dietary P to ova (e.g. copepods, Laspoumaderes et al. 2010), thereby contributing to offspring growth and survival. Dietary phospholipids are the main constituents of embryonic yolk (Wiegand 1996) and thus serve as both an energy source and component of structural growth in developing embryos (Bell 1989, Wiegand 1996). Furthermore, phospholipids are abundant in the membranes of neural tissues and are thus integral for growth of larvae, which have a high percentage of neural tissue relative to their body mass (Bell et al. 1997). Since lipids (and phospholipids) are important for storage, structure and reproduction of aquatic organisms, herbivory may be favored over ominivory and carnivory if essential lipids are obtained from available algal sources.

V. Disease avoidance hypothesis

The disease avoidance hypothesis maintains that herbivory is advantageous because it reduces disease transmission via animals.

Many secondary consumers such as piscivores are definitive hosts for parasites, with primary consumers (i.e. invertebrates or small vertebrates) serving as intermediate hosts (Covich et al. 1999, Marcogliese 2002). Furthermore, phylogenetic relatedness and similarity in biological traits between hosts has been shown to be a useful predictor of parasite prevalence in many taxa (see discussion in Huang et al. 2014). Specifically, carnivores that are phylogenetically and ecologically similar were shown to harbor similar parasite assemblages (Huang et al. 2014), suggesting that diet affects the probability of parasitic infection. Furthermore, a meta-analysis by Choudary and Dick (2000) showed that freshwater piscivorous fishes have rich parasite communities as compared to herbivores and zooplanktivores (Choudhury and Dick 2000; see Dogiel 1961 for examples). Although herbivores can contract a variety of parasites that do not originate from the diet (see Hoffman 1999 for a full review) and can experience negative effects as an intermediate host (e.g. Plaistow et al. 2001), herbivory may mediate the effects of animal-facilitated parasites and thus, energy allocation to maintenance mechanisms that respond to such parasites.

Alternatively, consuming animal prey may facilitate the transmission of prions, also referred to as transmissible spongiform encephalopathies. These infectious agents are composed of protein and are responsible for mad cow disease in mammals (Dalla Valle et al. 2008). Although prions are not as common in aquatic systems as they are in terrestrial systems, prions have been discovered in some fish species (e.g. Rivera-Milla et al. 2003, Dalla Valle et al. 2008).

Animal tissues are built from proteins that are potentially harmed by these agents, thereby posing a significant threat to aquatic food webs. Because basal items are not protein-rich resources (Mattson 1980, Sterner and Elser 2002), herbivores may benefit from reduced exposure to infectious prions that could alter the functioning proteins comprising their somatic tissues.

Discussion

The presence of both ancestral (carnivory) and derived (herbivory and omnivory) diets in nature indicates that there are conditions that favor eating plants over animals. In support of the adaptive hypotheses presented here, the literature suggests that herbivory is favored when higher quality food is limiting. But, freshwater herbivore diets are not always inadequate as they can provide a different suite of important dietary elements (e.g. plant-derived lipids and sterols) that are deficient in carnivorous diets. Furthermore, these dietary elements are incorporated into both somatic and reproductive tissues and therefore may be related to fitness. Diet supplementation with heterotrophs also promotes growth and reproduction of freshwater herbivores. Testing these hypotheses will allow researchers to understand the circumstances that promote herbivory over nutritionally "better" diets.

With a few assumptions (Table 2.2), these hypotheses could be evaluated in current herbivory research programs. For example, the intake-efficiency hypotheses might be tested using a similar experimental design to Alvarez and Peckarsky's (2013). They measured growth rates of two grazers (caddisfly and mayfly), algal accrual rates and per capita effects of grazers on algae in chambers that differed in the presence of moss (submerged vegetation) and predation risk.

They found no differences in growth; however, when mayflies were exposed to predators, algae associated with moss accrued at a slower rate, suggesting that mayflies were using moss as both habitat and a source of food in the presence of predators. Comparable experiments could be designed to include additional life history trait estimates (e.g. herbivore survival) and estimates of energy expenditure versus energy gain (as in optimal foraging theory) of animals eating herbivorous versus carnivorous diets. See Table 2.3 for more examples.

I present a series of hypotheses with independent explanations for each; however, these mechanisms are unlikely to function independently in nature and our knowledge of diet evolution may be limited by approaching them as such. Factorial designs evaluating multiple hypotheses and their interactions simultaneously may be more biologically relevant. For example, the heterotroph facilitation hypothesis may be tested using a design that examines the effects of diets composed of various heterotrophic: autotrophic ratios on consumer life histories (e.g. Fuller et al. 2004). Heterotrophs and autotrophs have unique lipid profiles that can be traced to consumer somatic and reproductive tissues (Iverson et al. 2004, Belicka et al. 2012). Therefore, the results from this experiment may also be explained in reference to the lipid allocation hypothesis, where consumer reproduction is affected by differential concentrations (and sources) of essential lipids in the diet. In another example, the suboptimal habitat hypothesis could be invoked in a system with high food availability and low food quality. This could be the case for *Terapontid* fishes, where availability of resources is hypothesized to be the driving force for their transition from marine to freshwater (e.g. a "suboptimal habitat") and subsequent diet shift from carnivory to herbivory (Davis et al. 2012).

This hypothesis may explain *Terapontid* invasion and shift to herbivory, but any of the remaining four hypotheses (or others not proposed here) could further explain why herbivory was maintained and continues to exist in this group. Testing these as alternative hypotheses rather than single, independent ideas may improve our interpretation.

I explained these ideas using the freshwater herbivory literature, but testing these hypotheses in other systems would complement the existing works that draw comparisons between aquatic (freshwater and marine) and terrestrial herbivory. Recent terrestrial studies have begun to elucidate the evolutionary origins of herbivory and have found similar patterns of diet evolution to those in freshwaters. For example, Reisz and Frobisch (2014) found fossil evidence supporting the evolution of herbivorous Caseid reptiles from smaller carnivore lineages and suggested that herbivory began as a way to exploit untapped resources (e.g. suboptimal habitat hypothesis). Although relative patterns of herbivory are different between terrestrial and freshwater systems (e.g. Cyr and Pace 1993, Cebrian and Lartigue 2004; Burkepile 2013), invoking comparable mechanisms for the adaptive evolution of herbivory could imply similar patterns of diet evolution across ecosystems, thereby unifying these independent bodies of work.

Herbivory has been the focus of many ecological studies spanning many sub-disciplines, but there is a significant gap in knowledge pertaining to the adaptive evolution of herbivory in nature. With backgrounds in both theoretical and experimental ecology, the incorporation of these hypotheses to the current literature will provide information about diet evolution, where it is currently lacking. The proposed hypotheses represent a starting point that may lead to more comprehensive studies of diet evolution in freshwater and other systems.

Exploring these already established ideas from an adaptive perspective will establish a much-needed research framework, allowing us to more fully understand the evolution of diet in freshwater and other systems.

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Table 2.1. Description of the proposed hypotheses for the adaptive evolution of herbivory in freshwaters.

Name	Hypothesis	References
I. Intake-efficiency	Aquatic herbivores may use all or part of their food source as habitat. Herbivory may allow an organism to maximize intake energy by minimizing the time spent searching for food, energy consumed during prey capture, and energy costs avoiding predators.	Brönmark and Vermaat 1998
II. Suboptimal habitat	Herbivory may allow organisms to invade suboptimal or recently disturbed habitats. Such habitats are often characterized by having high primary production relative to consumer biomass.	e.g. Proulx and Mazumder 1998
III. Heterotroph facilitation	Herbivory may be adaptive because herbivores supplement their diets by indirectly consuming heterotrophic microbes that are associated with algae. These heterotrophs can provide nutrients that are not attainable by eating algae alone.	e.g. Martin-Creuzberg et al. 2011
IV. Lipid allocation	Some freshwater algae are sources of essential lipids and herbivorous organisms consume large quantities of these lipids relative to animal-consuming species. Because aquatic organisms use lipids for energy storage and reproduction consuming a diet rich in fatty acids may result in greater reproductive allocation. Herbivory may be adaptive because higher lipid consumption leads to higher reproductive allocation and thus, increased fitness.	Brett and Muller- Navarra 1997, Karasov and Martinez del rio 2007, Sharathchandra and Rajashekhar 2009
V. Disease avoidance	Animal prey may serve as intermediate hosts and facilitate transmission of parasites or prions through the diet. Herbivory may be adaptive because it reduces animal-facilitated disease transmission.	Covich et al.1999, Marcogliese 2002

Table 2.2. Assumptions of proposed hypotheses. Testing these hypotheses may be best accomplished by evaluating the assumptions necessary for them to be viable explanations for adaptive evolution of herbivory.

Hypothesis	Assumptions
I. Intake-efficiency	Freshwater herbivores are relatively small and require refuge from predators, usually in the form of submerged aquatic vegetation. Submerged aquatic vegetation is associated with more palatable plants like algae that are consumed by herbivores.
II. Suboptimal habitat	Herbivores are able to detect food availability and/or quality in the current habitat and make dispersal decisions accordingly.
III. Heterotroph facilitation	Heterotrophic microbes (heterotrophic bacteria, protoza, etc.) are in close association with freshwater primary producers and herbivores consume them indirectly.
IV. Lipid allocation	At least some essential lipids come from freshwater primary producers.
V. Disease avoidance	Parasites and prion diseases are only transmitted via animal vectors.

Table 2.3. Examples of experimental designs that could be used as tests of the posed hypotheses.

Hypothesis	Example
I. Intake-efficiency	See text (Alvarez and Peckarsky 2013).
II. Suboptimal habitat	Jiang and Morin (2004) constructed microcosms with a productivity gradient and subjected plankton communities to invading species. Invaders and resident species increased their abundances with resource enrichment. This hypothesis could be tested by replacing herbivores as "residents" and carnivores as "invaders" and measuring herbivore and carnivore abundances as a function of increasing productivity and/or disturbance levels.
III. Heterotroph facilitation	Jäger et al. (2014) examined the interactions between *Daphnia spp.*, phytoplankton and bacteria using three algal species compositions. *Daphnia* grew to high densities with a mixed diet and high light conditions. Similar field or lab feeding experiments could be designed by manipulating the autotrophic: heterotrophic ratio of the herbivorous diet and measuring life history effects relative to those resulting from a carnivorous diet. A norm-of-reaction may be used to assess the conditions where a mixed autotrophic and heterotrophic diet is equal to or better than a carnivorous diet (in terms of fitness).
IV. Lipid allocation	Wacker and Martin-Cruezburg (2007) fed *Daphnia spp.* either algae with high lipid content or algae with low lipid content and measured lipid allocation to somatic and reproductive tissue. They found that essential lipids were preferentially allocated to offspring when provided foods with high lipid content. Gergs et al. (2014) measured growth and survival of amphipods fed diets with or without essential lipid supplementation and found that both were positively affected by the addition of lipids. Comparable feeding experiments should be conducted with these and other herbivores using natural dietary items. Life history effects of non-herbivorous diets that vary in lipids should also be assessed. Identifying the source of lipids allocated to somatic and reproductive tissues will provide further support for the lipid allocation hypothesis.
V. Disease avoidance	Huang et al. (2014) examined factors that influence parasite sharing between carnivore hosts using a large data set on reported parasites and previously published phylogenies. They found that viruses and helminths infect phylogenetically related carnivores more than expected by chance. Similar comparative analyses could be implemented to determine patterns of parasite and prion infection across diet types.

CHAPTER 3

FRESHWATER-TO-MARINE TRANSITIONS MAY EXPLAIN THE EVOLUTION OF HERBIVORY IN THE SUBGENUS *MOLLIENESIA* (GENUS *POECILIA*)

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Abstract

The ability of organisms to cross ecosystem boundaries is an important catalyst of evolutionary diversification. The genus *Poecilia* is an excellent model system for studying ecosystem transitions because species display a wide range of salinity affiliations. Furthermore, *Poecilia* species exhibit a variety of diet preferences, with herbivory concentrated in the subgenus *Mollienesia*. It has been suggested that herbivory may be an adaptive strategy to allow organisms to invade habitats with decreased resource quality, also known as the Suboptimal Habitat Hypothesis. I evaluated this hypothesis by reconstructing ancestral states of habitat and diet across a phylogeny of the genus *Poecilia* to identify patterns of diet evolution and habitat transition, and then used phylogenetically independent contrasts to identify patterns of diet evolution in response to habitat transition.

I found that the subgenus *Mollienesia* had freshwater or euryhaline roots and crossed ecosystem boundaries at least once following the divergence of the three recognized species complexes (*P. mexicana, P. sphenops* and *P. latipinna*). Increased salinity affiliation explained 26% of the decrease in animal material in the gut, and jaw morphology was associated with percent animal material in the gut, but not with percent of species occupying saline habitats. These findings suggest that in the genus *Poecilia*, herbivory evolved in response to transitions from fresh to euryhaline habitats, and jaw morphology evolved in response to the appearance of herbivory. These results support herbivory as an adaptation for invading less productive saline habitats, which is consistent with the Suboptimal Habitat Hypothesis.

Keywords: Diet evolution, herbivory, *Poecilia,* habitat transition, freshwater habitat, marine habitat, adaptive evolution, phylogeny, ancestral state reconstruction, *Mollienesia*

Introduction

The ability of organisms to cross habitat and ecosystem boundaries and invade new space is an important driver of evolutionary diversification. Habitat shifts by organisms may provide new foraging opportunities with little competition and decreased predation threats for organisms (Betancur-R et al. 2012). In addition, invading a new habitat can have significant evolutionary consequences for the invading species by enhancing the possibility for novel phenotypes to spread. These novel phenotypes can promote new ecological interactions between species, ultimately resulting in species radiation (Lee 1999; Betancur-R et al. 2012; Davis et al. 2012). However, there are physiological costs associated incurred by organisms that transition between habitats, because the ability of an organism to transition requires a suite of specialized adaptations suited for the new environment (Vermeij and Dudley 2000; Betancur-R 2009). Many metazoans are derived from ancestors that have crossed ecosystem boundaries, suggesting that the relative costs of transitioning can be outweighed by the ecological opportunities afforded to those with the ability to do so.

In aquatic systems, the interface between marine and freshwater habitats represents a boundary that creates a physiological challenge for potential invaders (Lee 1999). As a result, approximately half of marine animal phyla have failed to colonize freshwater habitats (Betancur-R 2009).

Annelids, crustaceans, mollusks and fish are among those that were able to make the marine-to-freshwater transition (e.g. Lee and Bell 1999; Lovejoy et al. 2001; Lovejoy et al. 2006; Augusto et al. 2009; Betancur-R 2009; Yamanou et al. 2011). Following their incursion from marine waters, these groups experienced rapid radiation resulting in high diversification in the freshwater clades relative to their marine counterparts (Bloom et al. 2013). For example, fish from the family Terapontidae originated in marine habitats, but after a single marine-to-freshwater transition, 40 out of 54 extant species are restricted to freshwaters (Davis et al. 2012). In addition, the freshwater Terapontids experienced three times faster diversification than the marine clade, accompanied by a shift from a carnivorous diet in marine habitats to an herbivorous diet in freshwaters (Davis et al. 2012).

While marine-to-freshwater transitions are relatively common in fishes (Betancur-R 2009), colonization of marine habitats by freshwater organisms, or reinvasion of freshwater by secondary marine clades, have occurred less frequently (McDowall 1997; Vermeij 2000; Betancur-R 2009). In addition, diversification of marine fishes tends to be slower than diversification of freshwater fishes, likely because of the heterogeneity offered by freshwater habitats (Bloom et al. 2013). Despite these slower rates of colonization and diversification, several clades have moved into marine habitats from fresh waters (e.g., catfish, Ferraris 2002, Sullivan et al. 2006). One of these families, *Poeciliidae* (Cyprinodontiformes), has evolved the ability to disperse across marine water barriers, and extant species inhabit both fresh and euryhaline habitat types (Meffe and Snelson 1989).

As a result, this group has undergone a significant evolutionary radiation (Hrbek et al. 2007), resulting in a multitude of endemic taxa (Palacios et al. 2016). One genus, *Poecilia*, is an excellent model system for studying transitions across ecosystem boundaries, because it consists of species with limited ranges and species with wide, overlapping distributions (Palacios et al. 2016). In addition, *Poecilia* species occupy several continents, informing phylogeographic analyses that have provided insights into the historical processes that shaped distribution patterns of this group (Palacios et al. 2016).

Phylogeographic analyses of the genus *Poecilia* have suggested that it originated in South America and dispersed to the Greater Antilles via the Aves land bridge (Hrbek et al. 2007; Palacios et al. 2016; Reznick et al. 2017). Through migration and vicariance events, *Poecilia* species also dispersed into Middle America (Central and North America) approximately 2-7 mya, where they underwent significant evolutionary radiation (Hrbek et al. 2007; Ho et al. 2016). Extant species inhabit these continents, but they have experienced divergence that is linked to their biogeography (Ho et al. 2016). Uncovering these biogeographical patterns (e.g., Alda et al. 2013; Ho et al. 2016; Palacios et al. 2016) has allowed researchers to improve previously unresolved phylogenies of the genus *Poecilia* (e.g., Ptacek and Breden 1998; Breden 1999; Mateos 2005; Hrbek 2007; Meredith 2010), but no studies have used these phylogenetic relationships to trace characters related to habitat or diet of *Poecilia* species.

Although all members of the genus have some capacity to survive in both fresh and euryhaline waters, some species thrive in freshwater habitats, whereas others fare better in brackish and/or marine habitats (Meffe and Snelson 1989). In addition, all *Poecilia* species exhibit some degree of herbivory, however, I hypothesize that obligate herbivory is concentrated in the subgenus *Mollienesia* (pers. obs.). Most of the species comprising this subgenus inhabit Middle America and occupy both fresh and euryhaline habitats (Ho et al. 2016). I hypothesize that dispersal of the subgenus *Mollienesia* into Middle America resulted in habitat transitions across the freshwater-marine barrier (e.g., David et al. 2012) that potentially drove the evolution of herbivory in this group.

Herbivory is generally thought to be an inefficient feeding strategy relative to omnivory and carnivory (see Sanchez and Trexler 2016 for a review). However, many herbivorous metazoans have evolved from carnivorous/omnivorous ancestors, so there is some adaptive value associated with eating plants (Sanchez and Trexler 2016). It has been suggested that herbivory is an adaptive strategy to allow organisms to invade habitats with decreased resource quality, to escape the negative effects of competition and/or predation (i.e., 'Suboptimal Habitat Hypothesis', Sanchez and Trexler 2016). Although marine systems cover 99% of the Earth's surface, these habitats are less productive per unit area than freshwater aquatic habitats (e.g., Colinvaux 1980; May and Godfrey 1994; Vermeij and Grosberg 2010) and could therefore be considered 'suboptimal' under the Suboptimal Habitat Hypothesis. As such, transitions from freshwater to less productive marine waters may have prompted the evolution of the herbivorous strategy in the *Poecilia* group, particularly in the *Mollienesia* clade.

My objective for this study is to reconstruct ancestral states of habitat and diet across a phylogeny of the genus *Poecilia* to identify patterns of diet evolution and habitat transition from freshwater to euryhaline systems (or vice versa) in the subgenus *Mollienesia*. This information will allow us to evaluate the Suboptimal Habitat Hypothesis by determining if habitat affiliations explain patterns of diet evolution.

Methods

Taxon Sampling

There are 44 documented species in the genus *Poecilia*, spread across 7 subgenera (Poeser et al. 2005; Ho et al. 2016): *Acanthophacelus*, *Poecilia* (subgenus), *Micropoecilia*, *Curtipenis*, *Psychropoecilia*, *Allopoecilia*, and *Mollienesia*. Currently, the most complete phylogenies of the genus *Poecilia* are based on gene sequences from 11-19 distinct species (Alda et al. 2013; Ho et al. 2016; Palacios et al. 2016), sampled from 1-5 of the described subgenera. Previous studies sampled between 1-14 *Poecilia* species, belonging to 1-3 subgenera, but resulted in unresolved phylogenetic relationships (Ptacek and Breden 1998; Breden 1999; Mateos 2005; Hrbek 2007; Meredith 2010). In this study, I sampled 36 distinct *Poecilia* species with at least one representative from all 7 of the described subgenera, as well as 2 species from the sister genus *Limia* to construct an updated topology. I chose *P. reticulata* as an outgroup taxon. Although this species is in the genus *Poecilia*, it has been shown to be a reliable outgroup taxon in previous studies focusing on the subgenus *Mollienesia* (e.g. Ptacek and Breden 1998), as well as the genus *Poecilia* (Alda et al. 2013; Ho et al. 2016). This sampling represents the highest number of representative species collected across all *Poecilia* subgenera to date.

I collected diet and habitat data (see methodology below) from a subsample of the collection, represented by 15 *Poecilia* species spread across 6 sampled subgenera (excluding *Curtipenis*). These were: *P. butleri*, *P. orri*, *P. mexicana*, *P. sphenops*, *P. gilli*, *P. caucana*, *P. hispaniolana*, *P. dominicensis*, *P. vivipara*, *P. latipinna*, *P. kyesis*, *P. velifera*, *P. picta*, *P. parae*, and *P. reticulata* (Table 3.1). Of these, 8 were representatives of the *Mollienesia* subgenus (*P. butleri*, *P. orri*, *P. mexicana*, *P. sphenops*, *P. gilli*, *P. latipinna*, *P. kyesis*, and *P. velifera*) and represent individuals from the three recognized *Mollienesia* complexes (*P. mexicana*, *P. latipinna*, and *P. sphenops*) listed in Ho et al. (2016).

Phylogenetic Analyses

Previous *Poecilia* phylogenies were constructed using several mitochondrial genes and one ribosomal gene (Alda et al. 2013; Ho et al. 2016; Palacios et al. 2016): 5' prime region of the cytochrome oxidase subunit I (*COI*; mtDNA), *ATPase* 8/6 (mtDNA), NADH dehydrogenase subunit 2 (*ND2*; mtDNA), and the nuclear S7-like ribosomal protein (*S7*). These previous topologies did not include all available *Poecilia* species sequences, as well as a few of the sub-sampled species (*P. velifera*, *P. dominicensis*, *P. parae*, and *P. picta*). To compare diet and habitat characteristics, it was necessary to create an updated tree that included all of the sampled species. I retrieved sequences (36 *Poecilia* species + 2 *Limia* species) for the same suite of genes used in previous works, as they were reliable at resolving phylogenetic relationships at both the genus (e.g., Alda et al. 2013; Ho et al. 2016) and subgenus (e.g., Palacios et al. 2016) level.

These sequences were obtained from data deposited in Dryad by the previous authors (Alda et al. 2013 and Ho. et al. 2016) and were supplemented with additional sequences not included in these previous works using GenBank (see Table S.3.1 for accession numbers and sample IDs). I assembled the sequences using MEGA 7 (Kumar et al. 2015). Pseudogenes were investigated by: 1) translating nucleotides to amino acids; 2) examining the sequences for stop codons; 3) and searching for insertions/deletions (mitochondrial and ribosomal genes). The sequences were aligned using the Muscle option in MEGA 7 and concatenated (*COI*+ *ATPase 8/6* + *ND2* + *S7*) using Sequence Matrix (Vaidya et al. 2011). I removed the first base of the *COI* sequences to set them in reading frame 1 (651bp) and split the *ATPase 8/6* sequences into the partial *ATPase 8* (158bp) segment and complete *ATPase 6* (684bp) sequence. We used PartitionFinder v2.1.1 (Lanfear et al. 2012) to identify the best partitioning scheme and models of evolution that fit the data. I used Bayesian Information Criterion (BIC) to evaluate the best-fit scheme and model with the greedy search algorithm, linked branch lengths, and models restricted to those that can be used in MrBayes. I repeated these methods to obtain the best-fit scheme for a second dataset comprised of the subsampled sequences (15 *Poecilia* species). All replicate sequences were included in the pruned tree except *P. mexicana*, *P. sphenops* and *P. reticulata*. For these species, I only included individuals that were sampled in the same country as the specimens I used to collect dietary data.

I used MrBayes v3.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) to create a Bayesian Inference (BI) phylogeny using the partitions and models specified in PartitionFinder for the concatenated datasets (all sequences and sub-sampled sequences). I constructed an analysis with uninformed priors, that ran for 1 x 10^6 generations, on four Markov chains. Trees were sampled every 100 generations. I performed three separate runs, each running 2 replicate runs. Following methods of Ho et al. (2016), I evaluated convergence of parameters using Tracer v1.6 (Rambaut et al. 2014) for each replicate and combined run, and found that all values for effective sample size were >200. Pairwise convergence of resulting tree topologies was evaluated using the RWTY package (Warren et al. 2017) in R v3.4.1 (R core team 2017), using a 25% burn-in. In addition, I visually verified that the 50% majority-rule consensus trees for the three separate runs had matching topologies with minor deviations in branch lengths. I randomly selected one of the independent runs and constructed a consensus tree, computed Bayesian posterior probabilities, and visualized the topologies using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Habitat Characterization

Because *Poecilia* species can survive in most both fresh and salt waters, they show marked intraspecific variation in habitats they occupy. However, the rate of occurrence of individual species in fresh, brackish and marine habitats varies among species, revealing subtle differences in species-specific habitat preferences (Meffe and Snelson 1989). I used the *Fishnet2* data base to estimate interspecific habitat preferences.

For each of the subsampled species, I performed a *Fishnet2* search using the species name and collected habitat information on the first 25 independent hits with logged lat/long coordinates. Using the field collection notes provided by *Fishnet2*, Google Earth searches, and accompanying geographical information, I determined if each sample was collected from a freshwater, brackish or marine site. I then calculated the proportion of samples collected from each habitat type for all species (Table 3.1). I verified the predicted habitat associations with data reported in the literature for well-studied species (e.g., *P. reticulata, P. mexicana*, *P. latipinna*; Trexler and Travis 1990, Nordlie et al. 1992, Bussing 1998, Miller 2005), but these classifications are approximate and do not take into account seasonal or climatic changes in salinity, migration/dispersal events to or from different habitat types, or effective population sizes at each site. I made the assumption that if a species was able to be collected at a site, it has established there.

Diet Characterization

Subsampled species were obtained from Florida Museum of Natural History (retrieved from the *Fishnet2* data base, http://www.fishnet2.net/), University of Michigan Museum of Zoology (*Fishnet2*), El Colegio de la Frontera Sur (ECOSUR) Ichthyology Collection (donations made to the authors), and collections made by the authors (Table 3.2). I used the most recent naming convention for *P. kykesis*, so the *Fishnet2* search was performed using the former species name, *P. petenensis* (Poeser 2002). Individuals of each species were sampled from 2 distinct populations (i.e., no gene flow likely) within their native range using methods that do not interfere with diet characterization (e.g., by seining or cast nets, but not minnow traps).

They were fixed in formalin and preserved in 70% Ethanol following capture. An analysis of several Poeciliid species found that jaw morphologies varied among genera with different diet habits, with more herbivorous species displaying a larger degree of intramandibular bending (IMB), larger gape angles (GA), and a large degree of neurocranial rotation (NCR; Gibb et al. 2008; Hernandez et al. 2008 & 2009). I measured these jaw angles to the nearest 0.01 mm standard length and placed them under a dissecting scope with an attached digital camera. Using ImageJ software, I measured the vertex of a line along the ventral margin of the dentary bone that forms the lower jaw, and a second line along the ventral margin of the angular-articular bone complex. I then subtracted the measured angle from 180° to obtain the degree of IMB. For GA, I measured the vertex of a line along the anterior-ventral margin of the upper jaw and a line along the anterior-dorsal margin of the lower jaw. Finally, I measured NCR by measuring the angle between a vertical line posterior to the eye, and a line along the top of the skull above the eye (modified methods of Gibb et al. 2008). I compared these measurements among and within species using Analysis of Variance (ANOVA) and Tukey's Post-hoc tests.

Following jaw measurements, I assessed gut contents and morphology for each of the sub-sampled species. I was unable to dissect any specimens of *P. parae,* or specimens of *P. butleri* from a second locality due to museum limitations; therefore, only jaw measurements were obtained for these individuals. I dissected all other fish to remove the gut tract. Once the tract was removed, I weighed it to the nearest 0.001g, stretched it out onto a petri dish lined with grid paper (6.35mm/grid) and recorded the length.

To standardize the length for comparison among species, I divided the length of the gut in mm by standard body length (mm). I compared standardized gut lengths among species using ANOVA followed by a Tukey test.

I removed a subsample from each gut (from the esophagus to the first bend of the gut tract) and weighed it to the nearest 0.0001g. I extracted the contents of the subsample onto a tared microscope slide using the blunt end of a razor blade. I then added a drop of DI water to each slide, mounted them with a coverslip, and sealed them using clear nail polish. I examined slides using a light microscope at 40x magnification and counted and identified all organisms (to genus) in 10 random fields of view (counted area = 2.37 mm). I grouped the organisms found in the guts by trophic group (diatoms, green algae, cyanobacteria, metazoans) and calculated relative abundance of each group for each fish species at both sampled localities. I did not quantify detritus and assumed that it marginally contributed to the diet, although this is probably not the case for *Poecilia* species (e.g., Sanchez and Trexler 2018). I used a hierarchical clustering procedure using the Sorensen (Bray-Curtis) distance measure (CLUSTER package in R; Maechler et al. 2017) to classify gut contents into diet categories (e.g., carnivore, omnivore, herbivore). All individuals of the same species (collected from different localities) clustered together, suggesting that intraspecific variation in gut contents was less than interspecific variation in gut contents. As such, I performed the clustering procedure again using the average gut content values for each species.

I used the morphological data (IMB, GA, NCR and standardized gut length) and gut content estimations to determine if these diet characters are potential adaptations for the herbivorous diet in *Poecilia* species. For simplicity, I converted gut content data into percent animal material in the gut. I then generated phylogenetically independent contrasts (PIC) between percent animal material in the gut and each morphological character with the ape package in R (Paradis et al. 2004) using branch lengths from the pruned topology (containing only subsampled species). Contrasts were used in linear-regression analyses, where the regression was forced through the origin (Felsenstein 1985). Any characters that significantly correlated ($p < 0.05$) with percent animal material in the gut were assumed to have evolved as an adaptation to herbivory. These were used as characters in ancestral state reconstruction.

Tracing the Evolution of Habitat and Diet

I used ancestral state reconstruction to trace the dietary habits and salinity affiliations of ancestral *Poecilia* species. First, I coded diet categories estimated from Hierarchical Cluster Analysis as categorical traits (0-5, and '?' for *P. parae*). Similarly, I coded the proportion of samples collected from each habitat type (estimated from *Fishnet2*) as categorical traits (0-2), where 0 = species with 100% of samples collected in freshwaters ("Low salinity affiliation"), 1= species with samples collected in both freshwater and brackish waters ("Medium salinity affiliation"), and 2= species with samples collected in fresh, brackish and marine habitats ("High salinity affiliation"). I created character matrices from these coded diet and salinity characters.

In addition, I created character matrices from the morphological characters (IMB, GA, NCR, standardized gut length) that had significant relationships with percent animal material in the gut (contrasts).

I uploaded the pruned consensus tree (subsampled species only) and character matrices into MESQUITE v3.2 (Maddison and Maddison 2017) and ran the "trace character" analysis using maximum parsimony (MP) and maximum likelihood (ML) methods for salinity affiliation and diet category. I was only able to run MP analyses for the morphology characters because these are continuous data and ML can only analyze categorical data. Parsimony ancestral state reconstruction minimizes the amount of character change over the tree topology based on the character state distribution and has thus been criticized for underestimating rates of evolutionary change (Cunningham et al. 1998; Royer-Carenzi et al. 2013). Maximum likelihood makes use of branch lengths and possible rates of character evolution to find the ancestral state that maximizes the probability that the observed character state (i.e., diet or salinity affiliation) would evolve under a stochastic model of evolution (Schluter et al. 1997). In this study, I used the symmetrical Mk1 model, which assumes equal forward and backward character transition rates (Lewis 2001). Because there has been some debate between using maximum parsimony (MP) and maximum likelihood (ML) methods, and because I was limited to more conservative MP methods for a subset of the data, I present the resulting reconstructions from both methods. The reconstructed states were plotted with the "balls and sticks" model, with ancestral states marked at each node.

Identifying Patterns of Diet Evolution in Response to Habitat Transitions

I used phylogenetic independent contrasts (derived from the pruned tree) to compare diet and habitat affiliations across the genus *Poecilia*. Because this method can only be performed on continuous data, I generated contrasts from the percent of samples collected from euryhaline habitats (brackish + marine; *Fishnet2* data) as a metric for salinity affiliation. I then used contrasts for salinity affiliation and all characters related to diet (% animal material in gut and the 4 measured morphological characters) in linear-regression analyses to identify the relationships between salinity affiliation, herbivory and the morphological adaptations related to herbivory.

Results

Phylogenetic Analyses

Full-Phylogeny (37 Poecilia species). I partitioned the dataset by genes and by codons for the mtDNA (*COI, ATPase 8/6, ND2*) genes. PartitionFinder identified four subsets of partitions (out of 13) for the complete *Poecilia* dataset (36 *Poecilia* species + 2 *Limia* species). Their estimated models of DNA substitution were as follows: 1) GTR + I + G for *COI* codon position 1, positions 2 and 3 of *ATPase 8*, *ATPase 6*, and *ND2*, 2) K80 + G for *COI* position 2 and complete *S7*, 3) F81 for *COI* codon position 3, and 4) HKY + G for position 1 of *ATPase 8/6* and *ND2*. I specified these data partitions and best-fit models of DNA substitution in subsequent phylogenetic analyses.

The Bayesian phylogenetic analysis derived from the concatenated mitochondrial *COI*, *ATPase 8/6*, *ND2*, and the ribosomal *S7* genes from 36 *Poecilia* species (and 2 *Limia* species) resulted in a well-supported consensus tree, with the exception of the node linking the subgenera *Poecilia* and *Micropoecilia* (85 % Posterior probability, PP). Furthermore, these subgenera grouped together as an unresolved polytomy, which is not a supported relationship in previous studies. The low nodal support and polytomy likely resulted from missing sequence data for individuals of the subgenus *Micropoecilia*, as only *ND2* sequences were available for these species.

Although the analyses resulted in a tree with high resolution, I found that *P. mexicana* species are not monophyletic as suggested by Ho et al. (2016). Their topology placed *P. salvatoris* and several *P. mexicana* morphs (Clades I-VI, yellow, and red morphs) in a monophyletic group.

In this study, Bayesian analysis placed *P. salvatoris*, *P. maylandi*, *P. limantouri*, *P. sulphuraria* and *P. thermalis* with *P. mexicana* species, resulting in paraphyly. Although monophyly was not supported, the position of these species within the *P. mexicana* complex is supported in this tree. The exception is *P. maylandi*, which is hypothesized to belong to the *P. sphenops* complex (Ho et al. 2016). Because no phylogenetic work has included *P. maylandi*, I am unable to conclude if this species is in fact part of the *P. mexicana* complex instead of the *P. sphenops* complex, or if missing data and/or misidentification of the voucher specimen has resulted in the incorrect assignment of this species.

Furthermore, *P. wandae* (sequences obtained from Ho et al. 2016) was included in the subgenus *Mollienesia*, although this species has been classified as belonging to the subgenus *Allopoecilia*. Correspondence with Ho et al. (2016) suggests that these vouchers were possibly misidentified and could be *P. koper*i, although this claim was never verified. All other deep nodes were highly supported (PP $\geq$ 90%) and congruent to those revealed in previous studies (Fig. 3.1).

Subsampled Phylogeny (15 Poecilia species). Similar to the full-phylogeny, I partitioned the dataset by genes and by codons for the mtDNA (*COI, ATPase 8/6, ND2*) genes. PartitionFinder identified four subsets of partitions (out of 13) for the subsampled *Poecilia* dataset (15 species). Their corresponding models of evolution were: 1) GTR + G for *COI* position 1 and position 3 of *ATPase 8/6* and *ND2*, 2) K80 + G for position 2 of *COI* and *ATPase 8*, and for complete *S7*, 3) HKY + I for *COI* codon position 3 and for position 2 of *ATPase 6* and *ND2*, and 4) HKY + G for codon position 1 of *ATPase 8/6* and of *ND2*. The phylogenetic analysis of the subsampled *Poecilia* species resulted in a well-supported consensus tree, with few nodes of low support. Sepcifically, the node linking species of the subgenus *Micropoecilia* (72% PP) and the node linking the subgenus *Poecilia* to the other subgenera (73% PP) had low support, likely due to missing sequence data (see previous section). However, unlike the full-phylogeny, the pruned tree placed *P. vivipara* (subgenus *Poecilia*) in a different clade than *P. parae* and *P. picta* (subgenus *Micropoecilia*), a relationship that is congruent with previous studies (e.g., Palacios et al. 2016). Unlike the full-phylogeny, I found that *P. mexicana* species formed a monophyletic clade with two sub-specific groups (100% PP).

The entire *P. mexicana* complex was comprised of three sub-groups: 1) *P. mexicana* species (including species listed above); 2) *P. orri* and *P.gilli*; 3) and *P. butleri*. This relationship, and all others were highly supported (PP $\geq$ 90%) and congruent to those revealed in previous studies (Fig. 3.2).

Habitat Characterization

Of the sub-sampled species, 7 were classified as having a low salinity affiliation based on the proportion of habitats they were collected from (data retrieved from *Fishnet2*). These are: *P. hispaniolana, P. parae, P. dominicensis, P. sphenops, P. caucana,* and *P. reticulata*. High salinity affiliation species are *P. mexicana, P. vivipara, P. velifera, P. butleri, P. picta* and *P. orri.* The species that emerged as having a medium salinity affiliation were *P. gilli, P. latipinna* and *P. kykesis* (Table 3.1).

Diet Characterization

I found differences in jaw and gut morphology among the sub-sampled species. Specifically, *P. reticulata* had the largest angles of neurocranial rotation, which was 75% more than the species with the smallest angles, *P. velifera* ($F_{15,587} = 23.314$, $p < 0.0001$). Intramandibular bending was greatest in *P. mexicana*, where the degree of IMB was 13% greater than *P. reticulata*, the species with the smallest IMB angle ($F_{15,587} = 32.109$, $p < 0.0001$). Gape angles showed a 53% difference between the species with the largest gape (*P. sphenops*) and the smallest gape (*P. picta*; $F_{15,559} = 3.658$, $p < 0.0001$). There were intraspecific differences in all 3 jaw measurements for *P. vivipara* where the Rio de Janiero population had 38% greater neurocranial rotation and 24% greater gape angles (NCR: $F_{1,49} = 30.824$, $p < 0.0001$; GA: $F_{1,49} = 13.325$, $p = 0.001$).

However, the Bahia population had 9% greater IMB ($F_{1,49} = 6.105$, $p = 0.017$). All other species did not differ in intraspecific jaw measurements. *Poecilia sphenops* had the longest standardized gut length, which was 43% longer than *P. reticulata*, the outgroup species, ($F_{14,391} = 13.787$, $p < 0.0001$; Table 3.3).

The hierarchical cluster analysis of gut content data produced six broad feeding categories (coded 0-5 in ancestral state reconstructions): Carnivore (≥ 50% animals), 2 omnivore categories ('cyanobacteria + animals', and 'cyanobacteria + diatoms + animals'), and 3 herbivore categories ('green algae', 'cyanobacteria', and 'cyanobacteria + diatoms'). Based on these groupings, *P. reticulata* (outgroup) were classified as carnivores, and *P. picta* and *P. caucana* were classified as omnivores ('cyanobacteria + animals' and 'cyanobacteria + diatoms + animals', respectively). All other *Poecilia* species were grouped as herbivores, although the plant items present in their gut varied (Fig. 3.3). Relative abundance of each gut item can be found in Table S.3.2 in the supplementary material.

Contrasts on jaw morphology characters (IMB, GA) and percent animal material in the gut were phylogenetically informative. Specifically, intramandibular bending and gape angles showed inverse relationships with percent animal material in the gut, irrespective of phylogenetic relationship among species (IMB: $y = -0.608x$, $r^2 = 0.38$, $p < 0.0001$; GA: $y = -0.312x$, $r^2 = 0.21$, $p < 0.0001$; Fig. 3.4a & b). Neurocranial rotation angles and standardized gut lengths were not driven by percent animal material in the diet once the phylogenetic relationships were accounted for (NCR: $y = -0.105x$, $r^2 = -0.015$, $p = 0.670$; Gut length: $y = -2.16x$, $r^2 = 0.08$, $p = 0.003$).

Tracing the Evolution of Habitat and Diet

Ancestral state reconstructions estimating habitat varied between the methods used. Specifically, MP analyses suggest that the most recent common ancestor (MRCA) of subgenera *Acanthophacelus, Micropoecilia, Psychropoecilia, Allopoecilia* and *Mollienesia* inhabited freshwater habitats, whereas the MRCA of the subgenus *Poecilia* had high salinity affiliation. The ML analyses revealed that the MRCA of subgenus *Acanthophacelus, Psychropoecilia* and *Allopoecilia* inhabited freshwater habitats, the MRCA of subgenus *Poecilia* inhabited high salinity habitats, and the MRCA of subgenera *Micropoecilia* and *Mollienesia* had medium-high salinity affiliations (Fig. 3.5). Both analyses suggest that the MRCA of the *P. mexicana* complex (within the subgenus *Mollienesia*) was associated with high salinity habitats, the MRCA of the *P. sphenops* complex inhabited freshwater habitats, and the MRCA of the *P. latipinna* complex had medium salinity affiliation.

Ancestral diet reconstructions suggest that ancestral *Poecilia* species displayed varying diet strategies. Both MP and ML analyses revealed that the MRCA of the subgenus *Acanthophacelus* was carnivorous (Fig. 3.6) and showed relatively small degrees of intramandibular bending and small gape angles (Fig. 3.7). The MRCA of the subgenus *Psychropoecilia* was herbivorous or omnivorous (cyanobacteria + diatoms + animals) and showed mid-range intramandibular bending and gape angles. The MRCA of the subgenus *Allopoecilia* was omnivorous (cyanobacteria + diatoms + animals) and had a low degree of intramandibular bending, but mid-range gape angles.

The MRCA of the subgenus *Micropoecilia* was omnivorous (cyanobacteria + animals) based on MP analyses, with relatively low intramandibular bending and gape angles. However, ML analyses suggest that the ancestral condition of the subgenus *Micropoecilia* was carnivory. Finally, the MRCA of the subgenus *Mollienesia* displayed obligate herbivory (cyanobacteria + diatoms), with mid-range intramandibular bending and gape angles.

Identifying Patterns of Diet Evolution in Response to Habitat Transitions

Phylogenetic independent contrasts on salinity affiliation (percent of species occupying saline habitats) and diet characters revealed contrasting patterns. Despite the relationship between percent animal material in the gut and jaw morphology (IMB and GA), salinity affiliation did not predict IMB or GA (IMB: $y = 0.001x$, $r^2 = -0.015$, $p = 0.684$; GA: $y = -3.8 \times 10^4 x$, $r^2 = -0.018$, $p = 0.921$; Fig. 3.8a & b). However, salinity affiliation explained 22% of percent of animal material in the gut ($y = -21.99x$, $r^2 = 0.267$, $p < 0.0001$), where increased salinity affiliation drives an increase in herbivory (decrease in animal material in the gut; Fig. 3.8c).

Discussion

Results revealed that herbivory may have evolved as an adaptation for invading less productive saline habitats, thereby supporting the Suboptimal Habitat Hypothesis (Sanchez and Trexler 2016). I found that the MRCAs of subgenera *Acanthophacelus*, *Micropoecilia*, *Psychropoecilia* and *Allopoecilia* had low salinity affiliations and were either omnivorous or carnivorous.

Furthermore, the divergence of the subgenera *Poecilia* and *Mollienesia* resulted in MRCAs with brackish/ marine roots, and the transition from low to high salinity affiliation drove diet diversification favoring the appearance of obligate herbivory in these groups. Salinity affiliation explained 26% of the total variation in the diet of *Poecilia* species (measured by percent animal material in the gut), and jaw morphology (IMB and GA) was associated with percent animal material in the gut, but not with percent of species occupying saline habitats. These findings suggest that in this genus, herbivory evolved in response to habitat transitions between fresh and euryhaline habitats, and jaw morphology evolved in response to the appearance of herbivory.

Incorporating additional *Poecilia* species for phylogenetic analyses did not reveal any novel relationships compared to previous studies, but instead verified the relationships between subgenera within the tree, allowing us to estimate the ancestral diets and salinity affiliations of *Poecilia* species. Ancestral reconstructions revealed that the MRCA of the subgenus *Mollienesia* likely originated in freshwater and remained in these habitats until the divergence of the three species complexes (MP analyses). At this time, species of the *P. mexicana* and *P. latipinna* complexes transitioned into euryhaline habitats, and species belonging to the *P. sphenops* complex remained in freshwaters. Alternatively, the ML model suggests that before the divergence of the MRCA, this group likely originated in freshwater, transitioned into euryhaline waters, and either remained in euryhaline habitats (*P. mexicana* and *P. latipinna* complexes), or crossed back into freshwaters (*P. sphenops* complex).

Dietary ancestral state reconstructions were more clear for the subgenus *Mollienesia*, as both MP and ML models suggest that all species belonging to this group displayed obligate herbivory, although the mode of herbivory varies throughout the clade. Three herbivorous strategies emerged ('green algae', 'cyanobacteria', and 'cyanobacteria + diatoms'), and these correspond to the salinity affiliations of each species, and the primary producer communities of the different habitat types. Tropical euryhaline primary producer communities are typically dominated by cyanobacteria (e.g., Flombaum et al. 2013), and these results show that the species with the highest salinity affiliations (*P. mexicana* and *P. orri*) have diets comprised of these organisms. Furthermore, freshwater producer communities are dominated by diatoms, and I found that herbivorous species with low-medium salinity affiliations (*P. latipinna, P. kykesis, P. gilli*) consume both cyanobacteria and diatoms. The exception was *P. butleri*, which showed a high-salinity affiliation and consumed a high proportion of green algae. I only sampled *P. butleri* gut contents from one locality, so these results may not be representative for the entire species.

Despite the uncertainty in ancestral habitat and diet estimations, I found that increased salinity affiliation explained 26% of the decrease in animal material in the gut. Because the MRCA of the subgenus *Mollienesia* was herbivorous, this evidence suggests a freshwater→euryhaline→freshwater transition rather than a euryhaline→freshwater→euryhaline transition in this group. Similar to species of the subgenus *Mollienesia*, *P. vivipara* shows a high salinity affiliation and an herbivorous feeding strategy.

But this species diverged from the MRCA of the subgenus *Poecilia* approximately 3 mya (Palacios et al. 2016), suggesting that both salinity affiliation and herbivory evolved multiple times before the appearance of the subgenus *Mollienesia*, which appeared approximately 0.25 mya (Palacios et al. 2017). In addition, *P. picta* (subgenus *Micropoecilia*) shows a high salinity affiliation with an omnivorous feeding strategy, and *P. parae* (subgenus *Micropoecilia*) inhabits mostly freshwater systems. These species appeared approx. 2.7 mya (Palacios et al. 2016) suggesting that a habitat transition might have occurred during the early evolution of the subgenus *Micropoecilia*, many years before the appearance of the subgenus *Mollienesia*.

Freshwater-to-marine transitions are relatively rare in fishes (McDowall 1997; Vermeij 2000; Betancur-R 2009), likely because of the decreased habitat heterogeneity offered by marine habitats (Bloom et al. 2013). In addition, herbivory is thought to be an energetically inferior diet compared to omnivory or carnivory, so coevolution of high salinity affiliation and an herbivorous feeding strategy seems maladaptive. The evolution of herbivory in Terapontid fishes is more intuitive, as this process was driven by the transition into heterogeneous freshwaters (Davis et al. 2012). In Cleupeoid fishes, the evolution of herbivory was not driven by habitat transitions, but was instead driven by latitude (Egan et al. 2018). These results support multiple transitions between freshwater and euryhaline habitats in the genus *Poecilia* (particularly in the subgenera *Poecilia* and *Mollienesia*), and I show that these transitions are related to the evolution of herbivory in the same species.

The Suboptimal Habitat Hypothesis posits that herbivory may be an adaptive strategy to allow organisms to invade habitats with decreased resource quality, where animal prey are scarce and plant abundance is high (Sanchez and Trexler 2016). Under this definition, a euryhaline habitat may be considered "suboptimal" relative to a highly productive freshwater habitat. Therefore, these data support the Suboptimal Habitat Hypothesis as an explanation for the appearance of herbivory in this group. It is important to note, however, that there may be other explanations supporting the evolution of herbivory in other metazoan groups (see Sanchez and Trexler 2016 for alternative hypotheses), and that multiple mechanisms may be working simultaneously to explain the appearance and subsequent maintenance of herbivory in nature (see Sanchez and Trexler 2018).

Conclusions

This study suggests that high salinity affiliation and herbivory are derived characters in the genus *Poecilia*. In addition, I show that salinity affiliation and herbivory evolved together, where increased habitat salinity results in increased degree of herbivory. This result is surprising because there is ample evidence that freshwater-to-marine transitions generally result in decreased diversification relative to transitions in the opposite direction (e.g., McDowall 1997; Vermeij 2000; Betancur-R 2009). Although productive freshwater systems offer increased foraging opportunities compared to marine systems, I found that invading a 'suboptimal' habitat triggered diet diversification in the subgenera *Poecilia* and *Mollienesia.* The ability to cross ecosystem boundaries coupled with an adaptive diet strategy could allow *Poecilia* species to rapidly expand their range, thereby increasing opportunities for ecological diversification, ultimately resulting in species radiation.

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Table 3.1. Complete list of sampled *Poecilia* specimens for gut and jaw morphology analyses. Asterisks indicate museum samples obtained from the *Fishnet2* data base (http://www.fishnet2.net/).

Sample ID	Species	Locality Description	State, Country	Latitude	Longitude	Gut content sample size	Jaw morph. sample size
UF 7333*	*P. sphenops*	Km marker 583 between Lerdo de Tejada and Santiago Tuxtlas	Veracruz, Mexico	18.5869100°N	95.3650980°W	25	25
UF 87585*	*P. sphenops*	Aguan River, on road CA 13, 44.6 miles W of Trujillo	Colon, Honduras	15.5281790°N	86.2305890°W	10	25
UF 15249*	*P. butleri*	Rio Quelite, 22.6 mi NNW of Mazatlan	Sinaloa, Mexico	23.5226570°N	106.4978210°W	-	4
UF 15253*	*P. butleri*	Mangrove swamp, 1.7 mi SE and 4.5 mi SW of Tecoman	Colima, Mexico	18.8703980°N	103.9322370°W	5	13
UF 19554*	*P. gilli*	Quepos, stream near Los Junta de Alregados, at Pan American Hwy bridge	Colima, Mexico	9.4515450°N	84.1680030°W	5	13
UF 19567*	*P. gilli*	Rio Corobici and canal trib, at La Pacifica Hotel, 5 km NW of Las Canas, near Pan American Highway	Guanacaste, Costa Rica	10.4721250°N	85.1226740°W	-	7
UF 23988*	*P. dominicensis*	River 14 km NW of Sabina Grande de Boya	San Cristobal, Hispaniola	19.0092590°N	69.9094420°W	15	25
UF 25044*	*P. dominicensis*	Rio Maimon, 7 km SW of Piedra Blanca, 250 m elevation	La Vega, Hispaniola	18.9022540°N	70.2830910°W	15	25

UF 25049*	*P. hispaniolana*	Rio Yaque del Sur 9 km SW of Jarabacoa	La Vega, Hispaniola	19.0780560°N	-70.7186420°W	25	25
UF 111695*	*P. hispaniolana*	Lago Enriquillo, 4km from Descubierta	Hispaniola	18.5150000°N	-71.6608330°W	15	25
UF 74903*	*P. picta*	Salybia River Bridge #3 1/2, E of 1.25 mi post between Salybia Bay and Galera Point	Trinidad, Trinidad and Tobagao	10.8339450°N	60.9206520°W	-	25
UF 112133*	*P. vivipara*	Tenesopolis Municipality; Guarani farm	Rio de Janeiro, Brazil	19.9000000°S	55.8000000°W	15	25
UF 188017*	*P. vivipara*	Itapicuru River off BA-381 between Filadélfia and Itiúba	Bahia, Brazil	-10.7041944°S	-39.8965278°W	15	25
UMMZ 55052*	*P. caucana*	Small pools in course of small stream, Rio Camarones, at Arroyo de Arena	Columbia	11.2624590°N	72.9197800°W	-	25
UMMZ 186930*	*P. caucana*	Rio Portillo, tributary called Rio Carache	Venezuela	9.61482222°N	70.54972222°W	15	25
UMMZ 233640*	*P. parae*	Rio Maguari near Maguary, Belem	Para, Brazil	1.2818030°S	48.4274700°W	-	11
UMMZ 247482*	*P. parae*	Canals at Anna Regina on Essiquibo coast	Guyana, Brazil	7.2596680°N	58.4848630°W	-	19
UF 24504*	*P. orri*	Below dam of reservoir on Salt Creek	Isla de Providencia, Columbia	13.3435810°N	81.3877640°W	25	24

ECOSUR donation 1	*P. orri*	Laguna Ubero	Quintana Roo, Mexico	19.0530250°N	-87.5739000°W	10	10
ECOSUR donation 2	*P. mexicana*	Close to Carraterra El Cafetal- Mahahual	Quintana Roo, Mexico	18.96838333° N	-87.9472611°W	20	20
POEMEX A	*P. mexicana*	Arroyo Escondido	Quintana Roo, Mexico	18.6111111°N	-088.8122222°W	4	4
ECOSUR donation 3	*P. kykesis*	Champton	Campeche, Mexico	19.2652972°N	-87.5739583°W	15	15
ECOSUR donation 4	*P. kykesis*	Arroyo Nuevo Loria	Quintana Roo, Mexico	19.3011111°N	88.5347222°W	10	10
POEVEL A	*P. velifera*	Homochen	Yucatan, Mexico	21.2001510°N	-089.9484400°E	25	25
POEVEL B	*P. velifera*	Ojo de Agua Ex Granja Pecis	Yucatan, Mexico	21.1834400°N	-089.9791300°E	21	25
POELAT A	*P. latipinna*	Water Conservation Area 3B, boatramp near S-333 water structure	Florida, USA	25.7623722°N	-80.6731833°W	16	19
POELAT B	*P. latipinna*	Mangrove area on the right of South-bound US 1, Everglades National Park	Florida, USA	25.2361583°N	80.4336722°W	20	20
POERET A	*P. reticulata*	Tacarigua River via Caura Royal Road	Trinidad, Trinidad and Tobagao	10.6789333°N	-61.3194666°W	24	23
POERET B	*P. reticulata*	Quare River	Trinidad, Trinidad and Tobagao	10.6000000°N	-61.1000000°W	22	20

Table 3.2. Proportion of habitat types occupied by each species based on collections logged in the *Fishnet2* data base (http://www.fishnet2.net/).

		PROPORTION OF COLLECTION SITES			
	Species	**Freshwater**	**Brackish**	**Marine**	**Sample Size (N)**
1	*P. reticulata*	100	0	0	25
2	*P. parae*	100	0	0	9
3	*P. picta*	67	17	17	12
4	*P. vivipara*	84	8	8	25
5	*P. dominicensis*	100	0	0	25
6	*P. hispaniolana*	100	0	0	25
7	*P. caucana*	100	0	0	16
8	*P. kykesis*	68	24	8	25
9	*P. latipinna*	58	21	21	25
10	*P. sphenops*	100	0	0	25
11	*P. gilli*	88	12	0	25
12	*P. mexicana*	80	8	12	25
13	*P. orri*	55	0	45	25

Table 3.3. Measured jaw angles of each sampled *Poecilia* species. IMB= Intramandibular bending (angle subtracted from 180°), GA= Gape angle, NCR= Neurocranial rotation.

	Species	IMB	GA	NCR	Sample Size (N)
1	*P.reticulata*	77.75 ± 6.10	66.48 ± 13.40	19.24 ± 7.96	43
2	*P. parae*	78.81 ± 6.87	69.39 ± 27.84	12.34 ± 4.81	30
3	*P. picta*	86.25 ± 7.34	50.76 ± 12.06	17.68 ± 6.05	25
4	*P. vivipara*	85.96 ± 11.72	73.44 ± 14.62	14.30 ± 5.47	50
5	*P. dominicensis*	89.52 ± 8.49	82.39 ± 11.37	9.41 ± 4.24	50
6	*P. hispaniolana*	88.50 ± 12.08	72.69 ± 12.17	7.88 ± 2.94	50
7	*P. caucana*	72.38 ± 16.70	81.16 ± 18.27	10.28 ± 5.42	50
8	*P. kykesis*	89.17 ± 10.00	101.00 ± 15.03	16.14 ± 3.86	25
9	*P. latipinna*	87.98 ± 15.89	95.43 ± 9.30	11.91 ± 4.96	39
10	*P. velifera*	84.40 ± 15.10	96.36 ± 29.53	4.73 ± 4.16	50
11	*P. butleria*	85.98 ± 7.86	74.98 ± 14.02	8.79 ± 2.59	17
12	*P. sphenops*	84.55 ± 11.00	108.54 ± 14.47	13.36 ± 3.29	50
13	*P. gilli*	80.03 ± 11.27	78.79 ± 25.11	12.73 ± 4.34	20
14	*P. mexicana*	89.60 ± 13.35	84.68 ± 14.28	16.16 ± 5.49	24
15	*P. orri*	82.80 ± 14.78	78.22 ± 14.99	13.01 ± 3.29	34

Figure Legends

Fig. 3.1. Bayesian phylogenetic tree (50% majority-rule) derived from concatenated mitochondrial *Cytochrome Oxidase subunit I*, *ATPase 8/6*, *NADH dehydrogenase subunit 2*, and *Ribosomal Protein* S7 genes for 36 *Poecilia* and 2 *Limia* species. Bullets at each node represent the Posterior Probability (PP). Nodes with posterior probabilities ≥ 99% are considered highly supported, those with posterior probabilities ≥ 95% are well-supported, nodes with posterior probabilities ≥ 75% are moderately supported, and those with posterior probabilities ≥ 75% have no support. Genbank ID for each species is listed in parentheses. Species are colored by subgenus.

Fig. 3.2. Pruned Bayesian phylogenetic tree (50% majority-rule) derived from concatenated mitochondrial *Cytochrome Oxidase subunit I*, *ATPase 8/6*, *NADH dehydrogenase subunit 2*, and *Ribosomal Protein* S7 genes for the 15 subsampled *Poecilia* species. Bullets at each node represent the Posterior Probability (PP). Nodes with posterior probabilities ≥ 99% are considered highly supported, those with posterior probabilities ≥ 95% are well-supported, nodes with posterior probabilities ≥ 75% are moderately supported, and those with posterior probabilities ≥ 75% have no support. Genbank ID for each species is listed in parentheses. Species are colored by subgenus.

Fig. 3.3. Classification of *Poecilia* diets using Sorensen (Bray-Curtis) distance measures with flexible beta linkage. Hierarchical Cluster analysis identified 6 diet categories.

Fig. 3.4. (A) Relationship between degree of intramandibular bending (IMB) and percent animal material in the diet for 15 *Poecilia* species plotted as phylogenetically independent contrasts. **(B)** Relationship between gape angle (GA) and percent animal material in the diet for 15 *Poecilia* species plotted as phylogenetically independent contrasts.

Fig. 3.5. Maximum Parsimony (left cladogram) and Maximum Likelihood (right cladogram) ancestral character reconstruction for the evolution of habitat (salinity affiliation) in the *Poecilia* group. Circles at terminal nodes represent the observed character states for extant species, and pie charts for ancestral nodes show estimated probabilities for reconstructed character states. Species are colored by subgenus and nodes with large circles indicate the most recent common ancestor for that subgenus. Genbank ID for each species is listed in parentheses.

Fig. 3.6. Maximum Parsimony (left cladogram) and Maximum Likelihood (right cladogram) ancestral character reconstruction for the evolution of diet in the *Poecilia* group. Circles at terminal nodes represent the observed character states for extant species, and pie charts for ancestral nodes show estimated probabilities for reconstructed character states. Species are colored by subgenus and nodes with large circles indicate the most recent common ancestor for that subgenus. Genbank ID for each species is listed in parentheses.

Fig. 3.7. Maximum Parsimony ancestral character reconstruction for the evolution of intramandibular bending (left cladogram) and gape angle (right cladogram) in the *Poecilia* group. Circles at terminal nodes represent the observed character states for extant species, and pie charts for ancestral nodes show estimated probabilities for reconstructed character states. Maximum likelihood could not be performed because jaw metrics are continuous data. Species are colored by subgenus and nodes with large circles indicate the most recent common ancestor for that subgenus. Genbank ID for each species is listed in parentheses.

Fig. 3.8. (A) The relationship between salinity affiliation and intramandibular bending (plotted as phylogenetically independent contrasts) suggests that IMB did not evolve as an adaptation to saline habitats. (B) The relationship between salinity affiliation and gape angle (plotted as phylogenetically independent contrasts) suggests that GA did not evolve as an adaptation to saline habitats. (C) The relationship between salinity affiliation and % animal material in the gut (plotted as phylogenetically independent contrasts) suggests that herbivory evolved in response to increased salinity.

Fig. 3.1.

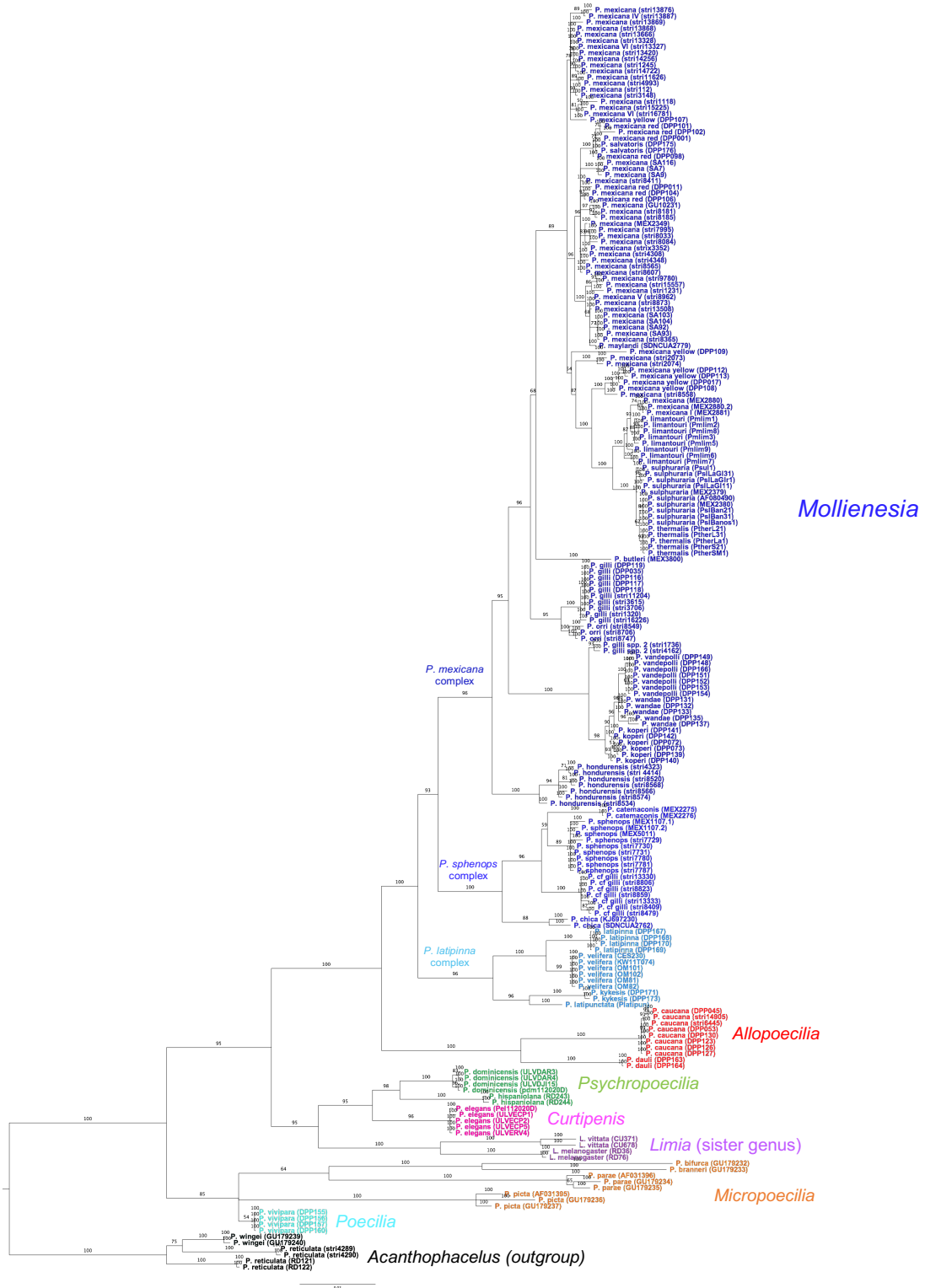
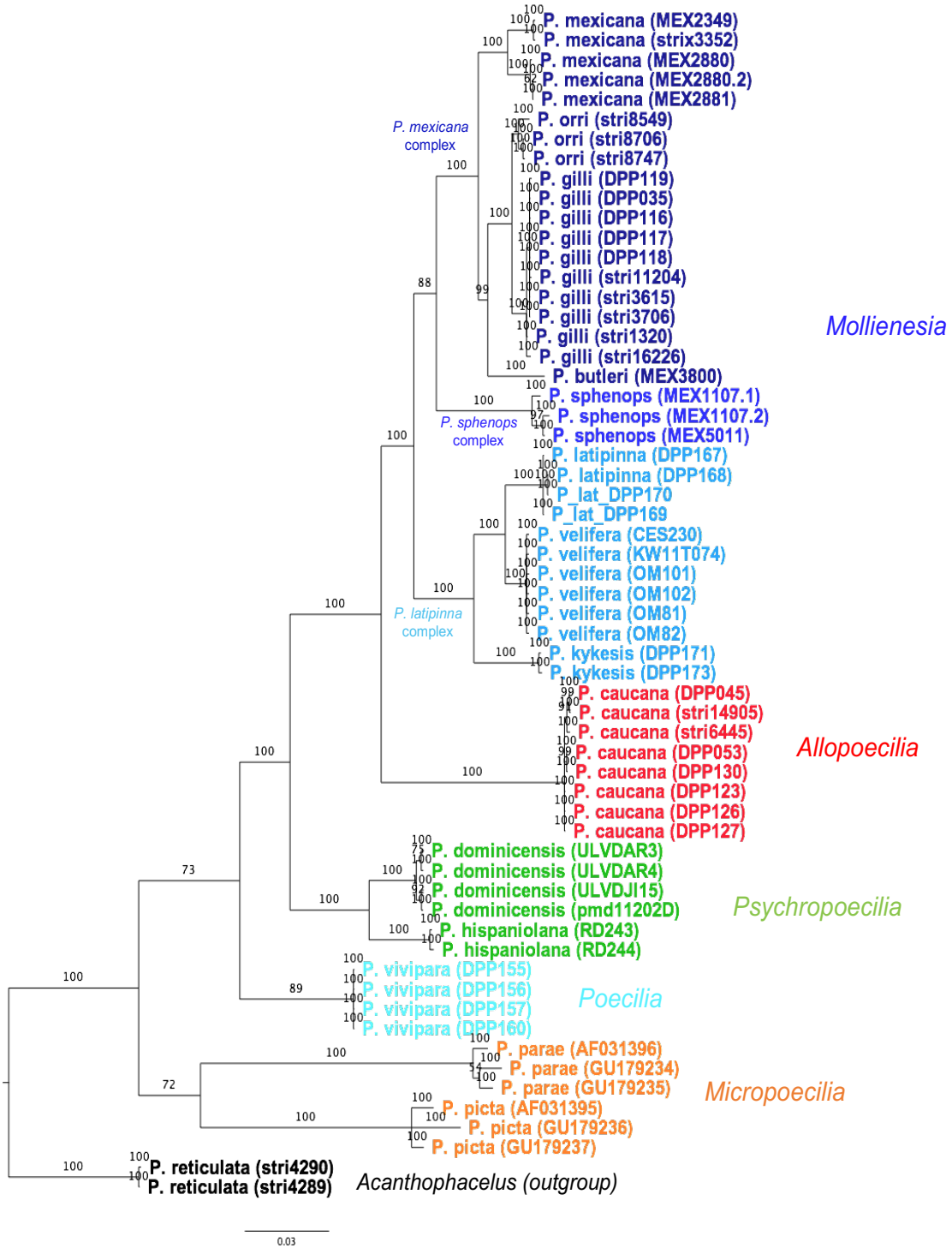

Mollienesia
P. mexicana complex
P. sphenops complex
P. latipinna complex
Allopoecilia
Psychropoecilia
Curtipenis
Limia (sister genus)
Micropoecilia
Poecilia
Acanthophacelus (outgroup)

Fig. 3.2.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P. mexicana (strix3352)
P. mexicana (MEX2880)
P. mexicana (MEX2880.2)
P. mexicana (MEX2881)
P. orri (stri8549)
P. orri (stri8706)
P. orri (stri8747)
P. gilli (DPP119)
P. gilli (DPP035)
P. gilli (DPP116)
P. gilli (DPP117)
P. gilli (DPP118)
P. gilli (stri11204)
P. gilli (stri3615)
P. gilli (stri3706)
P. gilli (stri1320)
P. gilli (stri16226)
P. butleri (MEX3800)
P. sphenops (MEX1107.1)
P. sphenops (MEX1107.2)
P. sphenops (MEX5011)
P. latipinna (DPP167)
P. latipinna (DPP168)
P_lat_DPP170
P_lat_DPP169
P. velifera (CES230)
P. velifera (KW11T074)
P. velifera (OM101)
P. velifera (OM102)
P. velifera (OM81)
P. velifera (OM82)
P. kykesis (DPP171)
P. kykesis (DPP173)
P. caucana (DPP045)
P. caucana (stri14905)
P. caucana (stri6445)
P. caucana (DPP053)
P. caucana (DPP130)
P. caucana (DPP123)
P. caucana (DPP126)
P. caucana (DPP127)
P. dominicensis (ULVDAR3)
P. dominicensis (ULVDAR4)
P. dominicensis (ULVDJI15)
P. dominicensis (pmd11202D)
P. hispaniolana (RD243)
P. hispaniolana (RD244)
P. vivipara (DPP155)
P. vivipara (DPP156)
P. vivipara (DPP157)
P. vivipara (DPP160)
P. parae (AF031396)
P. parae (GU179234)
P. parae (GU179235)
P. picta (AF031395)
P. picta (GU179236)
P. picta (GU179237)
P. reticulata (stri4290)
P. reticulata (stri4289)
P. mexicana complex
P. sphenops complex
P. latipinna complex
Mollienesia
Allopoecilia
Psychropoecilia
Poecilia
Micropoecilia
Acanthophacelus (outgroup)
0.03

Fig. 3.3.

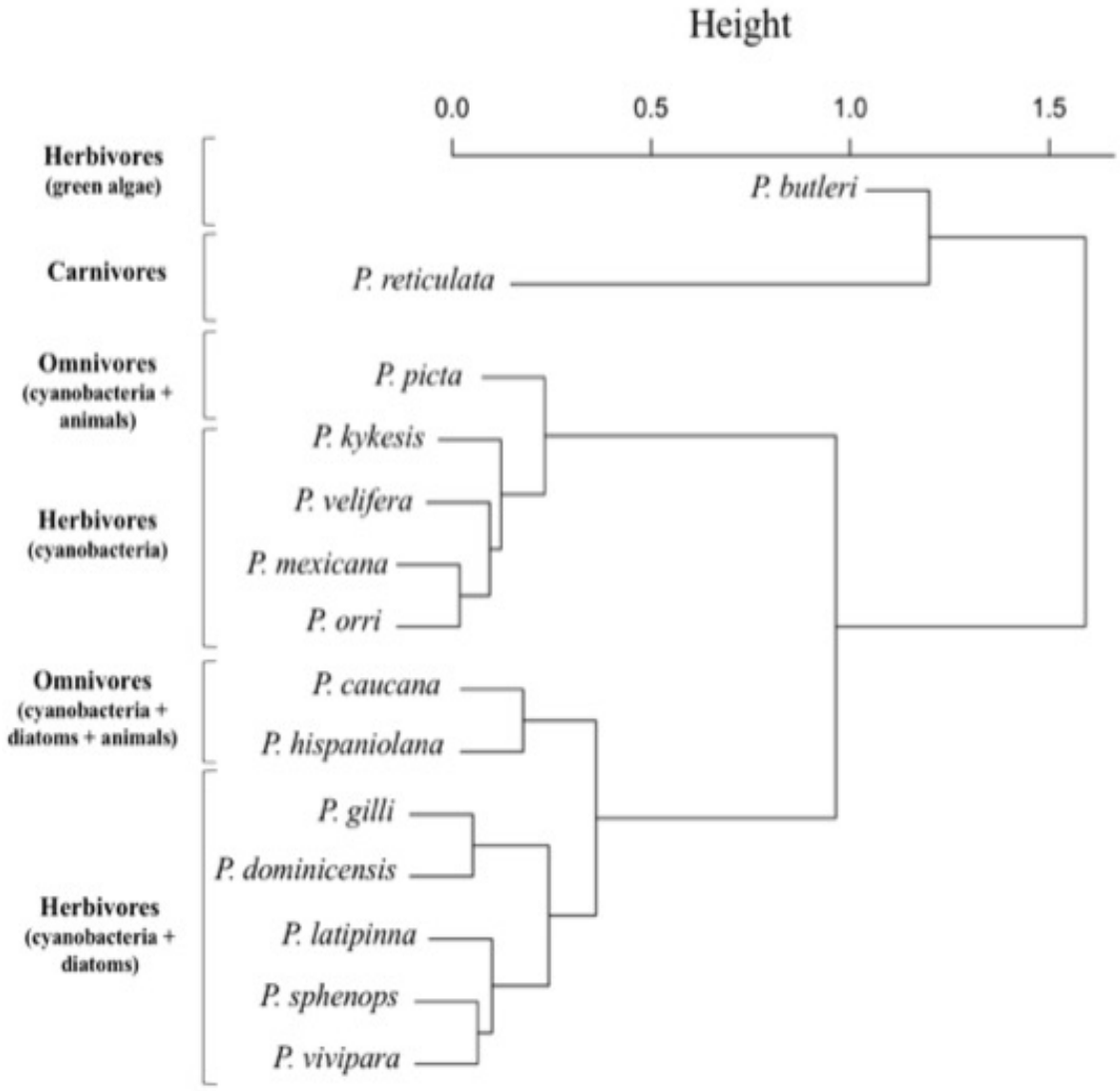

Height
0.0
0.5
1.0
1.5
Herbivores
(green algae)
Carnivores
Omnivores
(cyanobacteria +
animals)
Herbivores
(cyanobacteria)
Omnivores
(cyanobacteria +
diatoms + animals)
Herbivores
(cyanobacteria +
diatoms)
P. butleri
P. reticulata
P. picta
P. kykesis
P. velifera
P. mexicana
P. orri
P. caucana
P. hispaniolana
P. gilli
P. dominicensis
P. latipinna
P. sphenops
P. vivipara

Fig. 3.4.

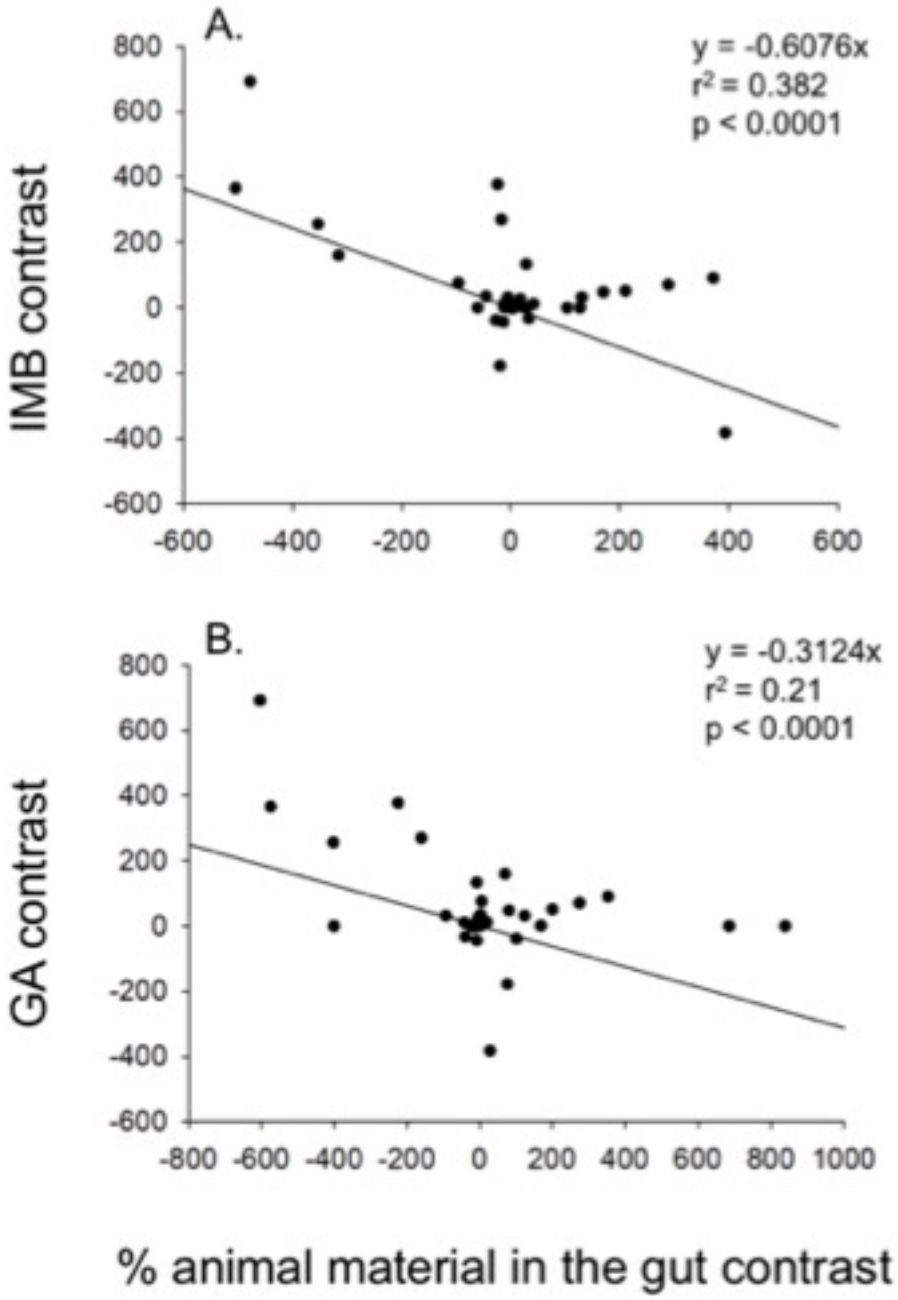

A.
y = -0.6076x
r² = 0.382
p < 0.0001
IMB contrast
800
600
400
200
0
-200
-400
-600
-600 -400 -200 0 200 400 600
B.
y = -0.3124x
r² = 0.21
p < 0.0001
GA contrast
800
600
400
200
0
-200
-400
-600
-800 -600 -400 -200 0 200 400 600 800 1000
% animal material in the gut contrast

Fig. 3.5.

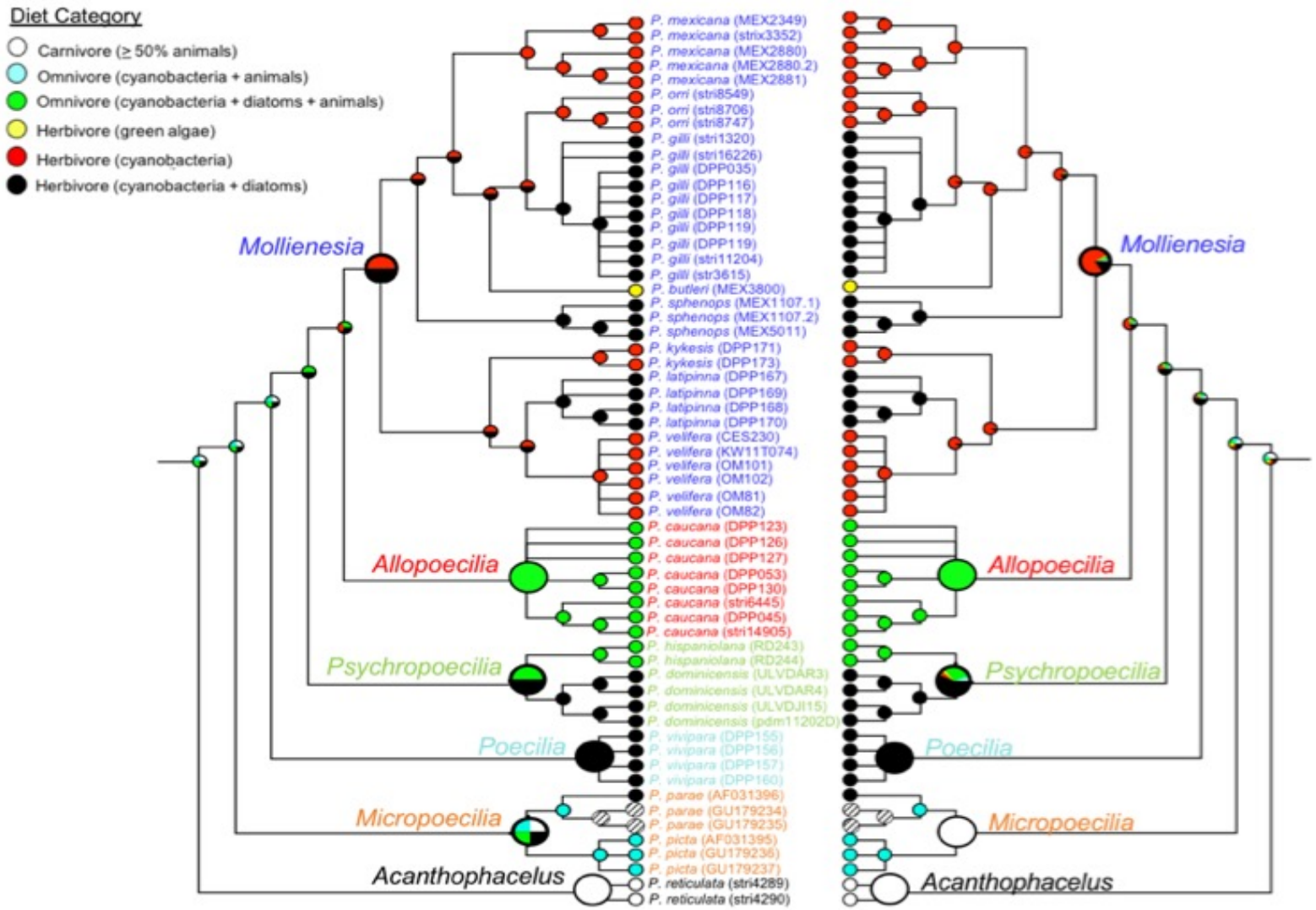

Diet Category
Carnivore (≥ 50% animals)
Omnivore (cyanobacteria + animals)
Omnivore (cyanobacteria + diatoms + animals)
Herbivore (green algae)
Herbivore (cyanobacteria)
Herbivore (cyanobacteria + diatoms)
P. mexicana (MEX2349)
P. mexicana (strix3352)
P. mexicana (MEX2880)
P. mexicana (MEX2880.2)
P. mexicana (MEX2881)
P. orri (stri8549)
P. orri (stri8706)
P. orri (stri8747)
P. gilii (stri1320)
P. gilii (stri16226)
P. gilii (DPP035)
P. gilii (DPP116)
P. gilii (DPP117)
P. gilii (DPP118)
P. gilii (DPP119)
P. gilii (DPP119)
P. gilii (stri11204)
P. gilii (str3615)
P. butleri (MEX3800)
P. sphenops (MEX1107.1)
P. sphenops (MEX1107.2)
P. sphenops (MEX5011)
P. kykesis (DPP171)
P. kykesis (DPP173)
P. latipinna (DPP167)
P. latipinna (DPP169)
P. latipinna (DPP168)
P. latipinna (DPP170)
P. velifera (CES230)
P. velifera (KW11T074)
P. velifera (OM101)
P. velifera (OM102)
P. velifera (OM81)
P. velifera (OM82)
P. caucana (DPP123)
P. caucana (DPP126)
P. caucana (DPP127)
P. caucana (DPP053)
P. caucana (DPP130)
P. caucana (stri6445)
P. caucana (DPP045)
P. caucana (stri14905)
P. hispaniolana (RD243)
P. hispaniolana (RD244)
P. dominicensis (ULVDAR3)
P. dominicensis (ULVDAR4)
P. dominicensis (ULVDJI15)
P. dominicensis (pdm11202D)
P. vivipara (DPP155)
P. vivipara (DPP156)
P. vivipara (DPP157)
P. vivipara (DPP160)
P. parae (AF031396)
P. parae (GU179234)
P. parae (GU179235)
P. picta (AF031395)
P. picta (GU179236)
P. picta (GU179237)
P. reticulata (stri4289)
P. reticulata (stri4290)
Mollienesia
Allopoecilia
Psychropoecilia
Poecilia
Micropoecilia
Acanthophacelus
Mollienesia
Allopoecilia
Psychropoecilia
Poecilia
Micropoecilia
Acanthophacelus

Fig. 3.6.

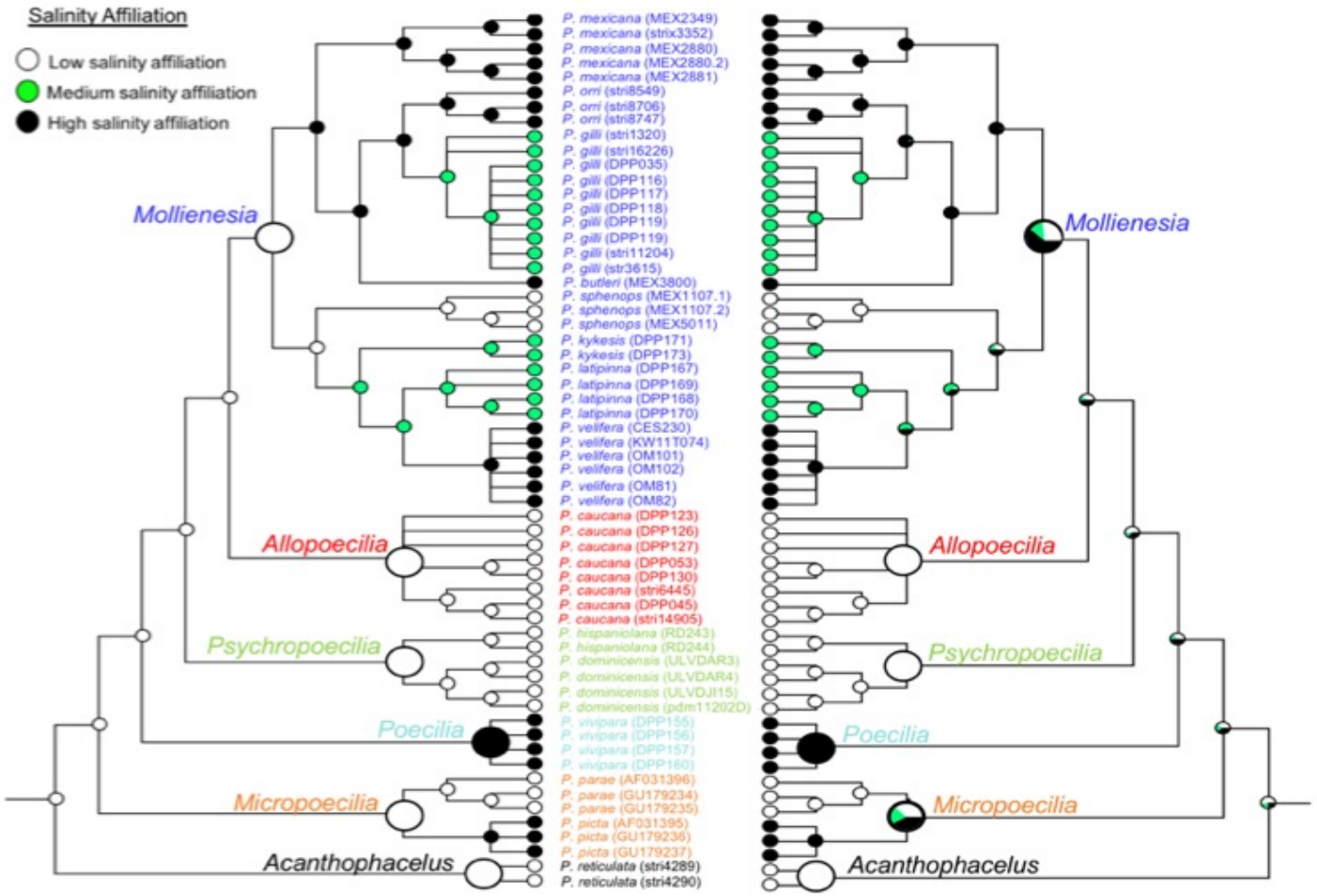
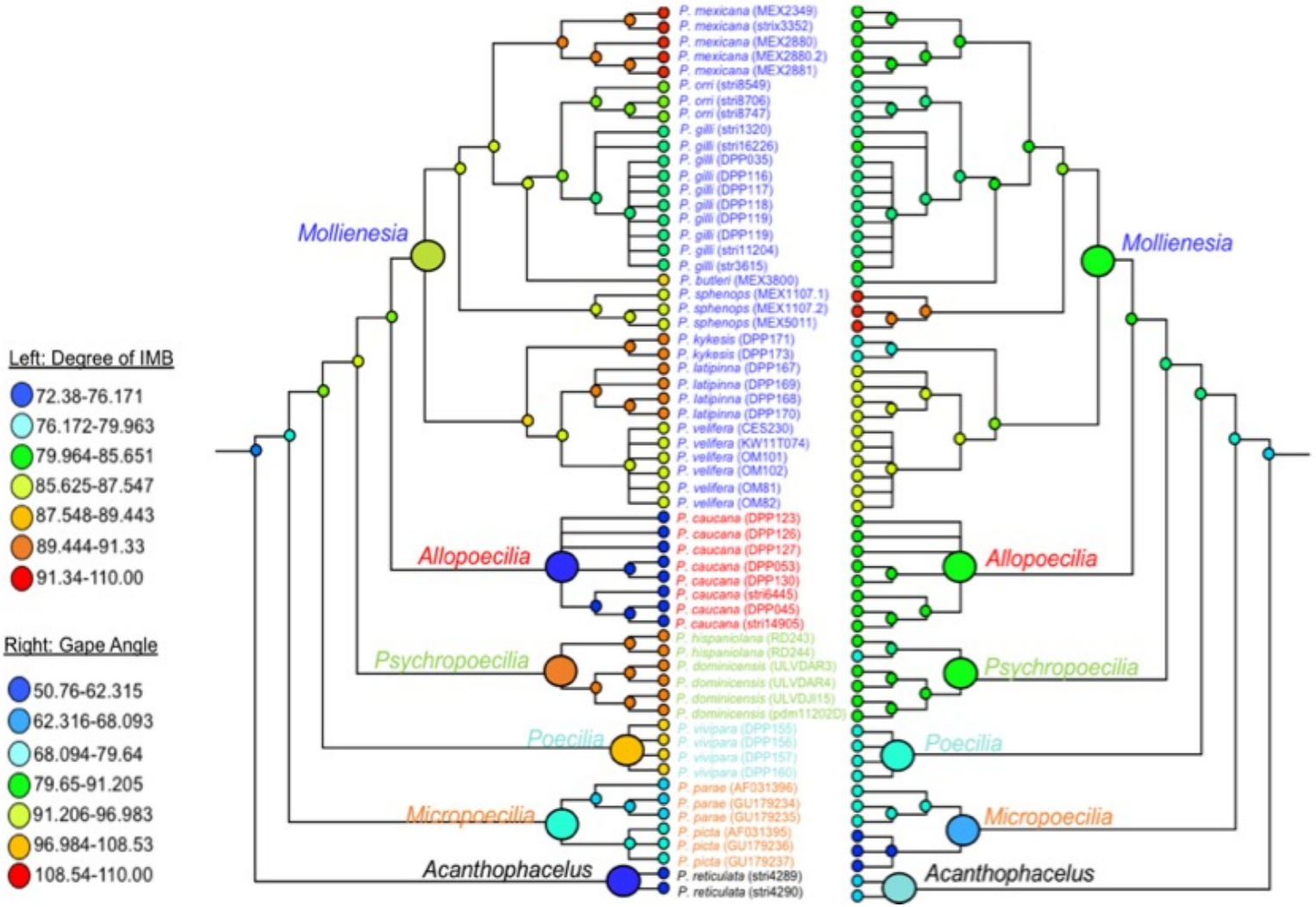

Salinity Affiliation
Low salinity affiliation
Medium salinity affiliation
High salinity affiliation
Mollienesia
Allopoecilia
Psychropoecilia
Poecilia
Micropoecilia
Acanthophacelus
P. mexicana (MEX2349)
P. mexicana (strix3352)
P. mexicana (MEX2880)
P. mexicana (MEX2880.2)
P. mexicana (MEX2881)
P. orri (stri8549)
P. orri (stri8706)
P. orri (stri8747)
P. gilli (stri1320)
P. gilli (stri16226)
P. gilli (DPP035)
P. gilli (DPP116)
P. gilli (DPP117)
P. gilli (DPP118)
P. gilli (DPP119)
P. gilli (DPP119)
P. gilli (stri11204)
P. gilli (str3615)
P. butleri (MEX3800)
P. sphenops (MEX1107.1)
P. sphenops (MEX1107.2)
P. sphenops (MEX5011)
P. kykesis (DPP171)
P. kykesis (DPP173)
P. latipinna (DPP167)
P. latipinna (DPP169)
P. latipinna (DPP168)
P. latipinna (DPP170)
P. velifera (CES230)
P. velifera (KW11T074)
P. velifera (OM101)
P. velifera (OM102)
P. velifera (OM81)
P. velifera (OM82)
P. caucana (DPP123)
P. caucana (DPP126)
P. caucana (DPP127)
P. caucana (DPP053)
P. caucana (DPP130)
P. caucana (stri6445)
P. caucana (DPP045)
P. caucana (stri14905)
P. hispaniolana (RD243)
P. hispaniolana (RD244)
P. dominicensis (ULVDAR3)
P. dominicensis (ULVDAR4)
P. dominicensis (ULVDJI15)
P. dominicensis (pdm11202D)
P. vivipara (DPP155)
P. vivipara (DPP156)
P. vivipara (DPP157)
P. vivipara (DPP160)
P. parae (AF031396)
P. parae (GU179234)
P. parae (GU179235)
P. picta (AF031395)
P. picta (GU179236)
P. picta (GU179237)
P. reticulata (stri4289)
P. reticulata (stri4290)

Fig. 3.7.


Left: Degree of IMB
72.38-76.171
76.172-79.963
79.964-85.651
85.625-87.547
87.548-89.443
89.444-91.33
91.34-110.00
Right: Gape Angle
50.76-62.315
62.316-68.093
68.094-79.64
79.65-91.205
91.206-96.983
96.984-108.53
108.54-110.00
Mollienesia
Allopoecilia
Psychropoecilia
Poecilia
Micropoecilia
Acanthophacelus
P. mexicana (MEX2349)
P. mexicana (strix3352)
P. mexicana (MEX2880)
P. mexicana (MEX2880.2)
P. mexicana (MEX2881)
P. orri (stri8549)
P. orri (stri8706)
P. orri (stri8747)
P. gilli (stri1320)
P. gilli (stri16226)
P. gilli (DPP035)
P. gilli (DPP116)
P. gilli (DPP117)
P. gilli (DPP118)
P. gilli (DPP119)
P. gilli (DPP119)
P. gilli (stri11204)
P. gilli (str3615)
P. butleri (MEX3800)
P. sphenops (MEX1107.1)
P. sphenops (MEX1107.2)
P. sphenops (MEX5011)
P. kykesis (DPP171)
P. kykesis (DPP173)
P. latipinna (DPP167)
P. latipinna (DPP169)
P. latipinna (DPP168)
P. latipinna (DPP170)
P. velifera (CES230)
P. velifera (KW11T074)
P. velifera (OM101)
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P. velifera (OM81)
P. velifera (OM82)
P. caucana (DPP123)
P. caucana (DPP126)
P. caucana (DPP127)
P. caucana (DPP053)
P. caucana (DPP130)
P. caucana (stri6445)
P. caucana (DPP045)
P. caucana (stri14905)
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P. hispaniolana (RD244)
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P. dominicensis (ULVDAR4)
P. dominicensis (ULVDJI15)
P. dominicensis (pdm11202D)
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P. vivipara (DPP156)
P. vivipara (DPP157)
P. vivipara (DPP160)
P. parae (AF031396)
P. parae (GU179234)
P. parae (GU179235)
P. picta (AF031395)
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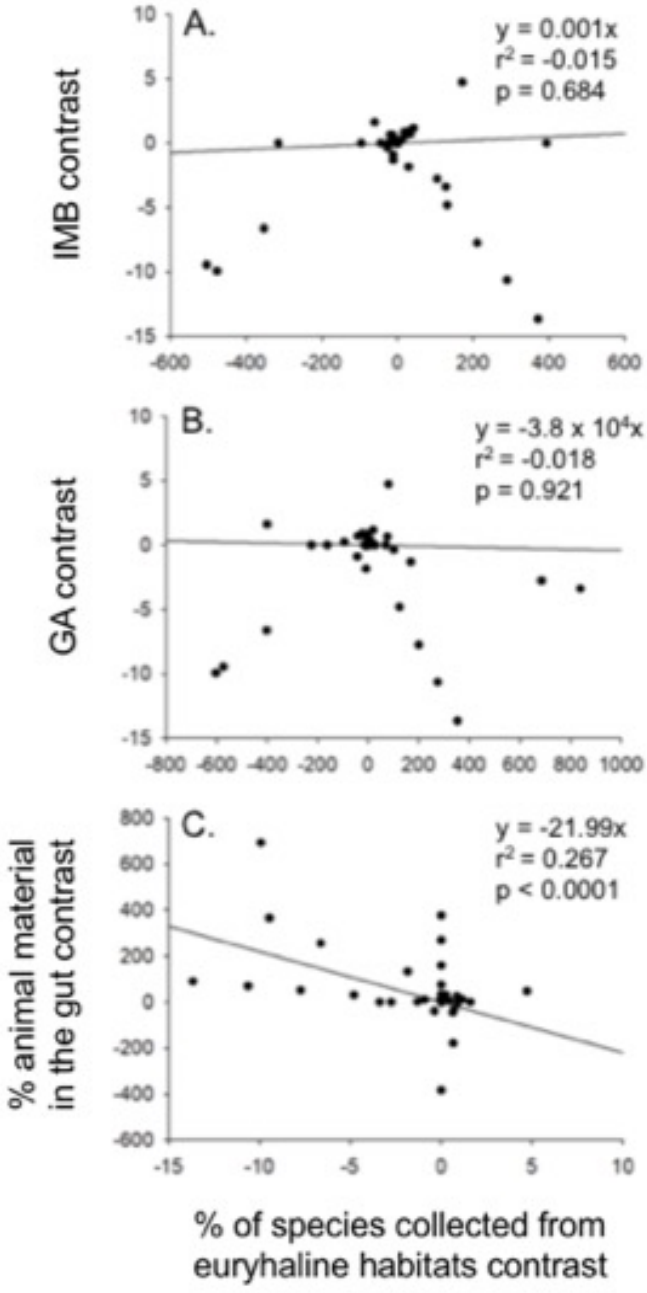
P. picta (GU179237)
P. reticulata (stri4289)
P. reticulata (stri4290)

Fig. 3.8.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r² = -0.015
p = 0.684
IMB contrast
B.
y = -3.8 x 10⁴x
r² = -0.018
p = 0.921
GA contrast
C.
y = -21.99x
r² = 0.267
p < 0.0001
% animal material in the gut contrast
% of species collected from euryhaline habitats contrast

Supplementary Information

Table S.3.1. GenBank accession numbers for genes used to reconstruct *Poecilia* phylogeny.

Sample ID	Species (mtDNA OTU)	*COI*	*ATPase 8/6*	*ND2*	*S7*	Reference
stri8479	*P. cf. gilli*		JX968594			Alda et al. 2013
stri8409	*P. cf. gilli*		JX968593			Alda et al. 2013
stri13333	*P. cf. gilli*		JX968613			Alda et al. 2013
stri8859	*P. cf. gilli*	JX968665	JX968592	JX968711	JX968760	Alda et al. 2013
stri8823	*P. cf. gilli*				JX968761	Alda et al. 2013
stri8806	*P. cf. gilli*	JX968664	JX968591	JX968710	JX968759	Alda et al. 2013
stri13330	*P. cf. gilli*				JX968776	Alda et al. 2013
GU179240	*P. wingei*			GU179240		Meredith et al. 2010
GU179239	*P. wingei*			GU179239		Meredith et al. 2010
DPP-137	*P. wandae*	KP761885	KP761835		KP761935	Ho et al. 2016
DPP-135	*P. wandae*	KP761884	KP761834		KP761934	Ho et al. 2016
DPP-133	*P. wandae*	KP761883	KP761833		KP761933	Ho et al. 2016
DPP-132	*P. wandae*	KP761882	KP761832		KP761932	Ho et al. 2016
DPP-131	*P. wandae*	KP761881	KP761831		KP761931	Ho et al. 2016
DPP-160	*P. vivipara*	KP761880	KP761830		KP761930	Ho et al. 2016
DPP-157	*P. vivipara*	KP761879	KP761829		KP761929	Ho et al. 2016
DPP-156	*P. vivipara*	KP761878	KP761828		KP761928	Ho et al. 2016
DPP-155	*P. vivipara*	KP761877	KP761827		KP761927	Ho et al. 2016
OM82	*P. velifera*	JQ667582				Khedkar et al. 2012
OM81	*P. velifera*	JQ667581				Khedkar et al. 2012
OM102	*P. velifera*	JQ667583				Khedkar et al. 2012
OM101	*P. velifera*	JQ667585				Khedkar et al. 2012
KW11T074	*P. velifera*	KU568973				Van der Walt et al. 2016
CES230	*P. velifera*	KJ669591				Hardy 2014
DPP-166	*P. vandepolli*	KP761869	KP761819		KP761919	Ho et al. 2016
DPP-154	*P. vandepolli*	KP761875	KP761825		KP761925	Ho et al. 2016
DPP-153	*P. vandepolli*	KP761874	KP761824		KP761924	Ho et al. 2016
DPP-152	*P. vandepolli*	KP761873	KP761823		KP761923	Ho et al. 2016
DPP-148	*P. vandepolli*	KP761870	KP761820		KP761920	Ho et al. 2016
DPP-151	*P. vandepolli*	KP761872	KP761822		KP761922	Ho et al. 2016
DPP-149	*P. vandepolli*	KP761871	KP761821		KP761921	Ho et al. 2016
PtherSM1	*P. thermalis*			KF276678		Palacios et al. 2016
PtherS21	*P. thermalis*			KF276679		Palacios et al. 2016
PtherLa1	*P. thermalis*			KF276675		Palacios et al. 2016
PtherL31	*P. thermalis*			KF276677		Palacios et al. 2016

PtherL21	*P. thermalis*			KF276676		Palacios et al. 2016
Psul1	*P. sulphuraria*			HQ677863		Tobler et al. 2010
PsILaGIr1	*P. sulphuraria*			KF276684		Palacios et al. 2016
PsILaGI31	*P. sulphuraria*			KF276686		Palacios et al. 2016
PsILaGI11	*P. sulphuraria*			KF276685		Palacios et al. 2016
PsIBanos1	*P. sulphuraria*			KF276681		Palacios et al. 2016
PsIBan31	*P. sulphuraria*			KF276683		Palacios et al. 2016
PsIBan21	*P. sulphuraria*			KF276682		Palacios et al. 2016
AF080490	*P. sulphuraria*			AF080490		Ptacek and Breden 1999
stri7787	*P. sphenops*				JX968756	Alda et al. 2013
stri7781	*P. sphenops*				JX968755	Alda et al. 2013
stri7780	*P. sphenops*	JX968661	JX968583	JX968707	JX968754	Alda et al. 2013
stri7731	*P. sphenops*	JX968660	JX968582	JX968706	JX968753	Alda et al. 2013
stri7730	*P. sphenops*				JX968752	Alda et al. 2013
stri7729	*P. sphenops*				JX968751	Alda et al. 2013
MEX5011	*P. sphenops*		JX968565			Alda et al. 2013
MEX1107.2	*P. sphenops*		JX968574			Alda et al. 2013
MEX1107.1	*P. sphenops*		JX968573			Alda et al. 2013
DPP-176	*P. salvatoris*		KR707737			Ho et al. 2016
DPP-175	*P. salvatoris*		KR707736			Ho et al. 2016
stri4290	*P. reticulata*	JX968696	JX968650	JX968742	JX968799	Alda et al. 2013
stri4289	*P. reticulata*	JX968695	JX968649	JX968741	JX968798	Alda et al. 2013
RD122	*P. reticulata*	JX968694	JX968648	JX968740	JX968797	Alda et al. 2013
RD121	*P. reticulata*		JX968647			Alda et al. 2013
GU179237	*P. picta*			GU179237		Meredith et al. 2010
GU179236	*P. picta*			GU179236		Meredith et al. 2010
AF031395	*P. picta*			AF031395		Breden et al. 1999
GU179235	*P. parae*			GU179235		Meredith et al. 2010
GU179234	*P. parae*			GU179234		Meredith et al. 2010
AF031396	*P. parae*			AF031396		Breden et al. 1999
stri8747	*P. orri*		JX968605			Alda et al. 2013
stri8706	*P. orri*	JX968671	JX968606	JX968717	JX968771	Alda et al. 2013
stri8549	*P. orri*	JX968670	JX968603	JX968716	JX968770	Alda et al. 2013
strix3352	*P. mexicana*		JX968566			Alda et al. 2013
stri8962	*P. mexicana*	JX968672	JX968607	JX968718	JX968772	Alda et al. 2013
stri8873	*P. mexicana*		JX968608			Alda et al. 2013
stri8607	*P. mexicana*		JX968604			Alda et al. 2013
stri8565	*P. mexicana*		JX968600			Alda et al. 2013
stri8558	*P. mexicana*				JX968764	Alda et al. 2013
stri8365	*P. mexicana*		JX968609			Alda et al. 2013
stri8185	*P. mexicana*		JX968581			Alda et al. 2013
stri8181	*P. mexicana*		JX968580			Alda et al. 2013

stri8084	*P. mexicana*	JX968659	JX968578	JX968705	JX968750	Alda et al. 2013
stri8033	*P. mexicana*		JX968577			Alda et al. 2013
stri7995	*P. mexicana*		JX968576			Alda et al. 2013
stri4993	*P. mexicana*		JX968623			Alda et al. 2013
stri4348	*P. mexicana*	JX968666	JX968596	JX968712	JX968762	Alda et al. 2013
stri4308	*P. mexicana*		JX968597			Alda et al. 2013
stri3148	*P. mexicana*		JX968627			Alda et al. 2013
stri2074	*P. mexicana*	JX968678	JX968622	JX968724	JX968782	Alda et al. 2013
stri2073	*P. mexicana*	JX968677	JX968621	JX968723	JX968781	Alda et al. 2013
stri16781	*P. mexicana*	JX968679	JX968630	JX968725	JX968783	Alda et al. 2013
stri15557	*P. mexicana*		JX968629			Alda et al. 2013
stri15225	*P. mexicana*		JX968631		JX968784	Alda et al. 2013
stri14722	*P. mexicana*		JX968618			Alda et al. 2013
stri14256	*P. mexicana*	JX968673	JX968610	JX968719	JX968773	Alda et al. 2013
stri13887	*P. mexicana*	JX968676	JX968615	JX968722	JX968778	Alda et al. 2013
stri13876	*P. mexicana*	JX968675	JX968615	JX968721	JX968777	Alda et al. 2013
stri13869	*P. mexicana*				JX968780	Alda et al. 2013
stri13868	*P. mexicana*				JX968779	Alda et al. 2013
stri13666	*P. mexicana*		JX968617			Alda et al. 2013
stri13508	*P. mexicana*		JX968616			Alda et al. 2013
stri13420	*P. mexicana*		JX968611			Alda et al. 2013
stri13328	*P. mexicana*				JX968775	Alda et al. 2013
stri13327	*P. mexicana*	JX968674	JX968612	JX968720	JX968774	Alda et al. 2013
stri1245	*P. mexicana*		JX968620			Alda et al. 2013
stri1231	*P. mexicana*		JX968619			Alda et al. 2013
stri11626	*P. mexicana*		JX968624			Alda et al. 2013
stri112	*P. mexicana*		JX968625			Alda et al. 2013
stri1118	*P. mexicana*		JX968628			Alda et al. 2013
SA93	*P. mexicana*		JX968587			Alda et al. 2013
SA92	*P. mexicana*		JX968586			Alda et al. 2013
SA9	*P. mexicana*	JX968663	JX968585	JX968709	JX968758	Alda et al. 2013
SA7	*P. mexicana*	JX968662	JX968584	JX968708	JX968757	Alda et al. 2013
SA104	*P. mexicana*		JX968590			Alda et al. 2013
MEX2881	*P. mexicana*	JX968653	JX968564	JX968699	JX968745	Alda et al. 2013
MEX2880.2	*P. mexicana*	JX968652	JX968563	JX968698	JX968744	Alda et al. 2013
MEX2880	*P. mexicana*		JX968562			Alda et al. 2013
MEX2380	*P. sulphuraria*	JX968656	JX968571	JX968702	JX968749	Alda et al. 2013
MEX2379	*P. sulphuraria*		JX968570		JX968748	Alda et al. 2013
MEX2349	*P. mexicana*		JX968567			Alda et al. 2013
GU10231	*P. mexicana*		JX968579			Alda et al. 2013
DPP-113	*P. mexicana VI*	KP761911	KP761811		KP761911	Ho et al. 2016
DPP-109	*P. mexicana VII**	KP761859	KP761809		KP761909	Ho et al. 2016

DPP-108	*P. mexicana VI*	KP761858	KP761808		KP761908	Ho et al. 2016
DPP-106	*P. mexicana V*	KP761868	KP761818		KP761918	Ho et al. 2016
DPP-104	*P. mexicana V*	KP761867	KP761817		KP761917	Ho et al. 2016
DPP-102	*P. mexicana V*	KP761866	KP761816		KP761916	Ho et al. 2016
DPP-098	*P. mexicana V*	KP761864	KP761814		KP761914	Ho et al. 2016
DPP-017	*P. mexicana VI*	KP761856	KP761806		KP761906	Ho et al. 2016
DPP-011	*P. mexicana V*	KP761863	KP761813		KP761913	Ho et al. 2016
DPP-001	*P. mexicana V*	KP761862	KP761812		KP761912	Ho et al. 2016
stri9780	*P. mexicana*		JX968626			Alda et al. 2013
stri8411	*P. mexicana*		JX968595			Alda et al. 2013
SA116	*P. mexicana*		JX968588			Alda et al. 2013
SA103	*P. mexicana*		JX968589			Alda et al. 2013
DPP-112	*P. mexicana VI*	KP761860	KP761810		KP761910	Ho et al. 2016
DPP-107	*P. mexicana VI*	KP761857	KP761807		KP761907	Ho et al. 2016
DPP-101	*P. mexicana*	KP761865	KP761815		KP761915	Ho et al. 2016
SDNCUA2779	*P. maylandi*	LC153119				Suzuki-Matsubara et al. 2016
Pmlim9	*P. limantouri*			HQ677848		Tobler et al. 2010
Pmlim8	*P. limantouri*			HQ677847		Tobler et al. 2010
Pmlim7	*P. limantouri*			HQ677846		Tobler et al. 2010
Pmlim6	*P. limantouri*			HQ677845		Tobler et al. 2010
Pmlim5	*P. limantouri*			HQ677844		Tobler et al. 2010
Pmlim3	*P. limantouri*			HQ677843		Tobler et al. 2010
Pmlim2	*P. limantouri*			HQ677842		Tobler et al. 2010
Pmlim1	*P. limantouri*			HQ677841		Tobler et al. 2010
PTR105	*P. latipunctata*	JQ935927				Mejia et al. 2012
Platipun	*P. latipunctata*	KP700519				Bagley et al. 2015
DPP-170	*P. latipinna*	KR707741	KR707733		KR707749	Ho et al. 2016
DPP-169	*P. latipinna*	KR707740	KR707732		KR707748	Ho et al. 2016
DPP-168	*P. latipinna*	KR707739	KR707731		KR707747	Ho et al. 2016
DPP-167	*P. latipinna*	KR707738	KR707730		KR707746	Ho et al. 2016
DPP-173	*P. kykesis*	KR707743	KR707735		KR707751	Ho et al. 2016
DPP-171	*P. kykesis*	KR707742	KR707734		KR707750	Ho et al. 2016
DPP-142	*P. koperi*	KP761855	KP761805		KP761905	Ho et al. 2016
DPP-140	*P. koperi*	KP761853	KP761803		KP761903	Ho et al. 2016
DPP-139	*P. koperi*	KP761852	KP761802		KP761902	Ho et al. 2016
DPP-073	*P. koperi*	KP761851	KP761801		KP761901	Ho et al. 2016
DPP-072	*P. koperi*	KP761850	KP761800		KP761900	Ho et al. 2016
DPP-141	*P. koperi*	KP761854	KP761804		KP761904	Ho et al. 2016
stri8574	*P. hondurensis*				JX968768	Alda et al. 2013
stri8568	*P. hondurensis*	JX968668	JX968601	JX968714	JX968765	Alda et al. 2013
stri8534	*P. hondurensis*				JX968766	Alda et al. 2013

stri8520	*P. hondurensis*	JX968669	JX968602	JX968715	JX968769	Alda et al. 2013
stri4414	*P. hondurensis*	JX968667	JX968598	JX968713	JX968763	Alda et al. 2013
stri4323	*P. hondurensis*		JX968599			Alda et al. 2013
stri8566	*P. hondurensis*				JX968767	Alda et al. 2013
RD244	*P. hispaniolana*	JX968691	JX968644	JX968737	JX968794	Alda et al. 2013
RD243	*P. hispaniolana*	JX968690	JX968643	JX968736	JX968793	Alda et al. 2013
stri16226	*P. gillii*		JX968632			Alda et al. 2013
stri4162	*P. gillii_spp 2*	JX968685	JX968638	JX968731	JX968789	Alda et al. 2013
stri1736	*P. gillii_spp 2*	JX968684	JX968637	JX968730	JX968788	Alda et al. 2013
stri3706	*P. gillii*	JX968682	JX968635	JX968728		Alda et al. 2013
stri3615	*P. gillii*	JX968683	JX968636	JX968729	JX968787	Alda et al. 2013
stri1320	*P. gillii*	JX968680	JX968633	JX968726	JX968785	Alda et al. 2013
stri11204	*P. gillii*	JX968681	JX968634	JX968727	JX968786	Alda et al. 2013
DPP-118	*P. gillii*	KP761848	KP761798		KP761898	Ho et al. 2016
DPP-117	*P. gillii*	KP761847	KP761797		KP761897	Ho et al. 2016
DPP-116	*P. gillii*	KP761846	KP761796		KP761896	Ho et al. 2016
DPP-035	*P. gillii*	KP761844	KP761794		KP761894	Ho et al. 2016
DPP-119	*P. gillii*	KP761849	KP761799		KP761899	Ho et al. 2016
ULVECP1	*P. elegans*			KX024009		Weaver et al. 2016
ULVERV4	*P. elegans*			KX024012		Weaver et al. 2016
ULVECP5	*P. elegans*			KX024011		Weaver et al. 2016
ULVECP2	*P. elegans*			KX024010		Weaver et al. 2016
Pel11202D	*P. elegans*			KP943309		Palacios et al. 2016
ULVDJI15	*P. dominicensis*			KX023981		Weaver et al. 2016
ULVDAR4	*P. dominicensis*			KX023979		Weaver et al. 2016
ULVDAR3	*P. dominicensis*			KX023978		Weaver et al. 2016
Pdm11202D	*P. dominicensis*			KP943308		Palacios et al. 2016
DPP-164	*P. dauli*	KP761843	KP761793		KP761893	Ho et al. 2016
DPP-163	*P. dauli*	KP761842	KP761792		KP761892	Ho et al. 2016
SDNCUA2762	*P. chica*	LC153110				Suzuki-Matsubara et al. 2016
KJ697230	*P. chica*			KJ697230		Pollux et al. 2014
stri6445	*P. caucana*	JX968687	JX968640	JX968733	JX968790	Alda et al. 2013
stri14905	*P. caucana*	JX968686	JX968639	JX968732		Alda et al. 2013
DPP-130	*P. caucana*	KP761841	KP761791		KP761891	Ho et al. 2016
DPP-127	*P. caucana*	KP761840	KP761790		KP761890	Ho et al. 2016
DPP-126	*P. caucana*	KP761839	KP761789		KP761889	Ho et al. 2016
DPP-123	*P. caucana*	KP761838	KP761788		KP761888	Ho et al. 2016
DPP-053	*P. caucana*	KP761837	KP761787		KP761887	Ho et al. 2016
DPP-045	*P. caucana*	KP761836	KP761786		KP761886	Ho et al. 2016
MEX2276	*P. catemaconis*	JX968655	JX968569	JX968701	JX968747	Alda et al. 2013
MEX2275	*P. catemaconis*	JX968654	JX968568	JX968700	JX968746	Alda et al. 2013

MEX3800	*P. butleri*	JX968651	JX968561	JX968697	JX968743	Alda et al. 2013
GU179233	*P. branneri*			GU179233		Meredith et al. 2010
GU179232	*P. bifurca*			GU179232		Meredith et al. 2010
CU678	*L. vittata*	JX968689	JX968642	JX968735	JX968792	Alda et al. 2013
CU371	*L. vittata*	JX968688	JX968641	JX968734	JX968791	Alda et al. 2013
RD76	*L. melanonotata*	JX968693	JX968646	JX968739	JX968796	Alda et al. 2013
RD36	*L. melanonotata*	JX968692	JX968645	JX968738	JX968795	Alda et al. 2013

Table S.3.2. Relative abundance of diet items in the gut of each sampled *Poecilia* species.

Species	Diatoms	Green Algae	Cyanobacteria	Animals	Sample Size (N)
P. reticulata	0.18	0.02	0.12	0.68	46
P. parae	NA	NA	NA	NA	0
P. picta	0.17	0.01	0.76	0.06	10
P. vivipara	0.41	0.03	0.50	0.06	30
P. dominicensis	0.57	0.01	0.40	0.02	30
P. hispaniolana	0.31	0.09	0.42	0.18	40
P. caucana	0.48	0.09	0.24	0.19	15
P. kykesis	0.01	0.12	0.85	0.02	25
P. latipinna	0.34	0.04	0.56	0.06	36
P. velifera	0.08	0.03	0.88	0.01	47
P. butleri	0.00	1.00	0.00	0.00	5
P. sphenops	0.45	0.04	0.51	0.00	35
P. gilli	0.54	0.00	0.46	0.00	5
P. mexicana	0.01	0.01	0.95	0.03	24
P. orri	0.02	0.02	0.95	0.01	35

Fig. S.3.1.

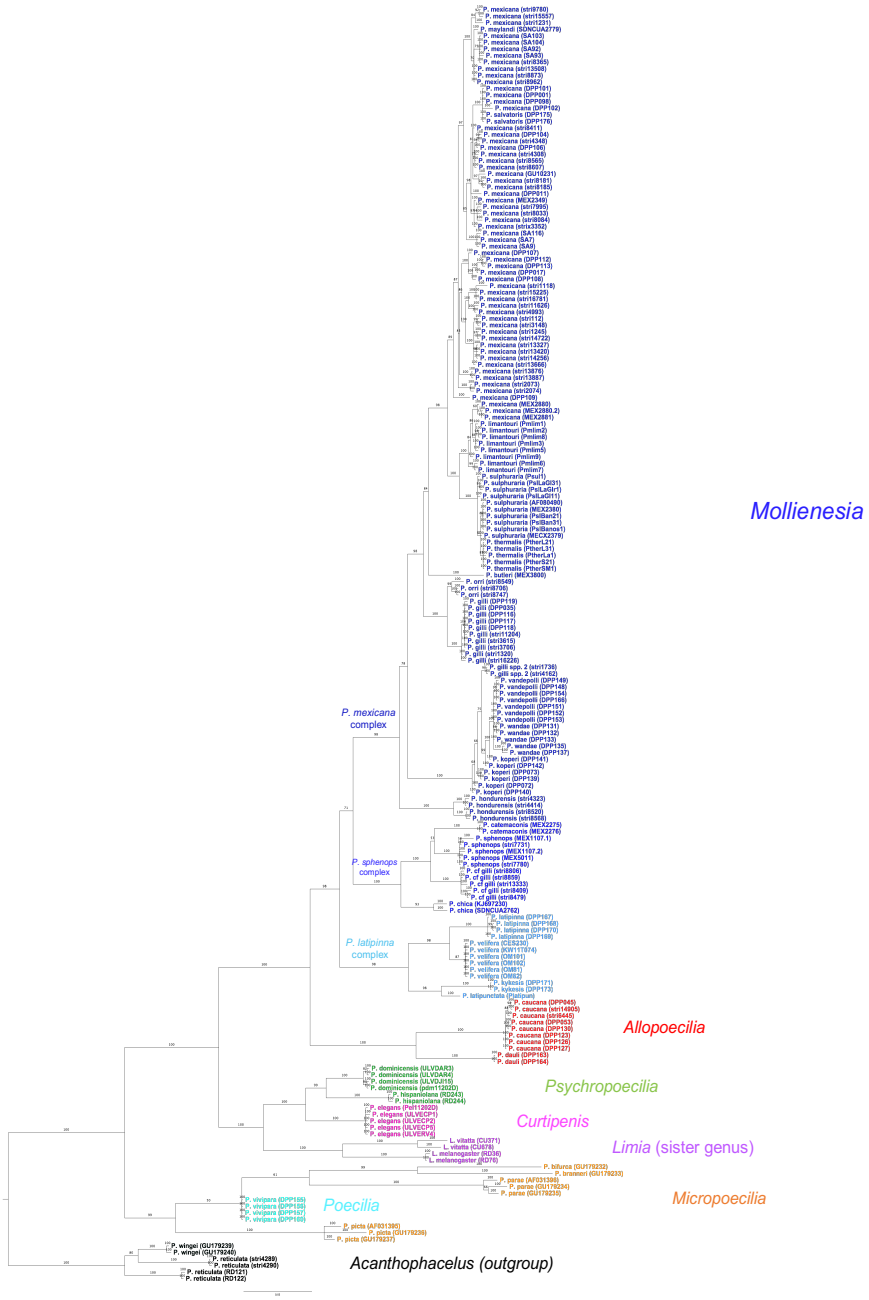

Fig. S.3.1. Bayesian phylogenetic tree (50% majority-rule) derived from mitochondrial genes *Cytochrome Oxidase subunit I*, *ATPase 8/6*, and *NADH dehydrogenase subunit 2* from 36 *Poecilia* and 2 *Limia* species. Genbank ID for each species is listed in parentheses. Species are colored by subgenus.

Fig. S.3.2.

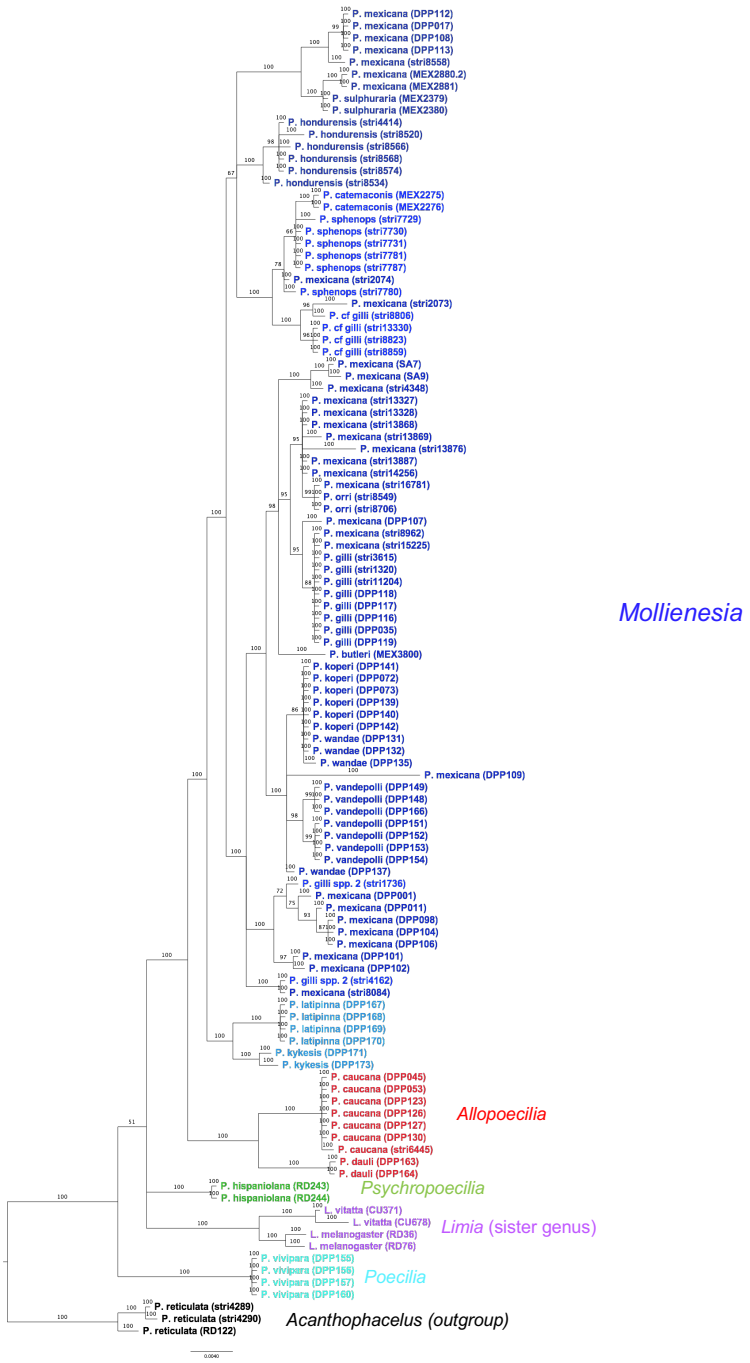

Fig. S.3.2. Bayesian phylogenetic tree (50% majority-rule) derived from ribosomal gene, *S7*, from 36 *Poecilia* and 2 *Limia* species. Genbank ID for each species is listed in parentheses. Species are colored by subgenus.

Fig. S.3.3.

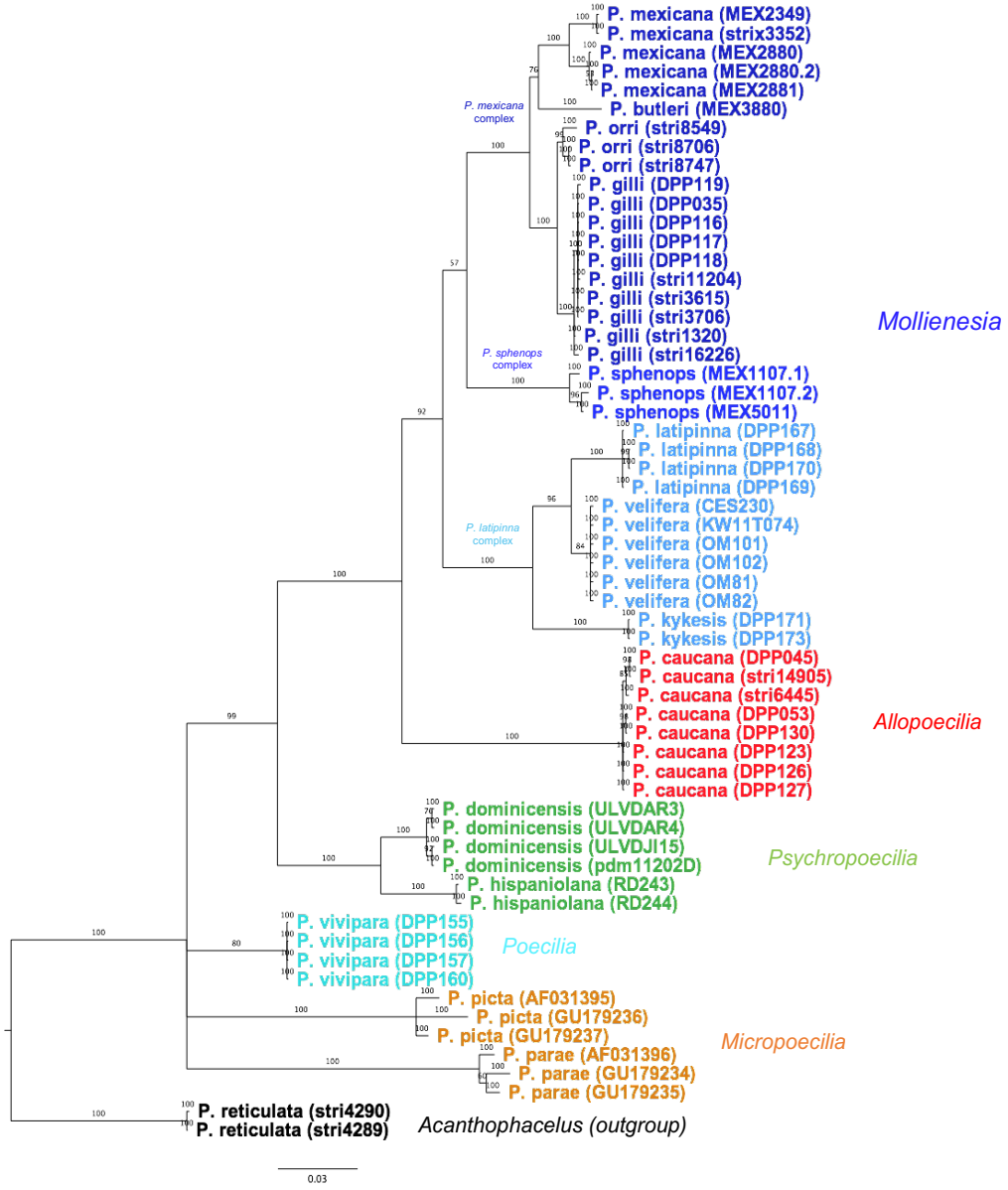

Fig. S.3.3. Pruned Bayesian phylogenetic tree (50% majority-rule) derived from mitochondrial genes *Cytochrome Oxidase subunit I*, *ATPase 8/6*, and *NADH dehydrogenase subunit 2* from 15 *Poecilia* species. Genbank ID for each species is listed in parentheses. Species are colored by subgenus.

Fig. S.3.4.

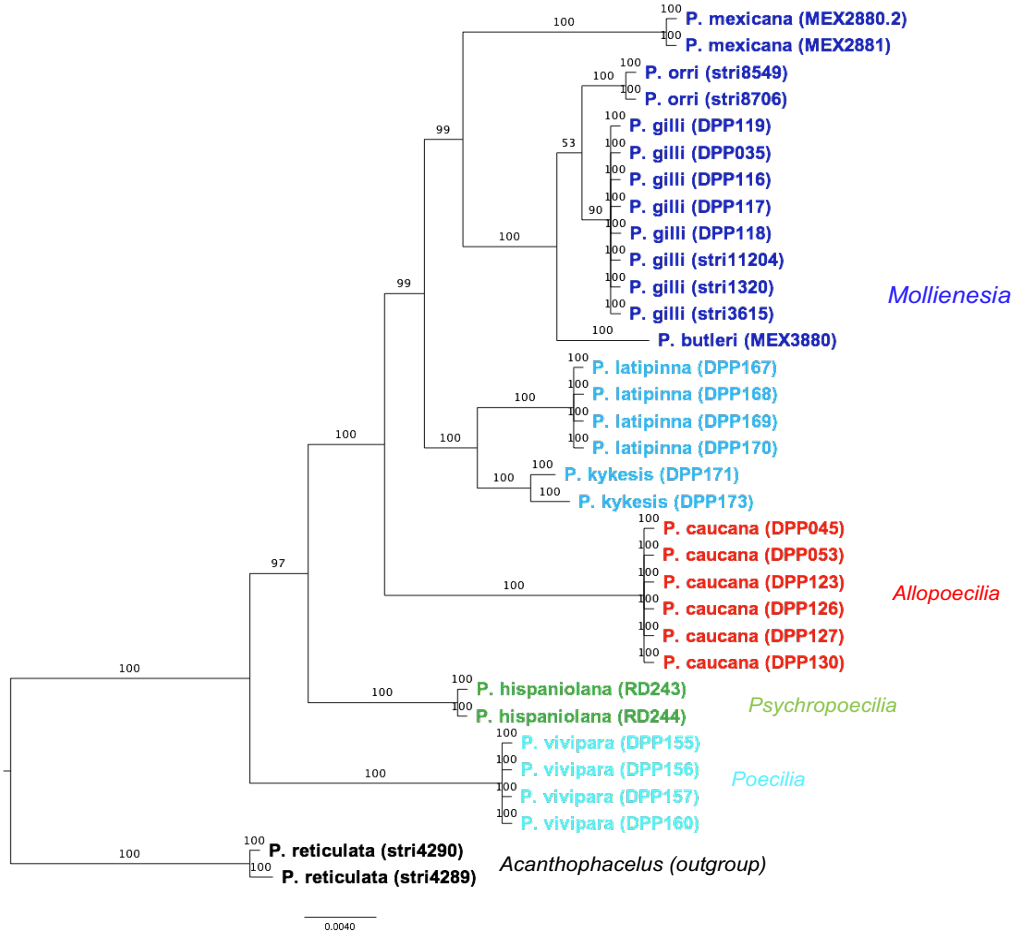

Fig. S.3.4. Pruned Bayesian phylogenetic tree (50% majority-rule) derived from ribosomal gene, *S7*, from 15 *Poecilia* species. Genbank ID for each species is listed in parentheses. Species are colored by subgenus.

Fig. S.3.5.

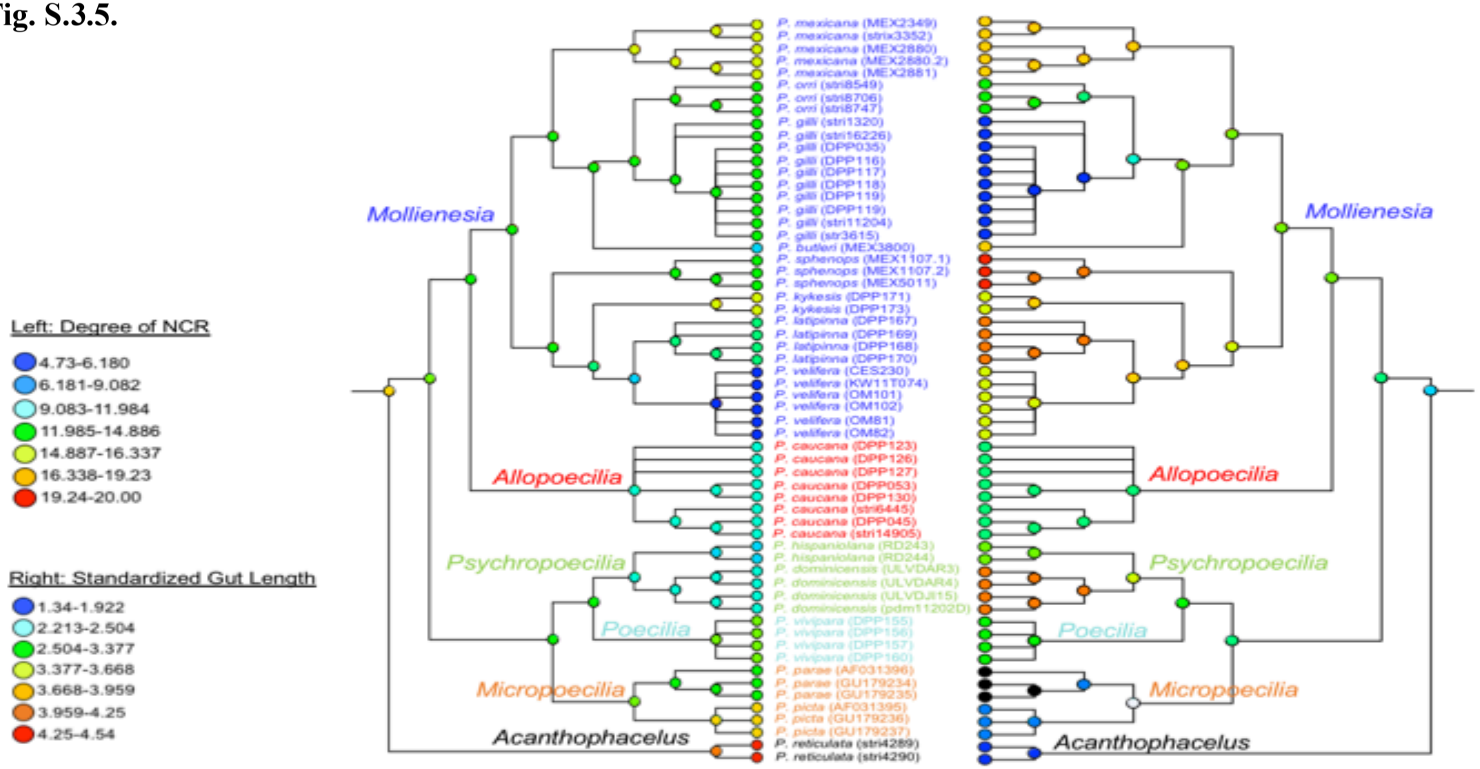

Fig. S.3.5. Maximum Parsimony ancestral character reconstruction for the evolution of neurocranial rotation (left cladogram) and standardized gut length (right cladogram) in the *Poecilia* group. Circles at terminal nodes represent the observed character states for extant species, and pie charts for ancestral nodes show estimated probabilities for reconstructed character states. Maximum likelihood could not be performed because jaw and gut metrics are continuous data. Genbank ID for each species is listed in parentheses. Species are colored by subgenus.

CHAPTER 4

WHEN IS AN HERBIVORE NOT AN HERBIVORE? DETRITIVORY FACILITATES HERBIVORY IN A FRESHWATER SYSTEM

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Abstract

Herbivory is thought to be an inefficient diet, but it independently evolved from carnivorous ancestors in many metazoan groups, suggesting that plant-eating is adaptive in some circumstances. In this study, I tested two hypotheses to explain the adaptive evolution of herbivory: 1) the Heterotroph Facilitation hypothesis (herbivory is adaptive because herbivores supplement their diets with heterotrophic microbes); and 2) the Lipid Allocation hypothesis (herbivory is adaptive because algae, which have high lipid concentrations, are nutritionally similar to carnivory). I tested these hypotheses using enclosure cages placed in the Everglades and stocked with Sailfin Mollies (*Poecilia latipinna*), a native herbivore. Using shading and phosphorus addition (P), I manipulated the heterotrophic microbe and lipid composition of colonizing epiphyton and examined the effects of varying food quality on Sailfin Molly life history.Epiphyton grown in 'shade only' conditions had a 55% increase in bacterial fatty acids and 34% lower ratios of saturated + monounsaturated to polyunsaturated fatty acids relative to the other treatments. Biovolume of heterotrophic microbes varied throughout the experiment, with a 697% increase at 3 weeks and 98% decrease at 6 weeks compared to the other treatments. Gut contents revealed that fish fed selectively on epiphyton to compensate for apparent deficiencies in the available food. Fish raised in 'shade only' cages experienced the highest survival, which was best explained by autotrophic abundance and algal- and bacterial-derived fatty acids at 3 weeks (2-6x more likely than alternative models with ΔAICc > 2.00), and by percentage of bacterial fatty acids in the diet at 6 weeks (3-8x more likely than alternative models with ΔAICc > 2.00).

There were no differences in fish growth among treatments. Autotrophic lipids play a role in early fish life history, but I did not find these to be the best predictors of life history later in the juvenile period. Instead, heterotrophic lipids facilitated the herbivorous diet and enhanced survival of juvenile fish in this experiment. Bacterial fatty acid content of the diet promoted herbivore survival, consistent with the Heterotroph Facilitation hypothesis. This is the first study to explicitly contrast Heterotrophic Facilitation and Lipid Allocation hypotheses for the adaptive evolution of herbivory in an aquatic system.

Keywords Diet evolution, diet quality, fatty acids, freshwater herbivore, herbivory, detritivory, structural equation model

Introduction

Herbivory appears to be at an evolutionary disadvantage compared to omnivorous or carnivorous strategies (Sanchez & Trexler, 2016). Omnivores and carnivores consume animal prey that are high in nutritional value (Mattson, 1980; Sterner & Hessen, 1994; Choat & Clements, 1998; Karban & Agrawal, 2002), and omnivores have the additional advantage of supplementing their diets with abundant and easy to obtain plant items (Coll & Guershon 2002; Diehl, 2003). Obtaining comparable energy from an exclusively herbivorous diet is difficult because food items are nutritionally variable and are usually accompanied by structural and/or biochemical barriers to assimilation (Mattson, 1980; Porter & McDonough, 1984; Horn 1989, Chivers & Langer, 1994; Sterner & Hessen, 1994; Choat & Clements, 1998; and others).

Furthermore, herbivores may be limited by foraging time and/or space by predators and competitors, by the ability to produce digestive or detoxifying enzymes (see Karban & Agrawal, 2002), or the amount of time it takes for food to pass through the gut (Horn, 1989; Bruggeman et al. 1994; Bellwood, 1995; Choat & Clements 1998). Despite these difficulties, there is evidence from many metazoan groups that herbivores evolved from carnivorous ancestors and that herbivory has been maintained alongside these animal-containing diets in the majority of these lineages (e.g. Vermeij, 1992; deMaintenon, 1999; Van Damme, 1999; Vermeij & Lindberg, 2000; Bellwood, 2003; Eubanks, Styrsky & Denno, 2003; Espinoza, Wiens & Tracy, 2004; Pauls et al. 2008, Bellwood et al. 2014; Reisz & Frobisch, 2014).

Because few studies have addressed the adaptive significance of the herbivorous diet, Sanchez and Trexler (2016) reviewed the freshwater herbivory literature to identify conditions where eating plants might be adaptive over eating animals. They defined freshwater "herbivory" as the consumption of algae and/or phytoplankton, and an "herbivore" as an organism that mainly eats these primary producers, but may indirectly consume detritus (consumes > 50% primary producers). Furthermore, they defined a "carnivore" as an organism that eats animals (consumes > 50% animal material) and refer to an "omnivore" as an organism that eats both plants and animals (see Sanchez and Trexler 2016 for a review). The term "food quality" is used to describe the nutritional worth of a diet item to a consumer and could be defined by macronutrient (e.g., nutritional ecology) or elemental (e.g., stoichiometry) composition, where food items are rich in protein or phosphorus, respectively.

However, elements may not be ideal currencies to answer questions about organismal diets since they form the basis of the molecules that animals often select for (e.g., proteins, carbohydrates and lipids; e.g., Sperfeld et al. 2017), and thus, I use the stoichiometric definition of food quality with caution. Food quality may also be defined as the ratio of food energy content to that assimilated by consumers. Regardless of the convention used, "food quality" is a relative term and can only be interpreted relative to other diets (e.g., a diet item can be both high and low quality depending on the comparison diet), and respective of organismal diet adaptations (e.g., "high quality" is defined differently for carnivores vs. herbivores). Under these designations, they concluded that herbivory is favored when higher quality food is limiting, or when plants provide important dietary elements that are unavailable in carnivore diets, such as lipids (e.g. Martin-Creuzburg, Beck & Freese, 2011) or antioxidants (e.g. Pike et al. 2007). Additionally, herbivores may overcome limiting resource quality by indirectly supplementing their diets with heterotrophic microbes that are associated with primary producers (see Sanchez & Trexler, 2016 for a review).

The idea that herbivores obtain nutrients from supplementary sources is well-established (see White, 1985). In aquatic systems, herbivores (e.g. macroinvertebrates) are nutrient-limited, and their nutrition is likely supported by detrital inputs (Hall, Likens & Malcolm, 2001). The heterotrophic microbes that decompose detritus promote higher growth in macroinvertebrate families, compared to algal diets in both lab (e.g. Fuller, Fry & Roelofs, 1988; Fuller & Fry, 1991; Fuller, Kennedy & Nielsen, 2004) and field studies (e.g. Mulla & Lacey, 1976; Edwards & Meyer, 1990).

Furthermore, growth rates of *Daphnia* spp. have been shown to increase when diets are supplemented with heterotrophic bacteria (e.g. Martin-Creuzburg, Beck & Freese, 2011), emphasizing the importance of heterotrophs in the herbivorous diet. However, diets composed only of heterotrophic bacteria are of poor quality for herbivores (e.g. *Daphnia magna*), suggesting that they also rely on autotrophs for essential lipids like sterols or polyunsaturated fatty acids (e.g. Goulden, Henry & Tessier, 1982; Tessier, Henry & Goulden, 1983; Schmidt & Jonasdottier, 1997; Weers & Gulati, 1997; Martin-Creuzburg, Wacker & von Elert, 2005; Martin-Cruezburg, von Elert & Hoffman, 2008; Martin-Creuzburg, Beck & Freese, 2011). The nutritional requirements of freshwater herbivores blur the distinction between herbivory and detritivory and emphasizes the idea that there are few "true" herbivores in nature (White 1985).

Although previous studies have shown that aquatic herbivores rely heavily on nutrients originating from both heterotrophic microbes and autotrophic bacteria and algae (e.g. Bowen, 1984; Martin-Creuzburg, Wacker & von Elert, 2005; Smoot & Findlay, 2010; Martin-Creuzburg, Beck & Freese, 2011; Belicka et al., 2012), none have explicitly identified these dietary elements as facilitators of the evolution of herbivory. Here, I test two alternative hypotheses for the adaptive evolution of the herbivorous diet: 1) Heterotroph Facilitation hypothesis, which states that herbivory may be adaptive by supplementing herbivory with heterotrophic microbes that are indirectly consumed along with primary producers; and 2) Lipid Allocation hypothesis, which states that autotrophic bacteria and algae, the primary source of essential fatty acids, may be as beneficial as a carnivorous diet (Sanchez & Trexler, 2016).

These hypotheses are not mutually exclusive, as the definition of heterotroph facilitation includes ingestion of autotrophic organisms. The key difference between these ideas lies in the nutritional source (heterotrophic vs. autotrophic microbes) that is the driver of life history.

The Florida Everglades is an ideal system to test these adaptive hypotheses because periphyton mats are the primary basal resource in this area (Browder, Gleason & Swift, 1994; Trexler et al., 2015) and are composed of complex assemblages of autotrophs (green algae, diatoms and cyanobacteria) and heterotrophs (fungi and bacteria; Gaiser et al., 2004). Both autotroph and heterotroph components of Everglades periphyton communities respond rapidly to changes in water chemistry (Pan et al., 2000; Noe, Childers & Jones, 2001; Gottlieb, Gaiser & Lee, 2015), such as when phosphorus is added, because the Everglades ecosystem is naturally oligotrophic (Gaiser et al., 2004). Furthermore, lipid profiles of Everglades primary and secondary consumers are comprised of both algal and bacterial-specific fatty acids (Belicka et al., 2012), suggesting that both items are important in their diet. One of these species is the native Sailfin Molly (*Poecilia latipinna)*, a small livebearing fish (Fig. 4.1). Most *Poecilia* fishes are omnivorous (*P. vivipara*, Andrade et al., 2000; *P. mexicana*, Tobler, 2008), but stable isotope and gut content studies indicate that Sailfin Mollies are primarily herbivorous (Loftus, 2000; pers. obs.) and incorporate prokaryotic resources into their diet (Belicka et al., 2012). I used Sailfin Mollies held in enclosures in an Everglades marsh to test the alternative hypotheses of the adaptive advantage of the herbivorous diet.

I predict that Sailfin Mollies will show increased growth and/or survival in response to increased dietary heterotrophic bacteria if the Heterotroph Facilitation hypothesis is the mechanism supporting the evolution of herbivory in the Everglades. Alternatively, Sailfin Mollies will show increased growth and/or survival in response to algal-derived fatty acids if the Lipid Allocation hypothesis is supported by this study.

Methods

I maintained juvenile Sailfin Mollies in cages in the Everglades from September 17, to October 29, 2015, to evaluate the effects of varying herbivorous diets on fish growth and survival. The 24 cages were 1-m^2 and had five surfaces covered in 1-mm mesh (sides and bottom) and were open at the top. The cages were randomly placed in a slough located in the central Everglades (25°49'41.23"N, 80°37'53.41"W), with an average depth of 30 cm and temperature of 29.4 $\pm$ 1.2 C. Light and temperature were tracked throughout the experiment using HOBO® data loggers. Artificial vegetation strips (2.54 cm wide) made of black plastic sheeting (0.154 mm thick) attached to wire frames for a total of 150 strips per frame, simulating natural stem density of this area (described in Chick et al. 2008), were added to each cage. The length of the strips was trimmed to water depth (approximately 28 cm) in the field so that they did not float on the surface and shade the water column. Periphyton was collected from the slough, cleaned of invertebrates, and 2000mL was placed into each cage to encourage growth of epiphytic algae on the artificial vegetation strips. An initial periphyton sample was brought back to the lab on ice and subsequently frozen for nutrient and lipid analyses (ambient periphyton).

Sailfin Mollies were born in the lab and raised on Tetramin® flake food for 6 weeks prior to the start of the experiment. They were measured (average standard length, SL) and transplanted to the field cages (n=6 fish per cage; N=36 total fish/treatment) 1 week following cage set-up. This lag-time allowed epiphyton to colonize the artificial vegetation strips prior to the addition of consumers. For detailed experimental set-up, refer to Figs. S.4.1-S.4.2 located in the supplementary material.

I manipulated colonizing epiphyton by adding phosphorus (P) and manipulating light (shade or light) to create a gradient of food quality for herbivores. Because the Everglades is a naturally oligotrophic system, both autotrophic and heterotrophic species within Everglades periphyton mats can be easily manipulated by addition of phosphorus. Each cage was randomly assigned to one of four treatments: 1) light + P; 2) light only; 3) shade + P; 4) or shade only. Phosphorus (Na_2HPO_4) was added at a concentration of 15 µg/L weekly to 'shade + P' and 'light + P' cages. Previous studies manipulated the concentration of P across the Everglades landscape to understand the resulting changes to basal resources (e.g. McCormick and O'dell 1996; McCormick et al. 1996; Noe et al. 2001; Gaiser et al., 2005). They found that low and intermediate P concentrations induced changes in Everglades primary producers, but high concentrations resulted in a phase shift (e.g. Gaiser et al., 2005). The lower and intermediate nutrient concentrations occur in nature, in areas where Sailfin Mollies are native. Therefore, I chose the intermediate concentration (15 µg/L) in order to manipulate epiphyton composition within the natural dietary range of Sailfin Mollies.

Following dosing, these cages were wrapped with 3-mm clear plastic to prevent P from seeping and potentially affecting nearby cages. Everglades periphyton incorporates P very quickly (Noe et al., 2003); therefore, plastic covers were removed after 24 hours to permit water circulation. Shading was accomplished by covering cages with 3 sheets of greenhouse shade cloth to achieve approximately 75% reduction in ambient light (modified methods of Fuller, Kennedy & Nielsen, 2004).

Epiphyton, periphyton and biofilms growing on the mesh cages were all potential herbivorous diet items available to grazing by fish. At 3 and 6 weeks, a sample of periphyton, a 5x5 cm scrape taken from the mesh wall inside the cage (herein referred to as 'biofilm'), and 30 plastic strips were removed from each cage and brought back to the lab. At 3 weeks, two fish from each cage were euthanized with an overdose of MS-222, and the remaining fish were returned to their respective cage. At 6 weeks, all remaining fish were measured, euthanized and brought back to the lab on ice. Fish lacking gonopodial development (gonopodium, the male sexual organ) were dissected to assess fecundity.

Potential food items were processed for molecular analyses in the laboratory. Because plastic strips were various lengths from field trimming, standardized 30.5 cm sections from each were scraped of epiphytic algae. Subsamples of epiphyton, periphyton, and biofilm scrapes were kept for heterotroph and autotroph abundance estimates. Known volumes of epiphyton, periphyton or biofilms were stained with either DAPI (4', 6-diamidino-2-phenylindole) for bacteria (Hobbie, Daley & Jasper, 1977), or labelled lectin (fluorescien-labelled wheat germ agglutinin) for fungal counts (e.g. Wanchoo, Lewis & Keyhani, 2009).

Heterotrophs were counted under a microscope at 40x using epifluorescence and autotrophs were counted using standard light microscopy at 40x magnification. Counts were transformed into total cells/mL of material. Volume of bacteria, fungi and common algal species were estimated by taking measurements from of 20-30 representative organisms for each from high-definition photos and multiplied by total cells/mL to yield biovolume (μm^3/mL) estimates.

The remaining samples (including fish) were freeze-dried and prepped for fatty acid (sent to Microbial ID laboratory, Newark, DE) and stoichiometric analyses (CNP; sent to Southeastern Research Center, Florida International University, Miami, FL). Elements (CNP) are likely not ideal currencies for nutrition, but I measured the ratio of carbon to phosphorus, C:P, and ratio of nitrogen to phosphorus, N:P (molar ratios) to compare nutritional and stoichiometric methodologies. Fatty acid data were categorized by diet tracers (Table 4.1; Belicka et al., 2012) and further organized into polyunsaturated fatty acids (PUFAs), saturated fatty acids (SAFAs), and monounsaturated fatty acids (MUFAs). Fatty acids were also organized by common essential fatty acids that are known to affect fish growth and development: eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic (ARA) (see Saikia & Nandi, 2010 for a review). In addition to fatty acid and nutrient analyses, algal, bacterial, and fungal biovolume were used to calculate a ratio of autotrophic to heterotrophic organisms (A:H biovolume ratio). These metrics were analyzed in fish tissues and potential food sources to evaluate their influence on fish life history.

Statistical analyses

Growth curves of poeciliid fishes are more strongly asymptotic in males than females (Snelson, 1989), a phenomenon well-described for Sailfin Mollies (Snelson, 1982; Travis et al., 1989). There were a few mature males at the end of the experiment; however, there were no developing embryos found in the ovaries of the females, so growth curves were treated as if fish had not yet matured. Fish standard length (mm) measurements at 0, 3 and 6 weeks were analyzed using 2-way analysis of variance (ANOVA). Fish standard length (mm) measurements by week were analyzed using the quadratic equation. Growth rates were estimated by dividing the slope at 2/3 of that curve by the number of days to obtain the growth of Sailfin Mollies per day in mm (following Trexler & Travis, 1990). A logit model with maximum likelihood was fit to fish survival data to predict the probability of survival, p, where logit(p) = log (p/1-p). Temperature and light availability, potential influences on fish growth and survival, were analyzed for each treatment using one-way ANOVA.

Multiple potential diet items were present in the experimental cages (biofilm, epiphyton, and periphyton described above), therefore, it was important to determine which diet items had the strongest influence on fish size and survival. I assumed that items that best predict fish life history were those that dominated the diets of fish in the experimental cages. Several food-quality variables were measured for all potential diet types.

These were: ratio of carbon to phosphorus (C:P), ratio of nitrogen to phosphorus (N:P), relative fatty acid content, percentage of algal- and bacterial- derived fatty acids, fatty acid class (PUFA, SAFA, MUFA, ratio of SAFA+MUFA: PUFA), essential fatty acids (EPA, DHA, ARA, ratio of EPA:DHA), A:H biovolume, and proportion of edible algae (proportion of green algae relative to cyanobacteria). Stoichiometry of algal types (C:P) was analyzed using one-way Analysis of Variance (ANOVA) and Tukey post hoc tests. Algal species from epiphyton, periphyton, and fish guts were analyzed using 2-way multivariate analysis of variance (MANOVA) with Tukey post-hoc tests. To determine the probability that a fish would eat a diet item based on its availability in the environment, I calculated Ivlev's Electivity Index, $E_i = (r_i - p_i)/ (r_i + p_i)$, where r_i = the proportion of the item found in the gut and p_i = the proportion of the item found in the environment. Calculated indices were rounded to the nearest whole number. A value of $E_i < 0$ suggests that fish are avoiding the dietary item, $E_i > 0$ suggests that the fish are actively selecting the item, and $E_i = 0$ means that items are eaten in proportion to their availability in the environment. These were calculated for each treatment at both 3- and 6- weeks.

Relative fatty acid content of all samples was calculated by dividing the mass spectrometry peak area for each by the mg of dry weight of each sample. Although not a quantitative measure, it allowed us to compare relative fatty acid content across experimental treatments. These relative values were analyzed using two-way ANOVA with post-hoc tests. Two- way ANOVAs were also used to assess any differences in the percentage of algal and bacterial- derived fatty acids across treatments.

Fatty acid classes (PUFA, SAFA, MUFA) and essential fatty acids (EPA, DHA, ARA) comprising each algal type were analyzed using MANOVA tests, followed by Tukey multivariate comparison tests (ln transformed). Ratios of fatty acid classes (SAFA+MUFA: PUFA, ln transformed) and essential fatty acids (EPA:DHA, log +1 transformed) were analyzed using two-way ANOVA. Biovolume of heterotrophs and autotrophs were converted to ratios (A:H biovolume), natural-log transformed (ln), and analyzed using 2-way ANOVA. Proportion of edible algal species comprising each of the diet type was analyzed using two-way ANOVA.

Epiphyton and biofilm were not statistically different from each other across all measured variables, so biofilm was dropped from future analyses. Of the 14 measured characteristics, variables that were statistically different ($\alpha \leq 0.05$) between epiphyton and periphyton were used as independent variables in Discriminant Function Analysis, with diet type as the grouping variable. These were: C:P, A:H biovolume, SAFA+MUFA:PUFA, EPA:DHA, and percent of bacterial fatty acids. Discriminant scores for the function explaining the most variance were used as input variables for Structural Equation Models (SEM; Grace, 2006), which were fit using AMOS (Arbuckle, 2014)). Using Principal Component Analysis, fish size and survival rates were collapsed into a single score that was also an input for SEMs.

I used SEMs to evaluate the information in alternative hypothesized pathways that the treatments (light and nutrient manipulation) may affect the consumers through their impact on primary producers. The first set of 3 models were designed to test the linkages between potential food items and fish life history.

Paths were varied between epiphyton, periphyton and fish life history in each model. Models were compared using Akaike's Information Criterion (AIC) by calculating ΔAICc (ΔAICc = AIC_i –min AICc, where i=model i), Akaike weight ($AIC_w = (e^{(-0.5 *\Delta AIC_i)}/\sum(e^{(-0.5 *\Delta AIC_r)}$), Relative likelihood ($L_r$), and Evidence Ratios ($w_{min}/w_j$, where w_{min} = AIC_w for the model with the smallest ΔAICc and w_j = AIC_w for the current model; Anderson & Burnham, 2002). Path coefficients (regression weights) were assessed to determine which variables best predicted life history. Following Anderson & Burnham (2002), models with ΔAICc < 2.0 were considered equally explanatory. These models were fit for both 3- and 6-weeks.

I tested the alternative adaptive hypotheses by determining which food quality parameter influenced fish life history. The Heterotroph Facilitation hypothesis predicts that heterotrophs in the diet promote herbivore life history, and the Lipid Allocation hypothesis predicts that algal-derived fatty acids are driving herbivore success. Therefore, I chose to evaluate A:H biovolume (measure of heterotroph and autotroph abundance), percentage of bacterial fatty acids (measure of bacterial quality), and SAFA+MUFA:PUFA ratios (algal-derived fatty acids; measure of algal quality) as independent variables in a second set of SEMs designed to test the adaptive hypotheses. Paths were varied between these 3 diet variables and fish life history to produce a total of 7 models. Similar to the first SEMs, models were compared using AIC.

Results

Epiphyton

3 weeks. The cages differed in phosphorus availability, but this did not translate to differences in epiphyton stoichiometry at 3 weeks. Ratios of C:P and N:P were similar for epiphyton grown in all treatments ($F_{3,8}$= 0.079, P= 0.970 and $F_{3,8}$= 0.367, P= 0.779, respectively).

Unlike stoichiometry, autotroph species composition was affected by light. Epiphyton samples collected at 3 weeks were comprised of similar algal species among treatments (Wilks' Lambda= 0.053, $F_{15,11}$= 0.912, P= 0.588), but differed in relative abundance of edible algal types. Specifically, light drove the proportion of edible algae comprising epiphyton (light: $F_{1,8}$= 11.487, P= 0.010), where epiphyton from the 'light only' treatments had 18% higher relative abundance of diatoms, solitary green, and filamentous green species than 'light +P' epiphyton, and 94% higher abundance of these species than the shaded treatments. Furthermore, the shaded treatments were comprised of 50% inedible species (filamentous and coccoid cyanobacteria), as compared to 3% and 18% for 'light only' and 'light +P', respectively.

Biovolume differed between light and shade treatments. The biovolume of heterotrophs ($F_{3,8}$=0.415, P=0.747) were not different between treatments, however, 'shade + P' and 'shade only' epiphyton was comprised of 238% and 887% greater autotrophs (respectively) compared to the other treatments (Light: $F_{1,8}$=5.430, P=0.048; P: $F_{1,8}$=5.913, P=0.041).

Consequently, the ratios of A:H biovolume for 'shade + P' and 'shade only' epiphyton were approximately 140% and 697% greater than the light treatments, respectively (Light: $F_{1,8}$= 8.820, P=0.018).

The relative abundance of types of fatty acids was affected by the both light and P treatments. There were no differences the relative fatty acid content of epiphyton ($F_{3,8}$= 1.348, P= 0.279), the percentage of algal-derived fatty acids ($F_{3,8}$= 1.534, P= 0.279) or the percentage of bacterial-derived fatty acids ($F_{3,8}$= 0.299, P= 0.825). The relative abundances of PUFA's and SAFA's comprising the 3-week epiphyton samples were driven by both light and nutrient addition (Wilks' Lambda= 0.162, $F_{9,15}$= 10.31, P= 0.009), where 'light only' epiphyton had approximately 59% higher PUFAs and 'light + P' epiphyton had 8% higher SAFAs than the other treatments. However, only light drove the relative abundance of MUFAs (Wilks' Lambda= 0.135, $F_{3,15}$= 12.811, P= 0.005). The shaded treatments had 10% higher MUFAs than the light treatments. Nutrient addition affected the SAFA+MUFA:PUFA ratios, where 'light + P' and 'shade + P' epiphyton had approximately 61% and 27% higher ratios relative to the other epiphyton types, respectively (phosphorus: $F_{1,8}$= 28.946, P= 0.002). Epiphyton grown in different treatments were not significantly different in EPA, DHA and ARA (Wilks' Lambda= 0.288, $F_{9,15}$= 0.915, P= 0.543). For a summary of results, refer to Table 4.2.

6 weeks. Stoichiometric differences between treatments were revealed at 6 weeks. The C:P ratio of epiphyton was influenced by nutrient addition (phosphorus: $F_{1,8}$= 5.316, P= 0.05), where epiphyton grown in 'light + P' and 'shade + P' cages had 28% and 3% lower C:P ratios than the other treatments.

However, there were no differences in N:P ratios of epiphyton growing in the different treatments ($F_{3,8}$= 2.703, P= 0.116).

Differences in autotroph species composition disappeared at 6 weeks. There were no differences in algal community structure (Wilks' Lambda= 0.004, $F_{15,3}$= 1.407, P= 0.433) or in edible algae proportions across treatments ($F_{3,8}$= 1.125, P= 0.395). Biovolume of autotrophs and heterotrophs was affected by both light and P at 6 weeks. Shaded treatments showed an 85% and 75% ('shade +P' and 'shade only', respectively) decrease in heterotroph biovolume relative to light treatments ($F_{3,8}$= 1.570, P=0.271). Conversely, 'light + P' treatments showed 65% decreased autotrophic biovolume (Light x P: $F_{1,8}$= 36.72, P<0.0001) relative to all other treatments. As a result, light treatments had relatively low A:H ratios (approx. 98% decrease) compared to shaded treatments (Light: $F_{1,8}$= 5.088, P=0.04). These ratios also increased in magnitude from 3-week epiphyton.

Similar to 3-week epiphyton, the relative abundance of types of fatty acids was affected by the both light and P treatments. There were no differences in the relative fatty acid content of 6-week epiphyton ($F_{3,8}$=0.254, P= 0.857) or the percentage of algal fatty acids ($F_{3,8}$= 1.580, P= 0.269). Differences in bacterial fatty acid composition became evident at 6 weeks (Light: $F_{1,8}$= 8.854, P=0.018), where the 'shade only' and 'shade + P' treatments had 55% and 28% higher percentages than the other treatments, respectively. The relative abundance of PUFA's, SAFA's and MUFA's and the SAFA+MUFA:PUFA ratios were the same (Wilks' Lambda= 0.415, $F_{9,15}$= 0.713, P= 0.690 and $F_{15,3}$= 0.075, P= 0.591, respectively).

In addition, epiphyton grown in different treatments were not significantly different in EPA, DHA and ARA (Wilks' Lambda= 0.234, $F_{9,15}$= 1.337, P= 0.299). For a summary of results, refer to Table 4.3. For detailed epiphyton results for both 3- and 6-week time periods, refer to Table S.4.1 located in the supplementary material.

Periphyton

3 weeks. At 3 weeks, stoichiometric ratios of periphyton were consistent across treatments. Ratios of C:P and N:P were not different across treatments or from ambient periphyton ($F_{3,8}$= 0.551, P= 0.662 and $F_{3,8}$= 0.231, P= 0.872, respectively). Periphyton C:P and N:P was different from that of epiphyton ($F_{1,8}$= 142.32, P< 0.001 and $F_{1,8}$= 19.83, P<0.001, respectively), as periphyton had 110% higher C:P and 23% higher N:P ratios. Autotroph species composition of 3-week periphyton was driven by light. Periphyton samples were similar in algal composition among treatments (Wilks' Lambda= 0.514, $F_{6,14}$= 0.920, P= 0.509), but differed in relative abundance of edible algal types. Light drove the proportion of edible algae comprising periphyton (Light: $F_{1,8}$= 5.23, P= 0.05), where the light treatments had approximately 63% higher abundance of edible species than the shaded treatments.

The A:H biovolume ratios of 3-week periphyton were driven by P-addition. Periphyton grown in the 'light + P' and 'shade + P' treatments had 24% and 425% higher A:H biovolume than 'light only' and 'shade only' periphyton, respectively (phosphorus: $F_{1,8}$= 0.129, P= 0.003).

The relative abundance of types of fatty acids in periphyton was similar across treatments at 3 weeks. The percentage of algal and bacterial-derived fatty acids (Wilks' Lambda= 0.743, $F_{6,14}$= 0.411, P= 0.884), and the relative fatty acid content ($F_{3,8}$= 0.919, P= 0.474) were not different among treatments. The proportion of PUFAs, SAFAs and MUFAs (Wilks' Lambda= 0.452, $F_{9,15}$= 0.633, P= 0.752) as well as the SAFA+MUFA:PUFA ratios of 3- week periphyton were similar across treatments ($F_{3,8}$= 1.392, P= 0.314). Essential fatty acid composition (EPA, DHA, ARA) of periphyton was not different across treatments (Wilks' Lambda= 0.635, $F_{6,14}$= 0.595, P= 0.730), but periphyton had non-detectable levels of DHA (i.e. 0.0% by weight), which was significantly lower than epiphyton ($F_{1,8}$= 88.17, P<0.0001). For a summary of results, refer to Table 4.2.

6 weeks. Similar to 3-week periphyton, stoichiometric ratios of periphyton were not different across treatments. Ratios of C:P and N:P were consistent across treatments ($F_{3,8}$= 0.487, P= 0.701 and $F_{3,8}$= 0.438, P= 0.732, respectively), however, ambient periphyton was stoichiometrically different than 6-week periphyton from the experimental treatments ($F_{4,9}$= 5.965, P= 0.013), with 64% and 2% greater C:P and N:P ratios, respectively.

Autotroph species composition of 6-week periphyton was not driven by light, in contrast to periphyton at 3 weeks. Periphyton samples collected at 6 weeks were similar in algal species composition among treatments (Wilks' Lambda= 0.191, $F_{15,3}$= 0.980, P= 0.510) and in the proportion of edible algal species ($F_{3,8}$= 0.757, P= 0.549). The A:H biovolume ratios of 6-week periphyton were driven by light and nutrients.

Periphyton in the 'light + P' cages had 96% lower A:H ratio than 'light only' periphyton, and 74% lower ratio than the shaded treatments (Light x P: $F_{1,8}$= 5.211, P= 0.05).

The relative abundance of types of fatty acids in periphyton were similar across treatments at 6 weeks. The percentage of algal and bacterial-derived fatty acids (Wilks' Lambda= 0.679, $F_{6,14}$= 0.499, P= 0.799) and the relative fatty acid content ($F_{3,8}$= 0.170, P= 0.913) were not different among treatments. The proportion of PUFAs, SAFAs and MUFAs (Wilks' Lambda= 0.453, $F_{9,15}$= 0.630, P= 0.755) as well as the SAFA+MUFA:PUFA ratios were similar across treatments ($F_{3,8}$= 0.961, P= 0.457). Essential fatty acid composition (EPA, DHA, ARA) of 6-week periphyton was not different across treatments (Wilks' Lambda= 0.703, $F_{3,15}$= 1.125, P= 0.395), but periphyton was significantly lower in DHA than epiphyton ($F_{1,8}$= 50.01, P<0.001). For a summary of results, refer to Table 4.3. For detailed periphyton results for both 3- and 6-week time periods, refer to Table S.4.2 located in the supplementary material.

Fish

3 weeks. Juvenile Sailfin Molly survival, but not growth rate, was affected by the treatments. There were no differences in the sizes of juvenile fish stocked in each cage at the start of the experiment ($F_{3,8}$= 0.207, P=0.891). The light cages were approximately 2 degrees warmer than the shaded cages ($F_{3,51}$= 7.617, P< 0.0001), but this did not translate into differences in fish growth, as all fish were similar sizes at week 3 ($F_{3,8}$= 1.597, P= 0.265). However, there were differences in fish survival among treatments. Specifically, fish in the 'shade only' had the greatest survival compared to all other treatments (X^2 = 14.979, P =0.001).

Fish reared in the 'light + P' treatment experienced the lowest survival, which was 30% less than fish in the 'shade only' treatment (Fig. 4.2b).

Stoichiometric differences in fish tissues were evident at 3 weeks. Fish reared in the experimental treatments had 81% greater C:P ratios and 73% greater N:P ratios in their tissues relative to initial, lab-reared fish fed commercial food (C:P, $F_{4,9}$= 5.293, P=0.018; N:P, $F_{4,9}$= 4.238, P=0.034). Furthermore, fish in the light treatments showed 28% higher C:P ratios than those reared in the shaded treatments (Light: $F_{1,8}$=6.557, P= 0.034), but there were no differences in N:P ratios in fish reared in the different treatments ($F_{3,8}$= 1.411, P= 0.309).

The experimental treatments did not affect autotrophic species composition of 3-week fish guts. The algal composition of 3-week fish guts (Wilks' Lambda= 0.253, $F_{18,37}$= 1.297, P= 0.245; Fig 4.3a) and the relative abundances of edible algae were similar across treatments ($F_{3,8}$= 0.414, P= 0.748). There were some fish with invertebrate parts present in guts at both time periods (< 1% of total gut material), but these values were not significantly different across treatments. Although these values were similar, Ivlev's Electivity Index varied for fish eating the different epiphyton types because available food varied among treatments. Indices suggested that fish reared in the light treatments consumed green algal species in proportion to their availability in the environment, whereas those in the shaded treatments actively selected green algae. In addition, fish reared in the 'light only' treatment proportionally consumed cyanobacteria as they were available, and fish in the other treatments selectively fed on cyanobacterial species.

Fish in all treatments selectively fed on diatoms, and consumed cyanobacterial filaments in proportion to their availability (Fig. 4.4a; Supplementary Table S.4.4).

The differences in relative abundance of fatty acids in fish tissues were subtle at 3 weeks. There were no differences in relative fatty acid content of fish tissues across treatments ($F_{3,8}$=1.362, P=0.322), or in the relative abundance of algal and bacterial-derived fatty acids in the fish tissues across experimental treatments (Wilks' Lambda= 0.728, $F_{6,14}$= 0.840, P= 0.533).

The relative amounts of PUFAs and SAFAs in fish tissues were marginally different (Wilks' Lambda= 0.102, $F_{9,15}$= 2.549, P= 0.054). The shaded treatments revealed a 10% increase in PUFAs, whereas 'light only' fish had 36% lower SAFA abundance in their tissues. Despite these differences, the SAFA+MUFA: PUFA ratios were the same for fish tissues at 3 weeks ($F_{3,8}$= 2.658, P= 0.120). There were no differences in essential fatty acids (EPA, DHA, ARA) in fish tissues (Wilks' Lambda= 0.277, $F_{9,15}$= 1.140, P= 0.396), but initial fish tissues had 91% higher DHA than fish tissues from experimental treatments ($F_{4,10}$=3.940, P=0.036). For a summary of results, refer to Table 4.2.

6 weeks. Similar to 3-week data, there were differences in Sailfin Molly survival, but not growth rate at 6 weeks. The light cages were still 2 degrees warmer than the shaded cages ($F_{3,51}$= 4.376, P= 0.007), but all cage temperatures decreased by 2 degrees in the second half of the experiment. This temperature change did not affect fish growth, as all fish grew at similar rates during time period 3-6 weeks ($F_{3,8}$=1.877, P= 0.212; Fig. 4.2a) and achieved similar sizes at 6 weeks ($F_{3,8}$= 1.425, P=0.305).

Fish raised in the 'shade only' treatment experienced 53% higher survival relative to fish reared in the nutrient addition treatments (X^2 = 15.837, P <0.0001). Fish reared in the 'light only' treatments experienced the lowest survival (Fig. 4.2b).

There were stoichiometric differences in fish tissues at 6 weeks. Similar to 3-week fish tissues, fish in the 'light +P' and 'light only' treatments had 32% and 45% higher ratios of C:P than 'shade +P' and 'shade only' fish, respectively ($F_{4,9}$= 24.22, P<0.001). Fish raised in the light treatments also had higher tissue N:P ratios, at 27% higher than 'shade +P' fish and 18% higher than 'shade only' fish ($F_{4,9}$= 8.481, P=0.006).

The algal composition of 6-week fish guts was marginally different across treatments. Fish reared in 'light + P' and 'light only' treatments had higher proportions of diatoms (200 % increase) and green algae (900% increase) in their guts (Wilks' Lambda= 0.179, $F_{18,37}$= 1.774, P= 0.077). These fish reared in light treatments also had 99% lower abundances of both coccoid and filamentous cyanobacteria in their guts than fish from the shaded treatments (Fig. 4.3b). However, the proportion of edible algal species present in the guts were not different across treatments ($F_{3,8}$=0.810, P= 0.523). There were some fish with invertebrate parts present in guts at both time periods (< 1% of total gut material), but these values were not significantly different across treatments. Ivlev's Electivity Index (E_i) reflected differences in fish guts at 6 weeks.

Indices suggested that fish reared in the 'light + P' treatment avoided diatoms, consumed green algae in proportion to their availability in the environment and avoided all other algal types.

Those in the 'light only' treatments consumed all algae in proportion to their availability, except cyanobacteria. Fish in both shaded treatments selectively consumed diatoms. 'Shade + P' also selectively chose green algae, and avoided cyanobacteria. But 'shade only' fish ate green and cyanobacterial species in proportion to their availability in the environment (Fig. 4.4b; Supplementary Table S.4.4).

Differences in relative abundance of fatty acids in fish tissues were revealed at 6 weeks. The abundance of fatty acids in fish tissues was influenced by light (Light: $F_{1,8}$=6.641, P=0.033), where 'light +P' fish were comprised of 3x greater fatty acid abundance than 'shade only' fish. But, there were no differences between experimental treatments and fatty acid content of initial fish ($F_{3,8}$=1.362, P=0.322), or in the relative abundance of algal and bacterial- derived fatty acids in the fish tissues across experimental treatments (Wilks' Lambda= 0.430, $F_{6,14}$= 1.051, P= 0.441). At 6-weeks, fish reared in the 'shade only' treatments had 19% lower abundances of MUFAs, whereas initial fish tissues were 76% higher in PUFAs compared to experimental fish (Wilks' Lambda= 0.009, $F_{12,15}$= 5.575, P= 0.002). Ratios of SAFA+MUFA: PUFAs were the same for experimental fish, but were 124% higher than those of initial fish ($F_{4,9}$= 12.203, P= 0.002). At 6 weeks, 'shade only' fish had higher abundances of both DHA (60% increase) and ARA (71% increase) in their tissues relative to fish in other treatments (Wilks' Lambda= 0.082, $F_{9,15}$= 2.931, P= 0.033). Still, initial fish tissues were 84% higher in DHA compared to the experimental treatments at week 6 ($F_{4,10}$=13.148, P=0.001). For a summary of results, refer to Table 4.3. For detailed periphyton results for both 3- and 6-week time periods, refer to Table S.4.3.

Testing adaptive hypotheses

Based on ΔAICc values and evidence ratios, SEMs suggested that epiphtyon was the primary food source for Sailfin Mollies in this study (Fig. 4.5; Table 4.4). In addition, Akaike weights for the alternative models ('epiphyton + periphyton' and 'periphyton only') suggest that the best-fit model is 3x more likely than the others. Path coefficients for the linkages between periphyton and fish life history were negative in all models, and those between epiphyton and life history were positive in all models, suggesting that epiphyton positively influenced fish life history and periphyton did not. Based on this evidence, I concluded that epiphyton, and not periphyton, was the preferred food source for fish in this study. This information was used to inform the second group of structural equation models that were designed to test the Heterotroph Facilitation and Lipid Allocation hypotheses.

To test the alternative hypotheses, I varied the paths between diet metrics (A:H biovolume, the percentage of bacterial-derived fatty acids, SAFA+MUFA:PUFA ratio) and fish life history to produce 7 models for each time period, and an additional set of models that linked 3-week epiphyton characteristics to 6-week fish. Based on ΔAICc values and evidence ratios, the best fit model suggests that all 3 diet metrics influence fish life history at 3 weeks. There are several equally supported models (Table 4.5), but based on the path coefficients, they all suggest that fish life history trait values increase in proportion to A:H biovolume ratio. Path coefficients also show that fish size and survival decrease with increasing bacterial fatty acid percentage and SAFA+MUFA:PUFA ratio at 3 weeks (Fig. 4.6).

According to their evidence ratios, these supported models are between 3-6x more likely than those with poor fit (ΔAICc > 2.00). However, at 6 weeks, 'A:H + Bac. FA %', 'Bac. FA % + FA ratio' and 'Bac. FA %' models were the best supported based on ΔAICc values. Evidence ratios and path coefficients suggest that bacterial fatty acid percentage alone predicts fish life history 3x better than the other supported models, and 3-9x better than the models with no support (Fig. 4.7; Table 4.5). Models comparing 3-week diets to diets of 6-week fish, have similar support as 6-week models, and also suggest that increased bacterial fatty acid percentage best predicted fish life history. (Fig. 4.8; Table 4.5).

Discussion

I found evidence that detritivory facilitates herbivory, supporting the suggestion that "true" herbivory is rare in nature (White 1985). This study indicated that herbivorous Sailfin Mollies benefit from a diet supplemented with heterotrophic microbes, consistent with the Heterotroph Facilitation hypothesis. In this experiment, increased algal biovolume, increased proportion of monounsaturated fatty acids, and decreased percentage of bacterial fatty acids in the diet best predicted early Sailfin Molly life history (6-9 weeks of age). However, later in development (9-12 weeks of age), cages with high heterotroph fatty acid production yielded the highest juvenile survival. These results indicate that prior to maturation, Sailfin Mollies benefit from a mixed diet of autotrophic and heterotrophic food sources. The Lipid Allocation hypothesis focuses on algal-derived lipids as the main driver of herbivore success, and was therefore not supported in this study.

Rather, I show that heterotrophs supplement algal diets, and the quality (e.g. fatty acid abundance) of these microbes strongly influences herbivore life history by increasing survival by up to 53%. However, because Sailfin Mollies did not reach sexual maturity at the end of this experiment, I am unable to determine any potential trade-offs between survival and reproductive output, or if heterotrophic bacteria are important in the reproductive phase. Furthermore, these findings do not explain why herbivory exists as an alternative to a carnivorous diet, although I do provide a justification for how herbivory is sustained in a natural setting. Finally, these findings confirm that "herbivory" in aquatic systems may routinely include detritivory and that 'green' food webs may be less common than thought (Moore et al. 2004).

Although some authors have examined the influence of dietary heterotrophs on herbivore life history (e.g. Bowen, 1984; Smoot & Findlay, 2010; Belicka et al., 2012), it is not typically recognized as a fundamental part of the herbivorous diet (White, 1985). Many studies have assessed diet quality effects on life history using stoichiometry, polyunsaturated fatty acids, or indices like algal edibility, but, these diet measures were not retained in the model that best fit these data. The ecological stoichiometry literature assumes that diets with lower C:P ratios are the highest quality for consumers, and consumer tissues will reflect these diets by having high C:P levels (Sterner & Elser, 2002). This was not the case in this study as fish with the highest survival ('shade only') were consuming epiphyton with high C:P ratios and had tissues with low C:P ratios, although P did not appear to be limiting in the diet of fish in the field cages.

This finding was not surprising because animals catabolize and metabolize molecules, not individual elements (Raubenheimer et al. 2009; Sperfeld et al. 2017). The nutritional ecology literature suggests that food items with high PUFA content are of higher quality (e.g. Müller-Navarra et al., 2004; Persson & Vrede, 2006), but I show that the highest surviving fish ('shade only') consumed epiphyton with low SAFA+MUFA: PUFA ratios, similar to 'light only' fish who showed relatively low survival. Edibility indices have also been used as a simple measure of food quality (e.g. Geddes & Trexler, 2003; Trexler et al., 2015), where higher proportion of green algae and diatoms relative to cyanobacteria indicates a higher quality food source (Lamberti, 1996; Steinman, 1996; Sullivan & Currin, 2000). In this study, fish with high survival ('shade only') were in cages with epiphyton with relatively high abundances of both filamentous and coccoid cyanobacteria. However, Ivlev's Electivity index showed that fish were feeding selectively on higher quality food items when they were not abundant in the environment. This suggests that estimations of food quality that are derived from edibility indices are compromised by feeding strategies, and are thus not reliable indicators of food quality. If this study had been conducted with a higher density of fish, increasing competition and precluding selective feeding, these results may have differed. The density used was reflective of ambient densities in the study area.

While this study did not find support for the Lipid Allocation hypothesis, algal-derived fatty acids are important to herbivores. Fatty acids originating from primary producers fuel growth, survival and reproduction of herbivores.

However, these results emphasize that life history characteristics are optimized when these diets are supplemented with heterotrophs (e.g. Martin-Creuzburg, Wacker & von Elert, 2005; Martin-Creuzburg, Beck & Freese, 2011). I found that diets with high levels of both bacterial-derived fatty acids and PUFAs (e.g. 'shade + P' epiphyton) were sub-optimal for herbivore survival. Similarly, diets with intermediate levels of PUFAs, and decreased bacterial-derived fatty acids (e.g. 'light only'), or diets with decreased levels of both fatty acid types (e.g. 'light + P') are not ideal for herbivores. Diets with intermediate levels of PUFAs (e.g. 'shade only') were the best available diets in this study, providing evidence that detritivory represents an important part of the herbivorous diet as predicted by the Heterotroph Facilitation hypothesis.

I began this research to explore the conditions that would favor the evolution of an herbivorous diet from a carnivorous or omnivorous one. This study suggests that including heterotrophic microbes in the diet can compensate for the generally poor quality of aquatic plant foods. However, this study does not address how other nutritional components (e.g., macronutrients, algal starch, etc.) may have changed in response to the experimental manipulations, or their interactive effects on herbivore life history. Furthermore, I am unable to conclude why carnivory would be largely abandoned in herbivore-detritivores like Sailfin Mollies. Other adaptive hypotheses outlined by Sanchez & Trexler (2016) may fill this gap. For example, ancestral herbivores may have invaded habitats with few predators and animal prey, but high in microbial and autotrophic biofilms.

Because the mechanisms supporting the evolution of herbivory remain unknown, I hope this study is a step in establishing a research framework that will allow us to more fully understand herbivory from an adaptation perspective.

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Table 4.1. Sources of fatty acid tracers used in this study (modified from Belicka et al. 2012).

Carbon Source (grouped by fatty acids used in this study)	References
Bacteria (15:0i, 15:0a, 15:0n, 17:0i, 17:0a, 17:0n, 18:1w7, 19:1)	
Odd carbon number fatty acids, 15:0i, 15:0a, 17:0i, 17:0a, 18:1w7	Findlay and Dobbs (1993); Napolitano (1999) and references therein; Volkman et al. (1980)
Algae (16:3, 18:3w3, 18:4, 18:3w6, 20:4w6, 20:5w3 (EPA), 20:4, 22:4w6, 22:5w3, 22:5w6, 22:6w3)	
14:0, 16:1w7: multiple sources, but high in diatoms and some cyanobacteria	Napolitano (1999) and references therein
C_{16} PUFA: green algae and diatoms	Kates and Volcani (1966); Cranwell et al. (1990); Napolitano (1999)
18:3w3: green algae, cyanobacteria	Ahlgren et al. (1992); Dalsgaard et al. (2003)
18:3w6: cyanobacteria	Napolitano (1999)
18:4w3, 18:5w3, 22:6w3: dinoflagellates	Ahlgren et al. (1992); Dalsgaard et al. (2003)
20:5w3, ratio of 20:5w3 to 22:6w3: diatoms	Napolitano (1999); Dalsgaard et al. (2003)

Table 4.2. Summary of results showing differences between experimental treatments for epiphyton, periphyton and fish tissues at 3 weeks. FA ratio= SAFA+MUFA:PUFA ratio. Upward facing triangles indicate relatively high values, whereas downward facing triangles indicate relatively low values. Values that are not statistically significant are indicated by "ns". Blanks indicate metrics that could not be measured.

	EPIPHYTON				PERIPHYTON				FISH TISSUES			
Metric	**Light + P**	**Light only**	**Shade + P**	**Shade only**	**Light + P**	**Light only**	**Shade + P**	**Shade only**	**Light + P**	**Light only**	**Shade + P**	**Shade only**
C:P	ns	ns	ns	ns	ns	ns	ns	ns	▲	▲	▼	▼
N:P	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
A:H biovolume	▼	▼	▲	▲	▲	▼	▲	▼	---	---	---	---
Relative FA content	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Percent algal FA (%/wt)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Percent bacterial FA (%/wt)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
FA ratio	▲	▼	▲	▼	ns	ns	ns	ns	ns	ns	ns	ns
EPA:DHA	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
ARA (%/wt)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Edible algal spp.	▲	▲	▼	▼	▲	▲	▼	▼	ns	ns	ns	ns

Table 4.3. Summary of results showing differences between experimental treatments for epiphyton, periphyton and fish tissues at 6 weeks. FA ratio= SAFA+MUFA:PUFA ratio. Upward facing triangles indicate relatively high values, whereas downward facing triangles indicate relatively low values. Values that are not statistically significant are indicated by "ns". Blanks indicate metrics that could not be measured.

	EPIPHYTON				PERIPHYTON				FISH TISSUES			
Metric	**Light + P**	**Light only**	**Shade + P**	**Shade only**	**Light + P**	**Light only**	**Shade + P**	**Shade only**	**Light + P**	**Light only**	**Shade + P**	**Shade only**
C:P	▼	▲	▼	▲	ns	ns	ns	ns	▲	▲	▼	▼
N:P	ns	ns	ns	ns	ns	ns	ns	ns	▲	▲	▼	▼
A:H biovolume	▼	▼	▲	▲	▼	▲	▼	▼	---	---	---	---
Relative FA content	ns	ns	ns	ns	ns	ns	ns	ns	▲	▲	▼	▼
Percent algal FA (%/wt)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Percent bacterial FA (%/wt)	▼	▼	▲	▲	ns	ns	ns	ns	ns	ns	ns	ns
FA ratio	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
EPA:DHA	ns	ns	ns	ns	ns	ns	ns	ns	▼	▼	▼	▲
ARA (%/wt)	ns	ns	ns	ns	ns	ns	ns	ns	▼	▼	▼	▲
Edible algal spp.	ns	ns	ns	ns	ns	ns	ns	ns	▲	▲	▼	▼

Table 4.4. Comparison of structural equation models used to predict diet type (epiphyton vs. periphyton). AIC_w = Akaike weights, w_{min}/w_j = Evidence ratios. ΔAICc values ≤ 2 are highlighted in bold.

Model	Description	ΔAICc	AIC_w	w_{min}/w_j
1	Epiphyton + Periphyton	2.19	0.20	0.33
2	**Epiphyton**	**0.00**	**0.61**	**1.00**
3	Periphyton	2.38	0.19	0.30

Table 4.5. Comparison of structural equation models used to test 'Heterotrophic facilitation' and 'Lipid allocation' hypotheses. A:H = A:H biovolume, Bac. FA = percentage of bacterial fatty acids, FA ratio= SAFA+MUFA:PUFA ratio. AIC_w = Akaike weights, w_{min}/w_j = Evidence ratios. ΔAICc values ≤ 2 are highlighted in bold.

	3 weeks			**6 weeks**			**3→6 weeks**		
Model Description	**ΔAICc**	**AIC_w**	***w_{min}/w_j***	**ΔAICc**	**AIC_w**	***w_{min}/w_j***	**ΔAICc**	**AIC_w**	***w_{min}/w_j***
A:H + Bac. FA+ FA ratio	**0.00**	**0.26**	**1.00**	3.95	0.05	0.14	3.28	0.07	0.19
A:H+ Bac. FA	**0.32**	**0.22**	**0.85**	**1.95**	**0.15**	**0.38**	**1.91**	**0.15**	**0.38**
A:H+ FA ratio	**0.62**	**0.19**	**0.73**	4.36	0.04	0.11	4.16	0.05	0.12
Bac. FA + FA ratio	2.36	0.08	0.31	**2.00**	**0.14**	**0.37**	**1.32**	**0.20**	**0.51**
A:H	**1.77**	**0.11**	**0.41**	2.36	0.12	0.31	2.23	0.12	0.33
Bac. FA	2.15	0.09	0.34	**0.00**	**0.39**	**1.00**	**0.00**	**0.38**	**1.00**
FA ratio	3.73	0.04	0.15	2.49	0.11	0.29	5.15	0.03	0.08

Figure Legends

Fig. 4.1. (A) Male Sailfin Molly (*Poecilia latipinna*). (B) Female Sailfin Molly (*Poecilia latipinna*). Images retrieved from the Florida Museum Ichthyology Collection, University of Florida, Gainesville, FL, © George Burgess.

Fig. 4.2. (A) Standard length (mm) of juvenile Sailfin Mollies raised on biofilms grown in various treatments. (B) Probability of survival (p') of juvenile Sailfin Mollies showing high survival of those grown in 'shade only' treatments.

Fig. 4.3. (A) Relative abundance of algal species comprising fish guts reared in various treatments at 3 weeks. Guts are composed of similar proportions of diet items across treatments, and are dominated by diatoms and cyanobacteria. (B) Relative abundance of algal species comprising fish guts reared in various treatments at 6 weeks. Fish guts from light treatments are composed of similar proportions of diet items, and are dominated by cyanobacteria. Those from shaded treatments also contain a high proportion of cyanobacteria, but also have higher proportions of green filamentous algal species than fish guts from the light treatments.

Fig. 4.4. (A) Ivlev's Electivity Index (L_i) calculated for fish reared in various treatments at 3 weeks. All fish expect those in 'Shade + P' cages are actively avoiding filamentous cyanobacteria. (B) Ivlev's Electivity Index (L_i) calculated for fish reared in various treatments at 6 weeks. Fish reared in 'Light + P' cages are avoiding all diet types, whereas, all other fish are only avoiding coccoid cyanobacterial species.

Fig. 4.5. The structural equation model with the best fit showing epiphyton at 3 weeks as the best predictor of fish life history at 3 weeks. Numbers indicate regression coefficients for each path analyzed.

Fig. 4.6. The structural equation model with the best fit showing A:H biovolume, the percentage of bacterial fatty acids and the ratio of SAFA+MUFA:PUFA (FA ratio) at 3 weeks as the best predictor of fish life history at 3 weeks. Numbers indicate regression coefficients for each path analyzed.

Fig. 4.7. The structural equation model with the best fit showing 6-week bacterial fatty acid percentage as the best predictor of fish life history at 6 weeks. Numbers indicate regression coefficients for each path analyzed.

Fig. 4.8. The structural equation model with the best fit showing 3-week bacterial fatty acids percentage as the best predictor of fish life history at 6 weeks. Numbers indicate regression coefficients for each path analyzed.

Figure 4.1.

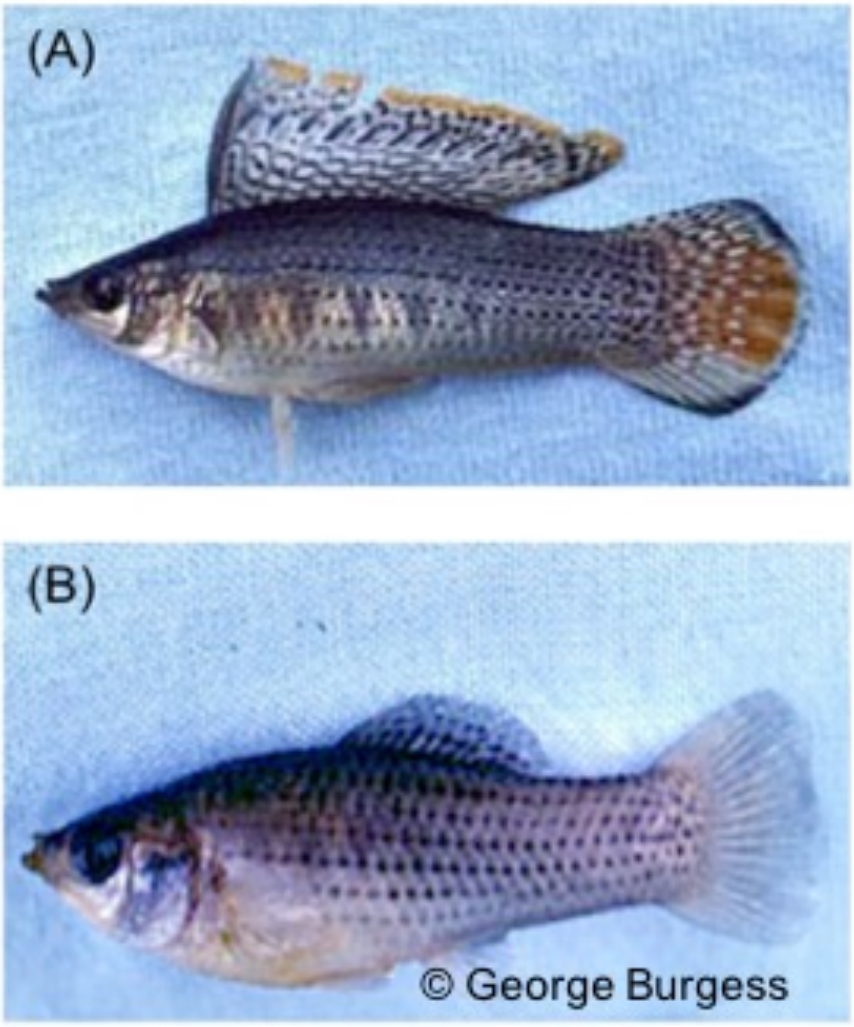


Figure 4.2.

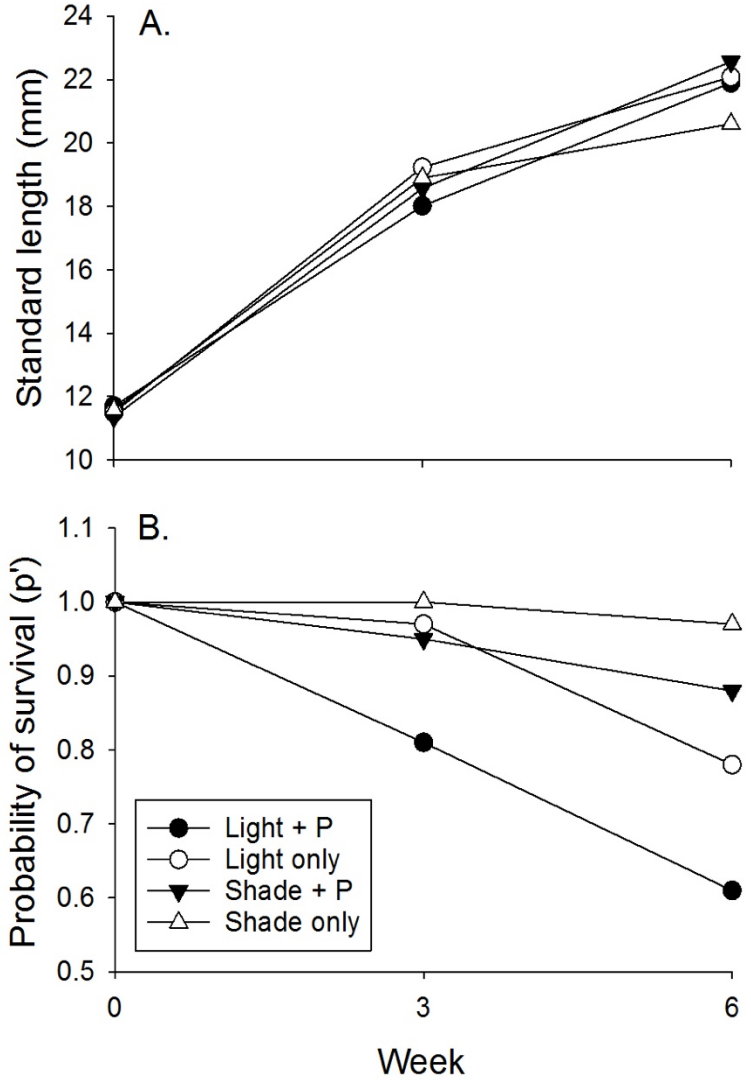

A.
Standard length (mm)
24
22
20
18
16
14
12
10
B.
Probability of survival (p')
1.1
1.0
0.9
0.8
0.7
0.6
0.5
Light + P
Light only
Shade + P
Shade only
0
3
6
Week

Figure 4.3.

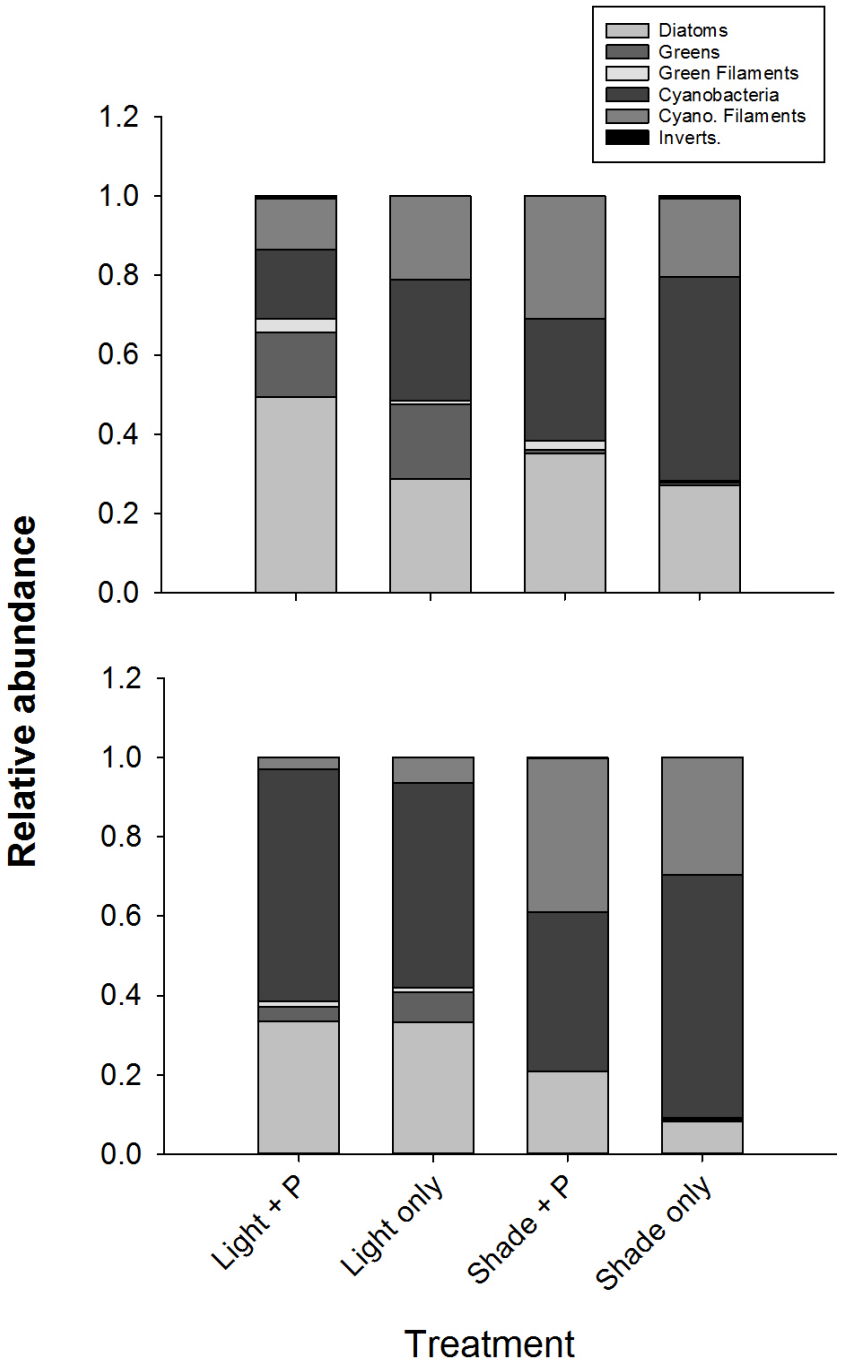

Diatoms
Greens
Green Filaments
Cyanobacteria
Cyano. Filaments
Inverts.
Relative abundance
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Light + P
Light only
Shade + P
Shade only
Treatment

Figure 4.4.

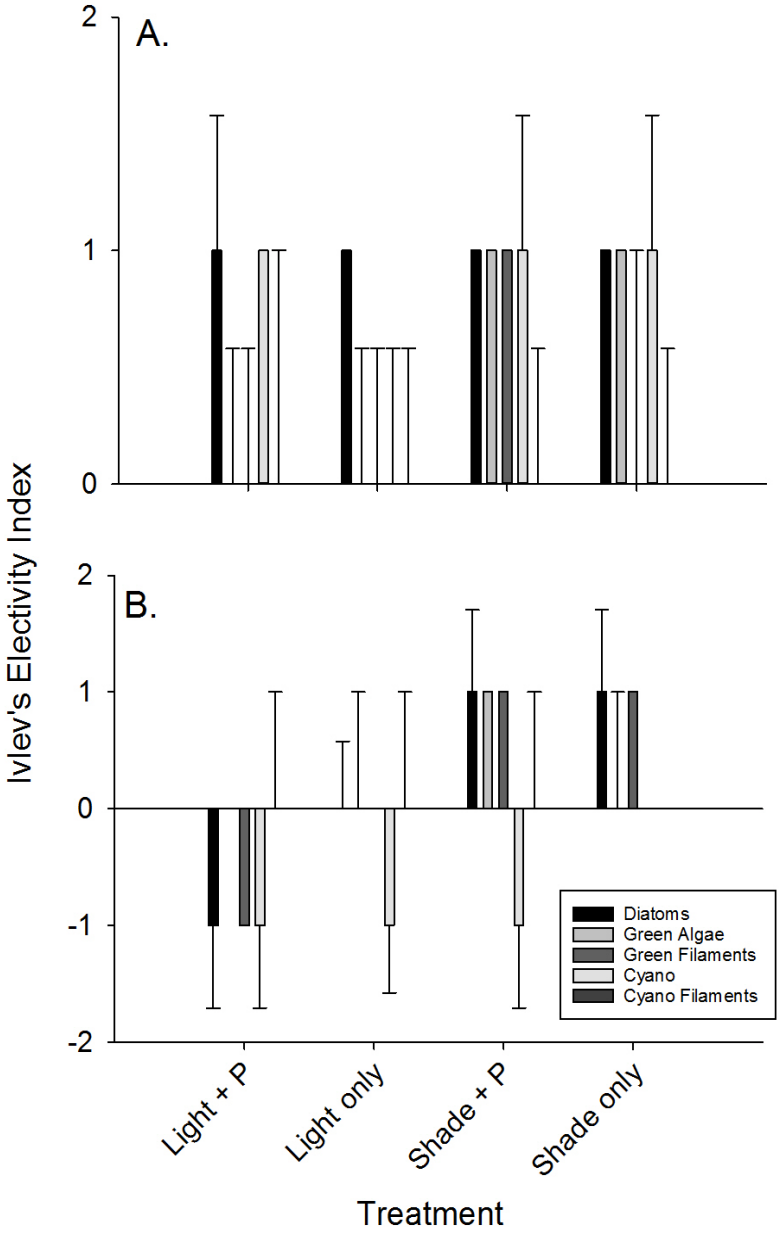

A.
B.
2
1
0
-1
-2
Ivlev's Electivity Index
Diatoms
Green Algae
Green Filaments
Cyano
Cyano Filaments
Light + P
Light only
Shade + P
Shade only
Treatment

Figure 4.5.

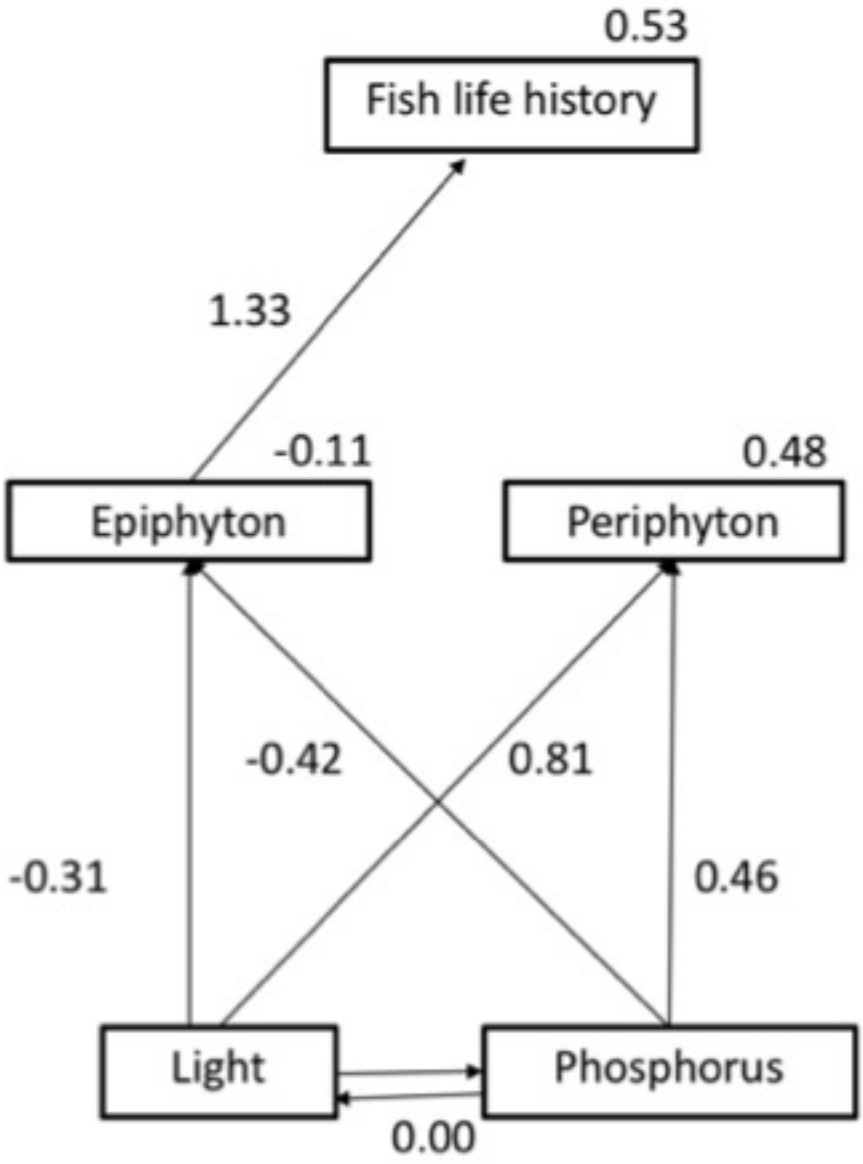

0.53
Fish life history
1.33
-0.11
0.48
Epiphyton
Periphyton
-0.42
0.81
-0.31
0.46
Light
Phosphorus
0.00

Figure 4.6.

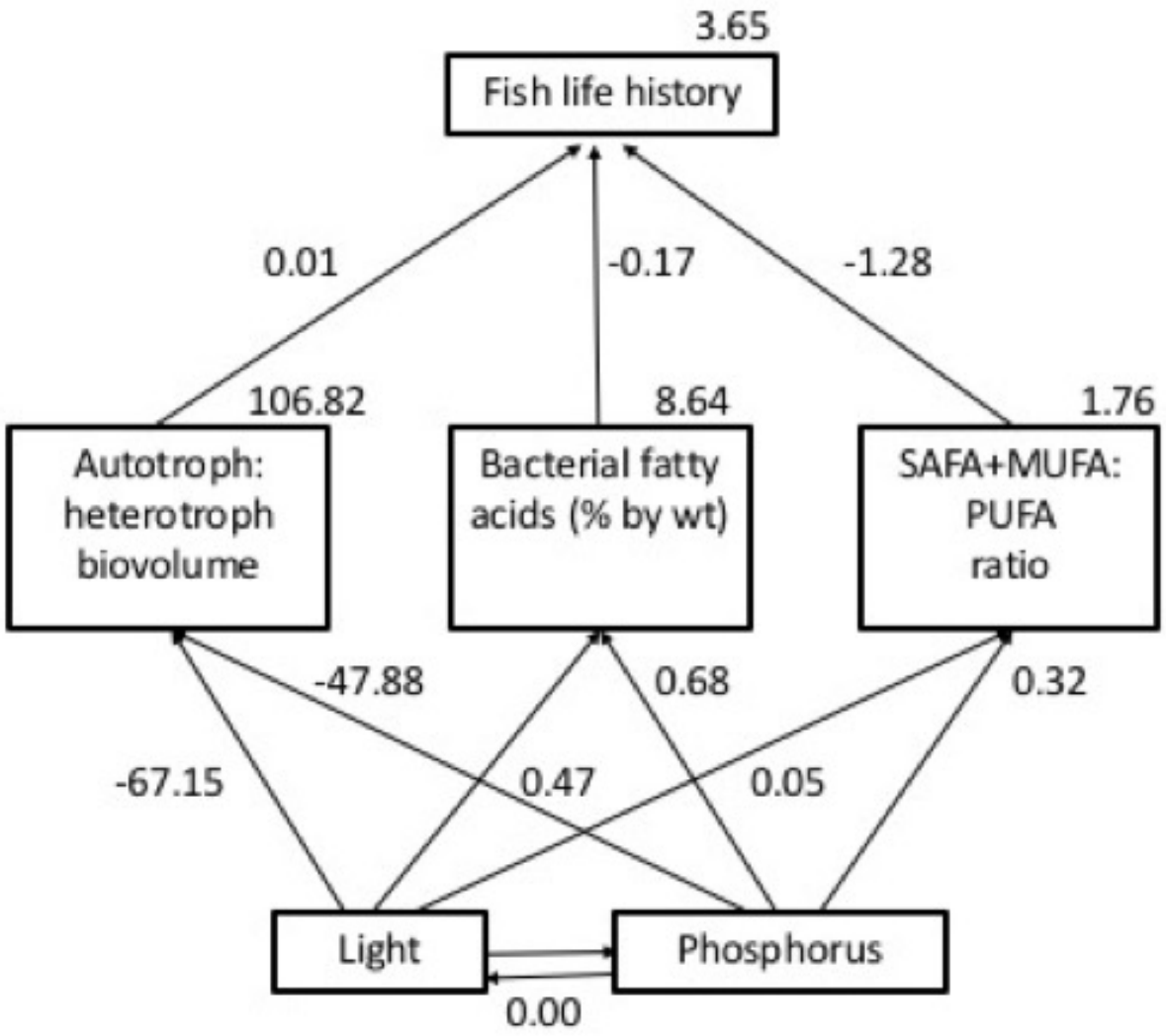

3.65
Fish life history
0.01
-0.17
-1.28
106.82
8.64
1.76
Autotroph: heterotroph biovolume
Bacterial fatty acids (% by wt)
SAFA+MUFA: PUFA ratio
-47.88
0.68
0.32
-67.15
0.47
0.05
Light
Phosphorus
0.00

Figure 4.7.

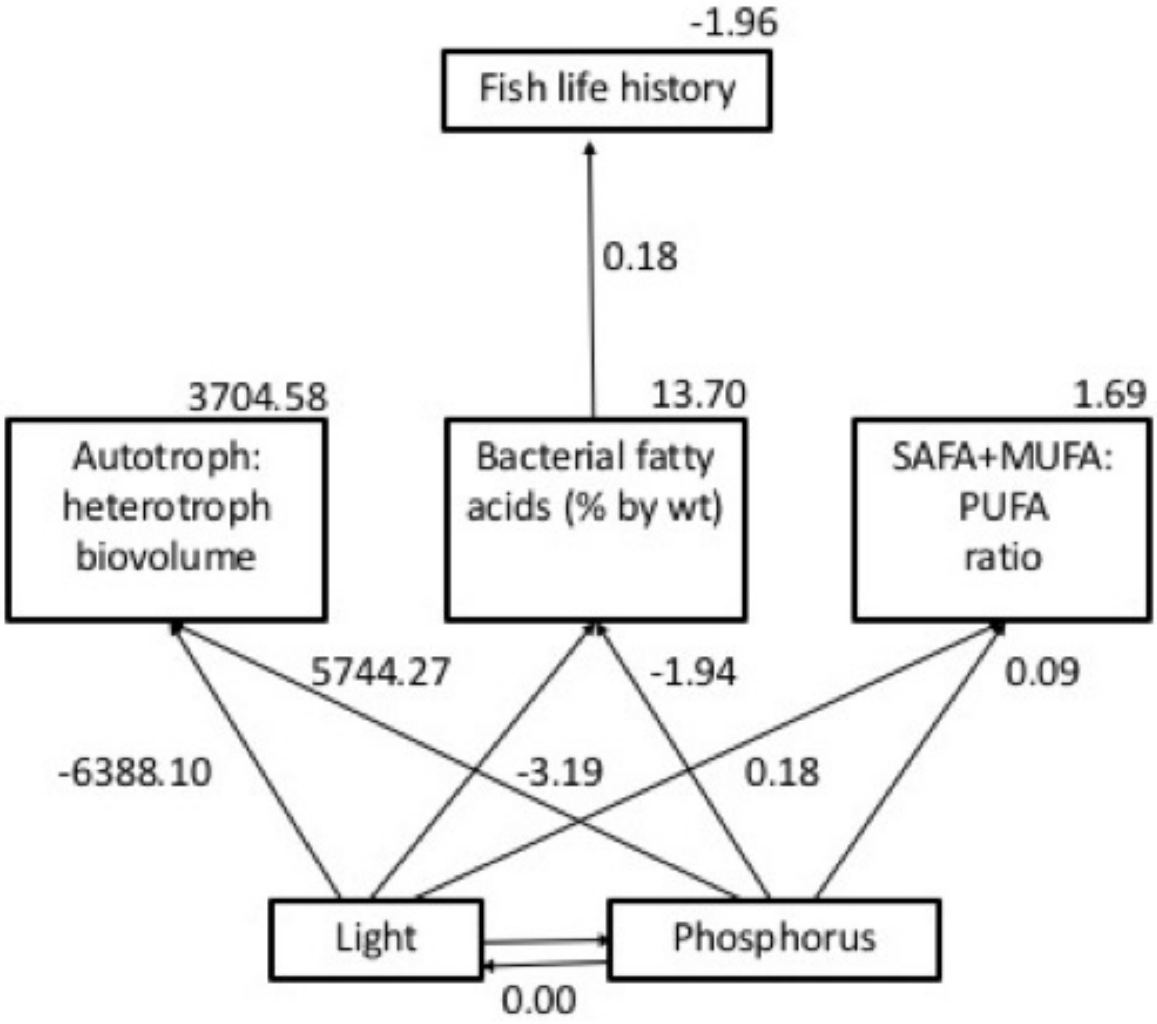
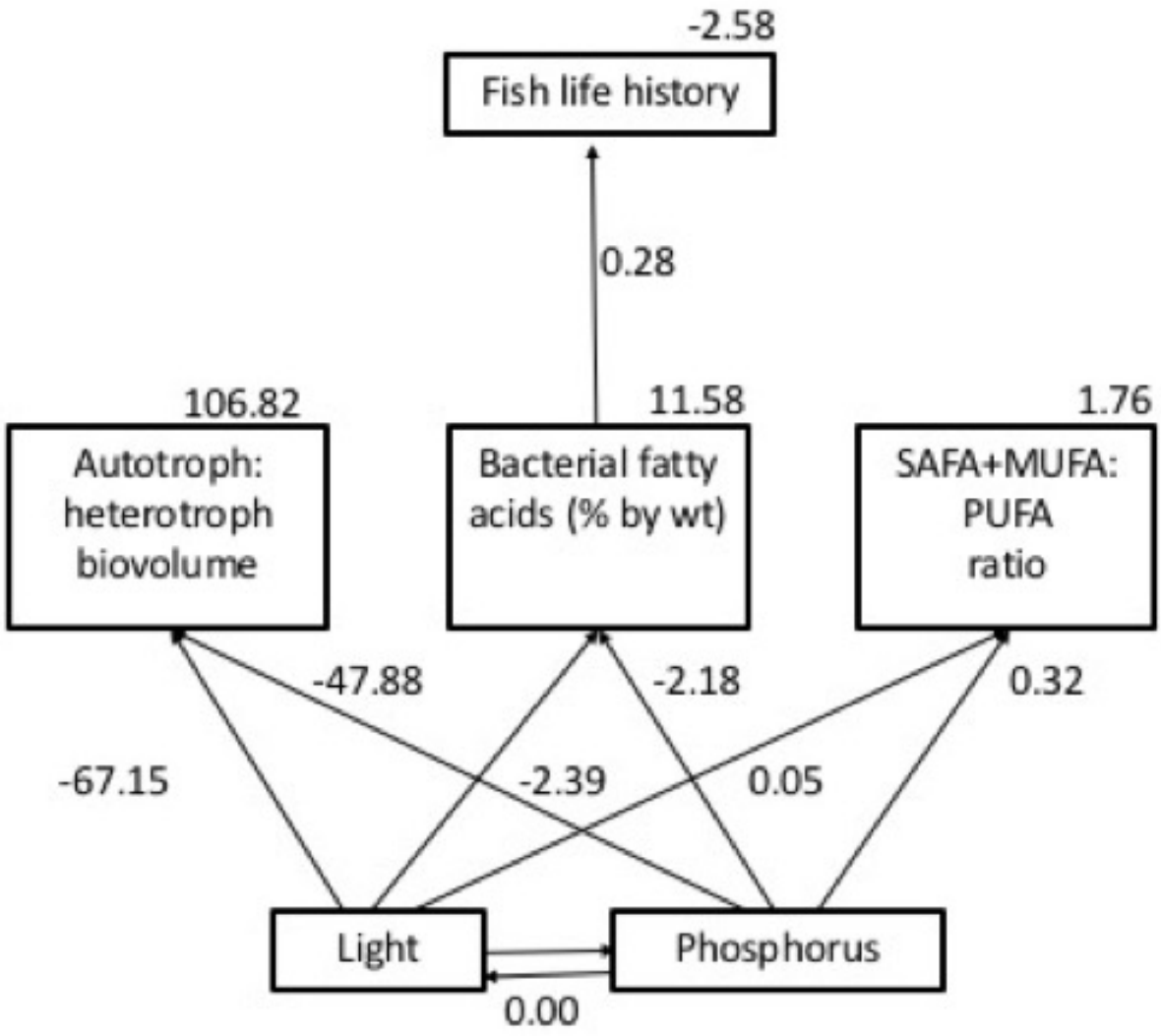

-1.96
Fish life history
0.18
3704.58
13.70
1.69
Autotroph: heterotroph biovolume
Bacterial fatty acids (% by wt)
SAFA+MUFA: PUFA ratio
5744.27
-1.94
0.09
-6388.10
-3.19
0.18
Light
Phosphorus
0.00

Figure 4.8.


-2.58
Fish life history
0.28
106.82
11.58
1.76
Autotroph: heterotroph biovolume
Bacterial fatty acids (% by wt)
SAFA+MUFA: PUFA ratio
-47.88
-2.18
0.32
-67.15
-2.39
0.05
Light
Phosphorus
0.00

Supplementary Information

Table S.4.1. Average values ± 1 SD for all measured epiphyton variables by treatment.

Treatment	C:P	A:H biovolume	Bac. FA (% by weight)	PUFA (% by weight)	SAFA (% by weight)	MUFA (% by weight)	EPA:DHA	ARA (% by weight)	Proportion of edible algal spp.
Light+ P									
3 weeks	2058.20 ± 301.82	12.44 ± 5.13	10.26 ± 3.54	9.39 ± 0.46	57.33 ± 0.14	33.08 ± 0.45	3.73 ± 4.35	1.60 ± 0.59	0.82 ± 0.09
6 weeks	1460.23 ± 424.87	147.83 ± 2.38	8.81 ± 1.05	12.71 ± 6.35	55.64 ± 9.99	31.65 ± 3.65	5.76 ± 6.86	2.40 ± 0.18	0.54 ± 0.33
Light only									
3 weeks	1995.80 ± 87.11	19.01 ± 9.02	8.63 ± 0.41	15.89 ± 3.21	53.69 ± 0.65	30.42 ± 2.58	3.95 ± 0.87	1.93 ± 0.16	0.97 ± 0.06
6 weeks	2119.64 ± 411.12	229.39 ± 73.52	10.27 ± 2.20	14.43 ± 2.43	54.46 ± 4.11	31.12 ± 2.14	4.44 ± 23.44	2.09 ± 0.33	0.95 ± 0.07
Shade + P									
3 weeks	1999.73 ± 272.93	38.29 ± 23.56	8.84 ± 2.04	12.50 ± 1.08	52.81 ± 0.97	34.69 ± 0.65	4.89 ± 2.55	1.65 ± 0.33	0.53 ± 0.30
6 weeks	1984 ± 87.55	12361.76 ± 9805.99	11.52 ± 2.44	15.49 ± 0.75	52.06 ± 3.03	32.44 ± 2.65	4.36 ± 2.44	1.97 ± 0.36	0.78 ± 0.03
Shade only									
3 weeks	2058.88 ± 108.58	127.47 ± 73.98	9.11 ± 2.03	13.11 ± 0.43	52.73 ± 1.40	34.15 ± 1.81	4.55 ± 1.09	1.68 ± 0.11	0.49 ± 0.13
6 weeks	2129.14 ± 89.84	791.66 ± 279.70	13.94 ± 2.20	14.59 ± 1.35	54.05 ± 0.95	31.35 ± 0.73	4.10 ± 7.54	1.84 ± 0.09	0.46 ± 0.09

Table S.4.2. Average values ± 1 SD for all measured periphyton variables by treatment.

Treatment	C:P	A:H biovolume	Bac. FA (% by weight)	PUFA (% by weight)	SAFA (% by weight)	MUFA (% by weight)	EPA:DHA	ARA (% by weight)	Proportion of edible algal spp.
Ambient	5298.94	8.34	13.43	12.38	60.28	27.34	NA*	1.16	0.12
Light+ P									
3 weeks	4585.99 ± 759.79	17.35 ± 7.31	10.34 ± 2.64	16.04 ± 3.20	51.77 ± 2.62	32.18 ± 1.85	65.33 ± 33.62	1.72 ± 0.43	0.74 ± 0.23
6 weeks	3343.42 ± 850.53	5.34 ± 3.53	14.95 ± 2.83	16.27 ± 3.47	53.05 ± 3.81	30.68 ± 3.16	57.53 ± 9.35	1.28 ± 0.17	0.30 ± 0.09
Light only									
3 weeks	4349.86 ± 829.44	21.65 ± 23.33	9.66 ± 0.88	18.63 ± 1.11	30.03 ± 0.93	30.03 ± 0.93	65.92 ± 24.11	1.60 ± 0.39	0.57 ± 0.10
6 weeks	3513.10 ± 540.84	133.19 ± 93.37	15.98 ± 1.44	14.01 ± 1.82	56.19 ± 5.33	29.80 ± 3.53	20.27 ± 85.12	1.29 ± 0.45	0.27 ± 0.09
Shade + P									
3 weeks	4027.97 ± 358.06	21.39 ± 8.11	7.97 ± 0.80	20.05 ± 2.90	51.22 ± 0.97	28.74 ± 3.85	59.60 ± 28.98	1.46 ± 0.52	0.35 ± 0.13
6 weeks	3020.67 ± 343.04	18.27 ± 14.07	15.13 ± 1.00	13.11 ± 0.75	57.66 ± 4.37	29.23 ± 3.64	35.96 ± 16.01	1.11 ± 0.11	0.19 ± 0.13
Shade only									
3 weeks	3995.32 ± 570.69	4.39 ± 2.32	8.62 ± 3.14	19.26 ± 2.81	51.23 ± 0.78	29.50 ± 3.15	52.02 ± 46.37	1.36 ± 0.18	0.52 ± 0.16
6 weeks	3019.93 ± 600.33	20.74 ± 11.02	15.63 ± 1.40	14.43 ± 1.74	54.89 ± 1.11	30.68 ± 1.46	23.85 ± 20.02	1.10 ± 0.15	0.24 ± 0.05

Table S.4.3. Average values ± 1 SD for all measured fish variables by treatment.

Treatment	Survival score (*p'*)	Size (mm)	C:P	Bac. FA (% by weight)	PUFA (% by weight)	SAFA (% by weight)	MUFA (% by weight)	EPA:DHA	ARA (% by weight)
Initial	NA	NA	54.37	5.09	37.13	31.77	31.09	0.09	3.43
Light+ P									
3 weeks	0.78 ± 0.10	18.30 ± 0.53	108.79 ± 18.57	10.90 ± 1.76	22.88 ± 4.25	45.59 ± 0.99	31.54 ± 3.64	0.18 ± .05	5.17 ± 1.56
6 weeks	0.95 ± 0.09	22.63 ± 2.34	95.33 ± 5.01	11.14 ± 3.15	19.39 ± 1.23	43.60 ± 1.96	37.01 ± 1.51	0.27 ± 0.18	3.49 ± 0.26
Light only									
3 weeks	0.83 ± 0.00	20.03 ± 1.40	110.62 ± 7.10	11.50 ± 1.19	21.93 ± 1.10	33.21 ± 1.32	33.21 ± 1.32	0.22 ± 0.69	4.14 ± 0.38
6 weeks	0.90 ± 0.001	22.1 ± 1.74	93.51 ± 5.58	12.04 ± 1.87	20.05 ± 1.70	44.72 ± 0.70	35.23 ± 2.41	0.27 ± 0.05	3.75 ± 0.49
Shade + P									
3 weeks	1.04 ± 0.06	18.80 ± 1.61	85.78 ± 17.88	10.35 ± 1.59	24.30 ± 1.61	45.46 ± 0.37	30.24 ± 1.39	0.15 ± 0.16	5.28 ± 0.69
6 weeks	0.91 ± 0.02	21.83 ± 0.81	70.63 ± 7.04	12.32 ± 1.39	21.66 ± 1.08	44.12 ± 1.76	34.23 ± 0.68	0.24 ± 0.10	4.02 ± 0.02
Shade only									
3 weeks	1.12 ± 0.01	19.10 ± 1.57	85.67 ± 18.37	10.48 ± 0.73	27.55 ± 1.50	45.31 ± 0.61	27.14 ± 1.04	0.12 ± 0.06	6.31 ± 0.37
6 weeks	1.39 ± 0.53	20.23 ± 0.70	64.54 ± 5.39	11.15 ± 0.24	24.66 ± 5.57	45.84 ± 1.55	28.61 ± 2.75	0.17 ± 0.11	5.99 ± 1.56

Table S.4.4. Average values ± 1 SD for Ivlev's Electivity Index by treatment. NA= variables that could not be measured for that treatment.

Treatment	Diatoms	Green Algae	Green Filaments	Cyano.	Cyano. Filaments
Light+ P					
3 weeks	1.00 ± 0.58	0.00 ± 0.58	0.00 ± 0.58	1.00 ± 0.00	0.00 ± 1.00
6 weeks	-1.00 ± 0.71	0.00 ± 0.00	-1.00 ± 0.00	-1.00 ± 0.71	0.00 ± 1.00
Light only					
3 weeks	1.00 ± 0.00	0.00 ± 0.58	0.00 ± 0.58	0.00 ± 0.58	0.00 ± 0.58
6 weeks	0.00 ± 0.58	0.00 ± 1.00	0.00 ± 0.00	-1.00 ± 0.58	0.00 ± 1.00
Shade + P					
3 weeks	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.58	0.00 ± 0.58
6 weeks	1.00 ± 0.71	1.00 ± 0.00	1.00 ± 0.00	-1.00 ± 0.71	0.00 ± 1.00
Shade only					
3 weeks	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 1.00	1.00 ± 0.58	0.00± 0.58
6 weeks	1.00 ± 0.71	0.00 ± 1.00	1.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Fig. S.4.1.

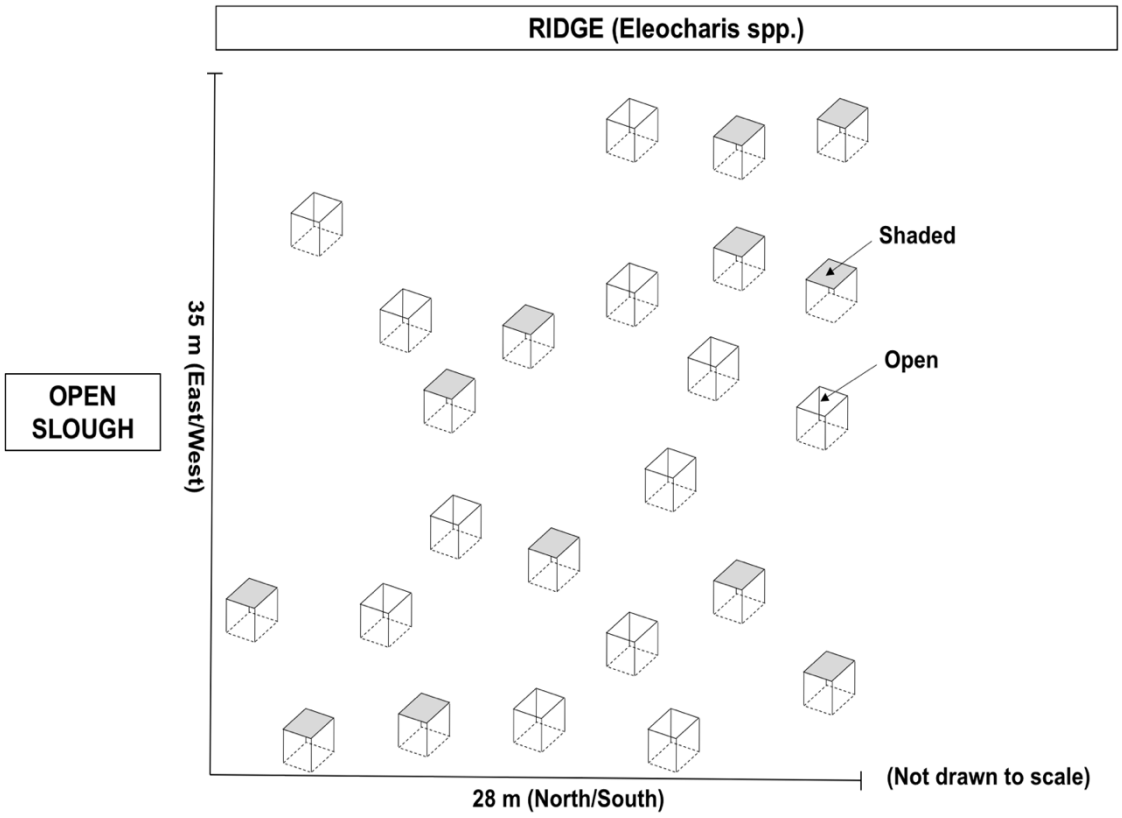


Figure S.4.1. Field experimental set-up. Boxes represent 1m^2 mesh cages (shaded and open) randomly distributed across a 980 m^2 plot located in an open Everglades slough (25°49'41.23"N, 80°37'53.41"W). Not drawn to scale.

Fig. S.4.2.

(a)

(b)

Figure S.4.2. (a) Photo showing mesh cages in the field. (b) Photo showing cages wrapped with 3mm clear plastic following nutrient dosing. Phosphorus (Na_2HPO_4) was added once per week and the cages remained wrapped for 24 hours to avoid seepage to cages without nutrient addition.

CHAPTER 5

CARNIVORY IS BEST, BUT HERBIVORY IS GOOD ENOUGH: A TEST OF THE HETEROTROPH FACILITATION AND LIPID ALLOCATION HYPOTHESES FOR DIET EVOLUTION

In review: *Freshwater Biology*

Abstract

Herbivorous diets are generally less nutritious than animal diets, but herbivory has evolved from carnivorous ancestors in many lineages, suggesting that herbivory can be adaptive. Using mesocosms stocked with periphyton from the Florida Everglades, I evaluated two hypotheses to explain the adaptive evolution of herbivory: 1) Heterotroph Facilitation (herbivores supplement their diet with nutrients derived from heterotrophic microbes); and 2) Lipid Allocation (herbivores consume algae that are similar in lipid concentration to animal prey). I manipulated the heterotrophic microbe and lipid composition of epiphyton using shading and phosphorus ('Light + P', 'Light only', 'Shade + P', and 'Shade only') and examined the effects of this varying food quality on growth and survival of juvenile Sailfin Mollies (*Poecilia latipinna*) compared to those raised on a reference carnivore diet.

I found that life history of Sailfin Mollies was driven by increased heterotrophic fatty acids in the diet (2.5x more likely than alternative models with ΔAICc > 2.00). When comparing herbivorous Sailfin Mollies to those raised on a carnivore diet, I found that carnivores showed 24%-34% higher survival than fish eating shaded epiphyton, and 44-100% higher survival than fish eating epiphyton grown in the light. Shaded epiphyton and carnivore diets were both comprised of relatively high levels of heterotrophic-derived lipids, suggesting that these organisms are important for fish survival. Although carnivory is the best diet, mixing autotrophs and heterotrophs can result in a diet almost as good as a carnivorous one, consistent with the Heterotroph Facilitation Hypothesis.

Key-words Diet evolution, diet quality, fatty acids, herbivory, detritivory

Introduction

Herbivory is a relatively inefficient feeding strategy compared to omnivory and carnivory (Mattson 1980, Sterner and Hessen 1994, Choat and Clements 1998, Sterner and Elser 2002, Laspoumaderes et al. 2010) because plants are nutritionally variable and often protected by structural and/or biochemical barriers to digestion (Mattson 1980, Montgomery and Gerking 1980, Porter and McDonough 1984, Hay and Fenical 1988, Horn 1989, Chivers and Langer 1994, Sterner and Hessen 1994, Choat and Clements 1998, and others). Furthermore, herbivores may be limited in foraging time and/or space by predators and competitors, in the ability to produce digestive or detoxifying enzymes (see Karban and Agrawal 2002), and in the amount of food they can process through their gut (Horn 1989, Bruggeman et al. 1994, Bellwood 1995, Choat and Clements 1998).

Despite these apparent disadvantages, there is evidence from many metazoan groups that herbivores evolved from carnivorous ancestors (e.g., Vermeij 1992, deMaintenon 1999, Van Damme 1999, Vermeij and Lindberg 2000, Bellwood 2003, Eubanks et al. 2003, Espinoza et al. 2004, Pauls et al. 2008, Bellwood et al. 2014, Reisz and Frobisch 2014). A review of the freshwater literature identified conditions where eating plants might be adaptive over eating animal prey (Sanchez and Trexler 2016).

In this review, "herbivory" was defined as the consumption of aquatic primary producers (algae and/or phytoplankton), and an "herbivore" as an organism that mainly eats autotrophic organisms, but may indirectly assimilate nutrients from organisms that decompose detritus (consumes > 50% autotrophs).

A “carnivore” was defined as an organism that consumes > 50% animal material, and an “omnivore” as an organism that eats both plants and animals in similar proportions (Sanchez and Trexler 2016, 2018). The term “food quality” describes the nutritional worth of a diet item to a consumer, or as the ratio of energy content of the food to energy assimilated by its consumers. Macronutrient (e.g., nutritional ecology) or elemental (e.g., stoichiometry) composition are commonly assessed as metrics of food quality, where high quality food items are rich in protein or phosphorus, respectively. Regardless of the convention used, “food quality” is a relative term and can only be interpreted relative to other diets, and respective of organismal diet adaptations (e.g., “high quality” is defined differently for carnivores and herbivores). Under these conditions, the review concluded that herbivory is favored when higher quality prey are limiting, or when plants provide important dietary elements that are limited in carnivore diets, such as essential fatty acids (e.g., Martin-Creuzburg et al. 2011) or antioxidants (e.g., Pike et al. 2007). Furthermore, herbivores may overcome limiting resource quality by passively supplementing their diets with heterotrophic microbes that are associated with primary producers (Sanchez and Trexler, 2016, 2018).

Many researchers recognize that herbivores obtain nutrients from supplementary sources, and as a result there are few “true” aquatic herbivores in nature (White 1985). In aquatic systems, primary consumers are nutrient-limited, and their diets depend on nutrients derived from both autochthonous and allochthonous inputs (Hall et al. 2001).

The heterotrophic organisms that colonize decomposing autochthonous and allochthonous material provide a rich source of dietary nutrients and promote higher growth in some invertebrate families (e.g., Mulla and Lacey 1976, Fuller et al. 1988, Edwards and Meyer 1990, Fuller and Fry 1991, Fuller et al. 2004). However, diets composed only of heterotrophs are of poor quality for herbivores (e.g., *Daphnia magna*), suggesting that they also rely on autotrophs for essential nutrients (e.g., Goulden et al. 1982, Schmidt and Jonasdottier 1997, Weers and Gulati 1997, Martin-Creuzburg et al. 2005, Martin-Creuzburg et al. 2011). In a recent study (Sanchez and Trexler 2018), I evaluated the relative importance of autotroph-derived lipids and heterotroph diet supplementation on herbivore success by testing two alternative hypotheses for the adaptive evolution of herbivory: 1) the Heterotroph Facilitation hypothesis, which states that herbivory may be adaptive by supplementing herbivore diets with heterotrophic microbes (bacteria and/or fungi) that are indirectly consumed along with primary producers; and 2) the Lipid Allocation hypothesis, which states that consumption of primary producers with high lipid concentrations may be as beneficial to individual life history as a carnivorous diet (Sanchez and Trexler 2016). I tested these hypotheses using field enclosures stocked with the herbivorous Sailfin Molly (*Poecilia latipinna*) and found that autotrophic lipids play an important role in early development of Sailfin Mollies, but when energy is re-directed to reproduction, heterotrophic lipids become an important driver of herbivore survival. Sailfin Mollies assimilate nutrients from heterotrophs, which were not the target diet item, but were consumed as a consequence of their close association with primary producers. This result was consistent with the Heterotroph Facilitation hypothesis (Sanchez and Trexler 2016, 2018).

Similar studies have shown that herbivores rely heavily on nutrients originating from heterotrophic microbes (see Sanchez and Trexler 2016), but no others have examined these diet components as potential mechanisms supporting the evolution of herbivory. Furthermore, it remains to be determined if a mixed herbivorous diet can be similarly nutritious for an herbivore as a carnivorous diet, as would be expected to facilitate a carnivorous ancestor giving rise to an herbivorous lineage.

I report a laboratory experiment designed to test the Heterotroph Facilitation and Lipid Allocation Hypotheses using a resource-consumer system that is native to the Florida Everglades, USA. The Everglades is an ideal system to test these hypotheses because periphyton mats, the primary basal resource in this area (Browder et al. 1994, Trexler et al. 2015), are composed of complex assemblages of autotrophs (green algae, diatoms and cyanobacteria) and heterotrophs (fungi, bacteria, protozoans and zooplankton) that are bound together by a calcium carbonate matrix (Gaiser et al. 2004). Because the Everglades is naturally oligotrophic, both autotroph and heterotroph components of periphyton mats are easily manipulated by nutrient addition (Gaiser et al., 2004, Bellinger et al. 2012). In addition, lipid profiles of Everglades primary and secondary consumers are comprised of both algal and bacterial fatty acids (Belicka et al. 2012), suggesting that both items are important in consumer diets. One of these native consumers is the Sailfin Molly (*Poecilia latipinna)*, which is herbivorous (Scharnweber et al. 2011), but incorporates prokaryotic resources (Belicka et al. 2012) and sometimes small invertebrates (Harrington and Harrington 1961, 1982) into its diet.

Methods

I kept juvenile Sailfin Mollies in tanks in the FIU Ecotoxicology Greenhouse Laboratory (North Miami, FL) from June- July 2015. The lab is covered by a clear canopy that blocks rain but allows exposure to sunlight with UV penetration, thus promoting growth of epiphytic algae, the food source for fish in this study. I constructed artificial vegetation strips (2.54 cm wide) made of black plastic sheeting (0.154 mm thick) attached to $1m^2$ wire frames for a total of 150 strips per m^2, simulating the natural stem density of the Everglades (described in Chick et al. 2008). Frames were placed into large mesocosms along with 151 liters of filtered freshwater (393.03 $\pm$ 4.74 uS/cm, 0.02 ppt). I collected periphyton from the Everglades (25°54'11.0"N, 80°39'43.2"W), cleaned the periphyton of invertebrates, and stocked 2000mL in each mesocosm. Periphyton was used to encourage growth of epiphytic algae on the artificial vegetation strips, and I manipulated the quality of colonizing epiphyton using phosphorus (P) and/or shade. At low levels of light, diatoms and cyanophytes are expected to dominate periphyton communities (Thomas et al. 2006, Vadeboncoeur and Power 2017), resulting in an increase in polyunsaturated fatty acids (Hill et al. 2011). In increased light conditions, green algae are expected to dominate (Thomas et al. 2006, Vadeboncoeur and Power 2017) and bacterial-derived saturated and monounsaturated fatty acids are also expected to increase in abundance (Hill et al. 2011). When light levels interact with P-addition, PUFAs are favored in low light and high P conditions, and SAFAs + MUFAs are favored in high light and low P conditions (Hill et al. 2011). Although heterotrophic responses to light and P is not well-established for periphyton communities, it is believed that increased nutrient input results in increased heterotrophy (McCormick et al. 1997).

Each mesocosm was randomly assigned to one of four treatments: 1) light + P; 2) light only; 3) shade + P; 4) or shade only. Light treatments were exposed to ambient sunlight. Phosphorus (Na_2HPO_4) was added at a concentration of 15 µg/L weekly to 'shade + P' and 'light + P' mesocosms. This concentration was chosen based on previous P dosing studies in the Everglades (Noe et al. 2002, Gaiser et al. 2005). Shading was accomplished by covering 'shade + P' and 'shade only' mesocosms with 2 sheets of greenhouse shade cloth to achieve approximately 50% reduction in ambient light (modified methods of Fuller et al. 2004), which is within the natural range of shading experienced in the field (10-65% reported by Armento et al. 2006). Light and temperature were tracked throughout the experiment using HOBO® data loggers. Mesocosm tanks were kept at approximately 28.72 $\pm$ 2.00°C.

Juvenile (< 12mm) Sailfin Mollies were born in the FIU indoor aquarium lab to wild-caught females, were separated by sib-groups, and were raised on Tetramin ® flake food for 3 weeks prior to the start of the experiment. They were then measured (average standard length, SL) and transplanted to 18.9 liter aquaria (filled with treated freshwater) located in the greenhouse lab (n=6 per tank; N=24 total fish/treatment) 3 weeks following mesocosm set-up (when fish were 6 weeks old). This allowed enough time for epiphyton to colonize the vegetation strips in mesocosms and to allow fish to acclimate to aquaria. I placed shade covers on top of the aquaria to keep them out of direct sunlight to prevent water from overheating (~25% decrease in ambient light). Tanks were maintained at 30.92 $\pm$ 2.64°C, were topped off with clean freshwater twice per week, and were cleaned (full-water change, tank walls wiped) once per week throughout the experiment. Fish were assigned to one of the above treatments, or a fifth 'carnivore' treatment.

Individual vegetation strips with newly colonized epiphyton were harvested from each mesocosm tank and provided to fish assigned to the same treatment (e.g., 'light only' epiphyton strips were provided to 'light only' fish tanks) for consumption. I provided fish 3 strips, 3 times per week; the number of strips provided was calculated based on how much epiphyton had grown on them, with a target goal of providing 20 mg dry weight of food per tank based on preliminary studies to estimate the maximum ration. Fish in the 'carnivore' treatment were provided with 20mg of Tetra ® Freeze-dried bloodworms 3x per week, along with 3 blank plastic vegetation strips to keep feeding trials as consistent as possible.

Multiple mesocosms with each epiphyton treatment (6 blocks of 4 tanks each) were maintained to create as consistent a "food supply" as possible throughout the study. For example, the first block was inoculated with stock periphyton on week 1, the second on week 2, the third on week 3, etc. On week 3, the artificial vegetation strips growing biofilms in the first block were ready to feed to fish. On week 4, strips from block 2 were ready; on week 5, strips from block 3 were ready, etc. Each block provided enough food to feed fish (4 replicates of 5 treatments, N=120 fish) for one week (feedings on Monday, Wednesday and Friday). Stock periphyton was collected from the field and brought back to the lab every 2 weeks so that water chemistry in the greenhouse minimally affected species composition of periphyton (e.g., Ruehl and Trexler 2015) and so that I could capture the natural variation in periphyton quality over the course of a season. Samples for nutrient and lipid analyses were taken from the ambient periphyton before placing it into the mesocosm tanks with artificial vegetation and applying nutrient/shading treatments.

Artificial strips were sampled at the beginning of the week they were fed to the fish (30 strips total per treatment).

The remaining samples (including carnivore diets and fish tissues) were freeze-dried and prepped for fatty acid analyses (sent to Microbial ID laboratory, Newark, DE). In addition, epiphyton, periphyton, bloodworms (carnivore diet), and fish tissues were processed for nutrient content (CNP; sent to Southeastern Research Center, Florida International University, Miami, FL). Stoichiometry data (CNP) were converted to molar ratios. Fatty acid data were categorized by diet tracers (Table 1; Sanchez and Trexler 2018) and further organized into polyunsaturated fatty acids (PUFAs), saturated fatty acids (SAFAs), and monounsaturated fatty acids (MUFAs). Fatty acid data were also organized by common Omega-3 and Omega-6 fatty acids (essential fatty acids) that are known to affect fish growth and development: eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic (ARA). These metrics were analyzed in fish tissues and the various diets to understand their influence on fish growth and survival.

Statistical analyses

Several food quality variables were measured for epiphyton and bloodworms: ratio of total carbon to phosphorus (C:P); ratio of nitrogen to phosphorus (N:P); relative fatty acid content; percentage of autotroph- and heterotroph- derived fatty acids; fatty acid class (PUFA, SAFA, MUFA; ratio of (SAFA + MUFA): PUFA); and essential fatty acids (EPA, DHA, ARA, ratio of EPA:DHA).

In addition, the ratio of autotroph to heterotroph (A:H) biovolume, algal species composition and the proportion of edible algae (proportion of green algae relative to cyanobacteria) were analyzed in the epiphyton samples only. Carnivore diets were compositionally different than the epiphyton diets, so I statistically examined the experimental diets by excluding bloodworm data from the analyses. This allowed me to determine if any differences existed among treatments, without biasing the analyses towards differences between bloodworms and epiphyton. All epiphyton diet characters were first analyzed to determine if there were effects of mesocosm block on variation in diet I found no differences across treatments that were attributed to growing epiphyton in blocks, suggesting that any observed differences were in response to the treatments themselves.

Fatty acid classes (PUFA, SAFA, MUFA), fatty acid ratios ((SAFA+MUFA): PUFA), and essential fatty acids (EPA, DHA, ARA) comprising each diet type were analyzed separately using two-way ANOVA tests, followed by Tukey tests. Biovolume of autotrophs and heterotrophs were converted to ratios (A:H biovolume) and were analyzed using two-way ANOVA. Algal species composition was analyzed using two-way MANOVA with Tukey multivariate comparison tests. Proportion of edible algal species comprising each diet type was analyzed using two-way ANOVA. Once I determined if any differences existed between experimental diets, I ran the same analyses (with the exception of A:H biovolume, algal species composition and edible algal abundance) with the bloodworm data.

My next goal was to test the alternative hypotheses by determining which food parameter influenced herbivore size and survival. The Heterotroph Facilitation hypothesis predicts that diet supplementation with heterotrophic organisms promotes herbivore life history, and the Lipid Allocation hypothesis suggests that fatty acids derived from algae are influencing herbivore success. Therefore, I choose to evaluate variables describing heterotroph and autotroph quality/quantity as independent variables to predict fish life history: A:H biovolume (measure of heterotroph and autotroph quantity), percentage of heterotrophic fatty acids (measure of bacterial and fungal quality), and (SAFA + MUFA): PUFA ratios (measure of autotroph quality). Using Principal Component Analysis, fish size and survival rates were collapsed into a single score, collectively called "fish life history". These PCA scores were used as response variables for structural equation models (SEM) created using AMOS (Arbuckle, J. L. 2014). The models were designed to test the strength between the 3 independent variables and fish life history to determine which suite of diet characteristics most strongly influences herbivore growth and survival (i.e., to determine which alternative hypothesis is best supported by the data).

Paths were varied between diet variables and fish life history in each model to produce a total of 7 models (Table 4) that were compared using Akaike's Information Criterion (AIC). I calculated ΔAICc (ΔAICc = AICi –min AICc, where i=model i). I also calculated Akaike weight (AICw = (e(-0.5 *ΔAICi)/∑(e(-0.5 *ΔAICr), (Lr), and Evidence Ratios (wmin/wj, where wmin = AICw for the model with the smallest ΔAICc, and wj = AICw for the current model; Burnham and Anderson 2002) for each model. Following Burnham and Anderson (2002), models with ΔAICc < 2.0 were equally supported by the data.

I was only interested in the carnivore diets as references, therefore, I excluded them from these analyses.

Results

Bloodworm Diet

Carnivore diets were compositionally different than epiphyton diets (Table 2; Table S1). Bloodworms had 84% lower C:P and 59% lower N:P ratios than the epiphyton with the lowest ratios ('shade + P' and 'light + P'), and the relative fatty acid content of this diet was 2-4x less than that of the epiphyton diets. Bloodworms contained 36% and 48% more heterotrophic fatty acids than 'shade + P' and 'shade only' epiphyton, respectively. Carnivore diets had 52% higher PUFAs and 51% fewer MUFAs than 'shade + P' epiphyton, which had the highest PUFA and lowest MUFA percentage of all epiphyton types. Furthermore, bloodworms were 75% lower in SAFAs than all epiphyton types. Carnivore diets were 95% higher in EPA, 98% higher in DHA, and 90% higher in ARA than the shaded diets.

Epiphyton Diet

Phosphorus addition influenced the stoichiometric ratios (C:P and N:P) of epiphyton in a predictable manner (Table 2; Table S1). Epiphyton grown in 'shade + P' and 'light + P' mesocosms had 52% lower C:P and 40% lower N:P ratios than the treatments without nutrient addition (Phosphorus: $F_{1,16}$= 15.739, P= 0.001; $F_{1,16}$= 0.664, P= 0.012, for C:P and N:P, respectively). Epiphyton grown in 'shade only' and 'light only' treatments had 33-50% lower relative abundance of green algal species than 'light + P' epiphyton, which had the most (Wilks' Lambda= 0.120, $F_{15,33}$= 2.586, P=0.011).

Phosphorus addition drove the percent of edible species comprising the epiphyton (phosphorus: $F_{1,16}$= 8.488, P= 0.010). In addition, the ratios of A:H biovolume for 'shade + P' and 'shade only' epiphyton were higher than the light treatments, by 50% and 37%, respectively (Light: $F_{1,16}$= 4.503, P=0.05). There was no difference in the percentage of autotroph-derived fatty acids ($F_{3,16}$= 1.534, P= 0.279) between treatments, but 'shade + P' and 'shade only' treatments had 42% and 14% more heterotrophic fatty acids than the light treatments, respectively (Light: $F_{1,16}$= 11.208, P= 0.004). The quantity of heterotrophs (A:H biovolume) and quality of heterotrophs (fatty acid percentage) were not correlated (R^2= -0.009, $t_{1,16}$ = -0.910, P = 0.375). 'Shade + P' epiphyton had 52% higher fatty acid content compared to the other treatments (Light: $F_{1,16}$= 15.48, P<0.0001).

The relative abundances of PUFAs and SAFAs comprising the epiphyton samples were not different between treatments, but light was correlated with the relative abundance of MUFAs (Table 2; Table S1). 'Light only' treatments showed 14% higher MUFAs than 'shade only', and 'light + P' treatments and 32% higher MUFAs than the 'shade + P' epiphyton (PUFA, $F_{3,16}$= 1.824, P=0.183; SAFA, $F_{3,16}$= 0.071, P=0.974; MUFA, $F_{1,16}$= 4.387, P=0.02). But, the (SAFA+MUFA): PUFA ratios were not statistically different between the treatments ($F_{3,16}$= 0.938, P= 0.445). Epiphyton grown in the 'shade + P' treatments had the highest percent of EPA ($F_{3,16}$= 13.093, P<0.0001), but lowest percent of ARA ($F_{3,16}$= 10.435, P=0.005). Epiphyton grown in 'light + P' mesocosms had the lowest EPA, which was 83% lower than 'shade + P'. 'Light only' epiphyton had 53% higher ARA than 'shade + P' epiphyton. The different epiphyton types were not significantly different in DHA ($F_{3,16}$= 0.481, P=0.70).

Ambient Periphyton

Periphyton varied in stoichiometry over the course of the experiment (Table 2; Table S1). Specifically, periphyton that stocked mesocosm blocks 1-3 had C:P ratios of approximately 4500, and periphyton from blocks 4-6 had C:P ratios of approximately 6000. Because stock periphyton for mesocosm blocks 1-2, 3-4, and 5-6, was collected on three separate occasions, variation in C:P is likely attributed to the natural variation in the field. Ratios of N:P were consistently between 195 and 235 for all mesocosm blocks. There were differences in epiphyton stoichiometry independent of mesocosm block (confirmed by randomized block ANOVA); therefore, I assumed that the inter-block variation in periphyton C:P did not influence colonizing epiphyton. The proportion of edible species comprising periphyton was similar across blocks, but was different than that of epiphyton (Table 2; Tables S1 & S2). Periphyton contained between 36% and 75% lower abundances of edible species than the experimentally manipulated epiphyton. Furthermore, periphyton contained 95% less heterotrophic biovolume and 82% less autotrophic biovolume than epiphyton. However, the A:H biovolume ratio of periphyton was within the range of epiphyton A:H ratios, which was 11.01 $\pm$ 5.86 for 'light + P' epiphyton (lowest ratio), 23.81 $\pm$ 12.16 for 'shade + P' epiphyton (highest ratio), and 16.26 $\pm$ 2.90 for periphyton. The relative fatty acid content of periphyton did not vary by block. In addition, periphyton contained 62% fewer fatty acids than 'shade + P' epiphyton, 36% fewer than 'shade only' epiphyton, and 16% fewer than epiphyton grown in the light treatments. Autotrophic fatty acid markers were present in similar amounts in periphyton and epiphyton.

But, heterotrophic fatty acids were 28% higher in periphyton than 'shade + P' epiphyton, which had the highest heterotrophic fatty acid content of the different epiphyton types. Periphyton had similar proportions of PUFAs compared to 'light only' epiphyton, but that amount was 49% lower than 'shade + P' epiphyton. Periphyton contained similar SAFA and MUFA content as epiphyton. Periphyton did not contain any EPA or DHA and contained 40% less ARA than epiphyton grown in the light treatments. However, periphyton had 85% and 25% higher ARA than 'shade + P' and 'shade only' epiphyton, respectively.

Fish Life History

There were no differences in the sizes of juvenile Sailfin Mollies stocked in each tank at the start of the experiment or at the end of the experiment. However, there were differences in growth rates between 0-3 weeks and 3-6 weeks. All fish were approximately 11.18 $\pm$ 0.37 mm at the beginning of the experiment ($F_{4,15}$= 2.141, P=0.126). Fish eating 'light only' epiphyton were approximately 12% larger than fish eating other epiphyton types, and 11% larger than the carnivores at 3 weeks ($F_{4,15}$= 4.482, P= 0.01; Fig. 1a). These size differences disappeared by 6 weeks ($F_{4,15}$= 0.662, P= 0.626; Fig. 1a). As such, growth rates were different at both time periods, where 'light only' fish grew the fastest from 0-3 weeks ($F_{4,23}$= 6.847, P= 0.001), but slowed growth from 3-6 weeks ($F_{4,23}$= 7.563, P< 0.0001). Carnivores showed the slowest growth at both time periods, and fish eating epiphyton grown in the shaded treatments displayed the fastest rates of growth from 3-6 weeks. Fish reared on 'light + P' epiphyton showed intermediate growth at both time periods.

Survival differences between treatments were not yet evident at 3 weeks (X^2 = 4.441, P =0.350; Fig. 1b), but at 6 weeks, carnivores showed 24% higher survival than 'shade only' fish, which were the highest surviving individuals of those eating epiphyton. Fish reared on 'light + P' epiphyton had no surviving individuals at 6 weeks, followed by 'light only' and 'shade + P' fish with 28% and 14% lower survival rates than 'shade only' fish (45% and 34% lower than carnivores). Because there were no surviving individuals from 'light + P' treatments at the end of the experiment, we were unable to perform nutrient analyses on tissues from fish reared in this treatment. However, there were differences in fish tissues that were evident across the remaining treatments (Table 3; Table S3). Specifically, the relative fatty acid content of fish tissues was highest in fish eating bloodworms and 'shade + P' epiphyton, and fish eating 'light only' and 'shade only' epiphyton had 14% fewer fatty acids comprising their tissues ($F_{3,16}$=3.564, P=0.038). The percentage of autotroph- and heterotroph- derived fatty acids in the fish tissues across experimental treatments were not different (Autotroph: $F_{3,16}$=0.54, P=0.662; Heterotroph: $F_{3,16}$=2.966, P=0.063). The relative amounts of PUFAs and MUFAs in fish tissues were the same (PUFA: $F_{3,16}$=1.169, P=0.353; MUFA: $F_{3,16}$=1.517, P=0.248); however, those reared in the 'shade + P' and 'light only' treatments had 6% more SAFAs in their tissues than the other fish ($F_{3,16}$=3.356, P=0.045). Despite these differences, the (SAFA+MUFA): PUFA ratios were the same for fish tissues ($F_{3,16}$= 1.095, P= 0.380). There were no differences in essential fatty acids (EPA, DHA, ARA) in fish tissues (EPA: $F_{3,16}$=0.696, P=0.568; DHA: $F_{3,16}$=0.946, P=0.442; ARA: $F_{3,16}$=2.324, P=0.114). For detailed results, refer to Table 3 and Supplementary Table 3.

Testing adaptive hypotheses

To test the Heterotroph Facilitation and Lipid Allocation hypotheses, I varied the paths between diet metrics (A:H biovolume, the percentage of heterotroph-derived fatty acids, and (SAFA + MUFA): PUFA ratio) and fish life history to produce 7 models for each time period (3 and 6 weeks). Based on ΔAICc values, and evidence ratios, low A:H biovolume ratios (high heterotroph biovolume) and low heterotrophic fatty acid percentage best predicted fish size and survival at 3 weeks. However, 6-week models show that increased A:H biovolume (low heterotroph abundance) and increased heterotrophic fatty acids best predicted fish life history (Fig. 2; Table 4).

Discussion

These results supported the Heterotroph Facilitation hypothesis, which suggests that diet supplementation with heterotrophic microbes diminishes the nutritional discrepancy between an herbivorous and carnivorous diet. In our mesocosm experiment, juvenile Sailfin Mollies (6-12 weeks of age) had high survival when fed diets high in heterotrophic fatty acids (carnivores, 'shade only', 'shade + P'), indicating that Sailfin Mollies benefit from a diet that incorporates heterotrophic food sources. However, the quality of these dietary heterotrophs played a more important role in fish survival than the quantity. Furthermore, the carnivorous fish in this study experienced the greatest life history benefits in terms of survival, likely as a result of a diet rich in heterotrophic fatty acids.

Although these results indicate that carnivory is a better diet than herbivory, herbivore survival increased with percent of dietary heterotrophic fatty acids, suggesting that an autotrophic diet rich in heterotrophic fatty acids can be adequate for fish growth and survival. It is important to note that I maintained these fish on a high-quality diet (Tetramin ® flakes) for their first three weeks post-partum to standardize their condition prior to assigning diet treatments, which may have diminished treatment differences, at least for the first three-week experimental interval. However, this does not undermine the finding that heterotrophs supplement and improve the herbivorous diet in both field (Sanchez and Trexler 2018) and mesocosm settings.

I found that heterotroph biovolume and percentage of heterotrophic fatty acids in the diet could be useful metrics for predicting herbivore success in nature. At 3 weeks, increased heterotroph abundance and decreased percentage of heterotrophic fatty acids in the diet predicted herbivore growth and survival, but 6-week models revealed the opposite pattern, suggesting that low heterotroph abundance and high heterotroph-derived fatty acids support fish growth and survival. The usefulness of these diet metrics depends on the life history of the study organism. For example, Sailfin Mollies mature approximately 21-68 days after birth (Snelson et al. 1986), and at the end of this study the fish were 63-70 days old. Because I found a few mature males at the end of the experiment, it was apparent that these fish were beginning to transition from juveniles to reproductively-capable adults. As such, their energetic requirements were shifting from growth to reproduction, and this shift was evidenced by the change in dietary requirements suggested by the models at 3- (growth phase) and 6- (reproductive phase) weeks.

Thus, heterotroph quantity in the diet may play a role in the early growth phase of Sailfin Mollies, but heterotroph quality (heterotrophic fatty acids) plays a larger role once fish approach the reproductive phase.

The (SAFA+MUFA): PUFA ratio was not found to be an important predictor of herbivore growth and survival in this study, although we found that an increase in PUFAs (autotroph-derived) promoted fish survival at 3 weeks in my field experiment. These results are not suggesting that autotroph-derived fatty acids are unimportant to herbivores, as several studies have proven the importance of these dietary elements for growth, survival and reproduction of aquatic organisms (e.g., Martin-Creuzburg et al. 2005, Martin-Creuzburg et al. 2011). Rather, I suggest that heterotrophs supplement autotrophic-based diets, and the quality (fatty acid abundance) of these microbes may influence herbivore life history.

The results from this mesocosm experiment are slightly different than the results from the previous field study, but both studies suggest that heterotrophs supplement the herbivorous diet in ways that benefit consumer life history, thereby supporting the Heterotroph Facilitation hypothesis. The Lipid Allocation hypothesis emphases autotrophic-derived lipids as the main driver of herbivore success, but this was not exclusively supported in either experiment. Previous studies support the idea that heterotroph supplementation is important for herbivore diets (e.g., Edwards and Meyer 1990, Fuller et al. 2004, Jäger et al. 2014) and have found that a diet consisting only of heterotrophs or autotrophs is suboptimal relative to a diet containing both in intermediate quantities (e.g., Martin-Creuzburg et al. 2005, Martin-Creuzburg et al. 2011).

Here, I found that diets with increased autotrophs do not benefit herbivores, but instead, diets with increased heterotrophic fatty acids promote herbivore growth and survival. The increased heterotroph biovolume in the 3-week models may suggest otherwise, but when considering overall growth and survival of fishes at the end of the experiment, it is evident that those consuming epiphyton with these qualities do not experience high survival past this time period. Fish that continued to survive and grow through the end of the experimental period were those consuming food with low heterotroph abundance and high percentages of heterotrophic fatty acids, providing evidence that heterotrophic quality (fatty acids) and not quantity (heterotrophic biovolume) represent an important part of the herbivorous diet.

Although several studies have examined the influence of dietary heterotrophs on herbivore life history (e.g., Bowen 1984, Smoot and Findlay 2010, Belicka et al. 2012), it is not recognized as a fundamental part of the herbivorous diet (White, 1985). Studies that examine the effects of diet quality have historically used stoichiometry or nutritional ecology to describe consumer life history, but neither framework solely explains these results. The ecological stoichiometry framework predicts that diets with lower C:P ratios are the highest quality for consumers (Sterner and Elser 2002). While this was true for the carnivore diets, 'light + P' epiphyton also had a low C:P ratio, and fish consuming epiphyton from this treatment experienced the lowest survival. The nutritional ecology framework predicts that high PUFA content represents a high-quality diet (e.g., Müller-Navarra et al. 2004, Persson and Vrede 2006), but variation in PUFAs (measured as (SAFA + MUFA): PUFA) was not retained in the models that best fit the data in this study, or in the field study.

This suggests that estimations of food quality assessed by stoichiometric ratios or from autotroph-derived fatty acids (PUFAs) may not be the most reliable metrics for all study systems (Trexler et al. 2015). However, these studies do not address how other nutritional components (e.g., macronutrients, algal starch, etc.) may have varied in response to our experimental manipulations, or their interactive effects on Sailfin Molly life history.

My goal for this research was to explore the conditions that would favor the evolution of an herbivorous diet from a carnivorous or omnivorous diet. The fitness peak of carnivorous consumers was higher than all herbivorous consumers in this study. However, diets comprised of mixed autotroph and heterotroph diets were sufficient in supporting fish survival. Compared to algae, carnivorous prey are in low abundance (Sanchez and Trexler 2016) and require elevated risk to obtain (reviewed by Milinski 1985). Supplementing the herbivorous diet with heterotrophic microbes can compensate for the generally poor quality of aquatic primary producers. This 'multichannel feeding' (Wolkovich et al. 2014) may allow consumers to expend less energy obtaining necessary nutrients to support growth and survival. Experimental tests of these hypotheses are valuable for establishing a research framework that will allow us to more fully understand the diet evolution and herbivory from an adaptation perspective.

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Table 5.1. Sources of fatty acid tracers used in this study (modified from Sanchez and Trexler 2018).

Carbon Source (grouped by fatty acids used in this study)	**References**
Bacteria (15:0i, 15:0a, 15:0n, 17:0i, 17:0a, 17:0n, 18:1w7, 19:1)	
Odd carbon number fatty acids, 15:0i, 15:0a, 17:0i, 17:0a, 18:1w7	Findlay and Dobbs (1993); Napolitano (1999) and references therein; Volkman et al. (1980)
Fungi (16:1w5, 18:3w9, 18:3w12)	Wang et al. (2017)
Algae (16:3, 18:3w3, 18:4, 18:3w6, 20:4w6, 20:5w3 (EPA), 20:4, 22:4w6, 22:5w3, 22:5w6, 22:6w3)	
14:0, 16:1w7: multiple sources, but high in diatoms and some cyanobacteria	Napolitano (1999) and references therein
C_{16} PUFA: green algae and diatoms	Kates and Volcani (1966); Cranwell et al. (1990); Napolitano (1999)
18:3w3: green algae, cyanobacteria	Ahlgren et al. (1992); Dalsgaard et al. (2003)
18:3w6: cyanobacteria	Napolitano (1999)
18:4w3, 18:5w3, 22:6w3: dinoflagellates	Ahlgren et al. (1992); Dalsgaard et al. (2003)
20:5w3, ratio of 20:5w3 to 22:6w3: diatoms	Napolitano (1999); Dalsgaard et al. (2003)

Table 5.2. Summary of results showing differences between experimental treatments for diet types (epiphyton and bloodworms) and periphyton. FA ratio= (SAFA+MUFA): PUFA ratio. For epiphyton diets, upward facing triangles indicate relatively high values, whereas downward facing triangles indicate relatively low values. Because bloodworms and periphyton were significantly different in quality than epiphyton, triangles for these variables represent relative comparisons rather than statistical comparisons. Values that are not statistically significant are indicated by "ns". Blanks indicate metrics that could not be measured.

				EPIPHYTON		
Metric	**Ambient Periphyton**	**Carnivore Diet**	**Light + P**	**Light only**	**Shade + P**	**Shade only**
C:P	▲	▼	▼	▲	▼	▲
N:P	▲	▼	▼	▲	▼	▲
A:H biovolume	ns	--	▼	▼	▲	▲
Relative FA content	▼	▼	▼	▼	▲	▲
Percent algal FA (%/wt)	ns	ns	ns	ns	ns	ns
Percent heterotrophic FA (%/wt)	▲	▲	▼	▼	▲	▲
FA ratio	▲	▼	ns	ns	ns	ns
EPA (%/wt)	▼	▲	▼	▲	▲	▼
DHA (%/wt)	▼	▲	ns	ns	ns	ns
ARA (%/wt)	▼	▲	▲	▲	▼	▼
Edible algal spp.	▼	--	▲	▼	▲	▼

Table 5.3. Summary of results showing differences between tissues from fish reared on different diets. FA ratio= (SAFA + MUFA): PUFA ratio. Upward facing triangles indicate relatively high values, whereas downward facing triangles indicate relatively low values. Values that are not statistically significant are indicated by "ns". There were no surviving fish from 'Light + P' treatments at the end of the experiment, therefore, I was unable to analyze tissues from these fish.

	FISH TISSUES			
Metric	**Carnivore**	**Light only**	**Shade +P**	**Shade only**
Relative FA content	▲	▼	▲	▼
Percent autotrophic FA (%/wt)	**ns**	**ns**	**ns**	**ns**
Percent heterotrophic FA (%/wt)	**ns**	**ns**	**ns**	**ns**
FA ratio	**ns**	**ns**	**ns**	**ns**
EPA (%/wt)	**ns**	**ns**	**ns**	**ns**
DHA (%/wt)	**ns**	**ns**	**ns**	**ns**
ARA (%/wt)	**ns**	**ns**	**ns**	**ns**

Table 5.4. Comparison of structural equation models used to test 'Heterotrophic facilitation' and 'Lipid allocation' hypotheses. A:H = A:H biovolume, Het. FA = percentage of heterotrophic fatty acids, FA ratio= (SAFA + MUFA): PUFA ratio. ΔAICc values ≤ 2 are highlighted in bold.

		3 weeks			6 weeks		
Model	**Description**	**ΔAICc**	**AIC_w**	***w_{min}/w_j***	**ΔAIC_c**	**AIC_w**	***w_{min}/w_j***
1	A:H + Het. FA+ FA ratio	**1.81**	**0.24**	**2.47**	**1.87**	**0.20**	**2.55**
2	A:H+ Het. FA	**0.00**	**0.58**	**1.00**	**0.00**	**0.52**	**1.00**
3	A:H+ FA ratio	7.57	0.01	44.04	3.85	0.08	6.86
4	Het. FA + FA ratio	5.11	0.05	12.87	8.42	0.01	67.36
5	A:H	5.58	0.04	16.28	2.28	0.17	3.13
6	Het. FA	3.91	0.08	7.06	6.53	0.02	26.18
7	FA ratio	9.42	0.01	111.05	9.20	0.01	99.48

Figure Legends

Fig. 5.1. (A) Standard length (mm) of juvenile Sailfin Mollies raised on experimental diets showing increased growth of fish consuming epiphyton grown in 'Light only' and 'Light + P' conditions at 3 weeks. (B) Survival scores (p') of juvenile Sailfin Mollies showing low survival of fish consuming epiphyton grown in 'Light + P' conditions.

Fig. 5.2. (A) The structural equation model with the best fit (ΔAICc = 0.00) showing autotroph: heterotroph (A:H) biolvolume and heterotrophic fatty acid percentage as the best predictors of fish life history at 3 weeks. Numbers indicate regression coefficients for each path analyzed, suggesting that decreased A:H biolvolume and decreased heterotrophic fatty acid percentage results in increased fish life history. (B) The structural equation model with the best fit (ΔAICc = 0.00) showing autotroph: heterotroph (A:H) biolvolume and heterotrophic fatty acid percentage as the best predictors of fish life history at 6 weeks. Numbers indicate regression coefficients for each path analyzed, suggesting that increased A:H biolvolume and increased heterotrophic fatty acid percentage results in increased fish life history. This is opposite of the 3-week model results.

FIG. 5.1.

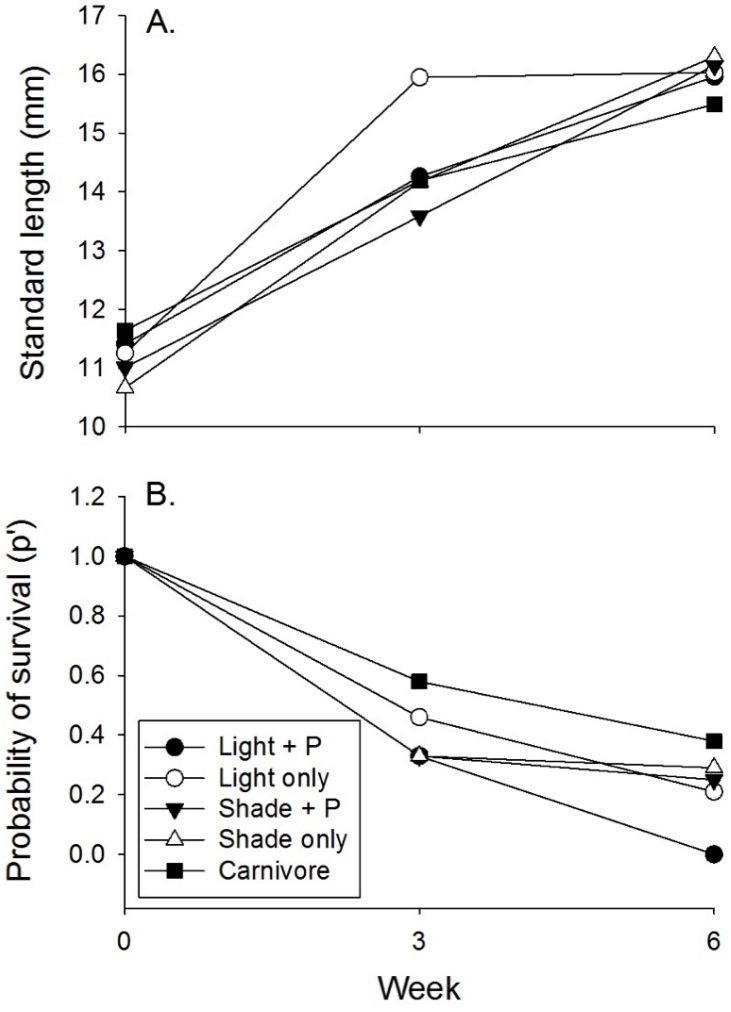
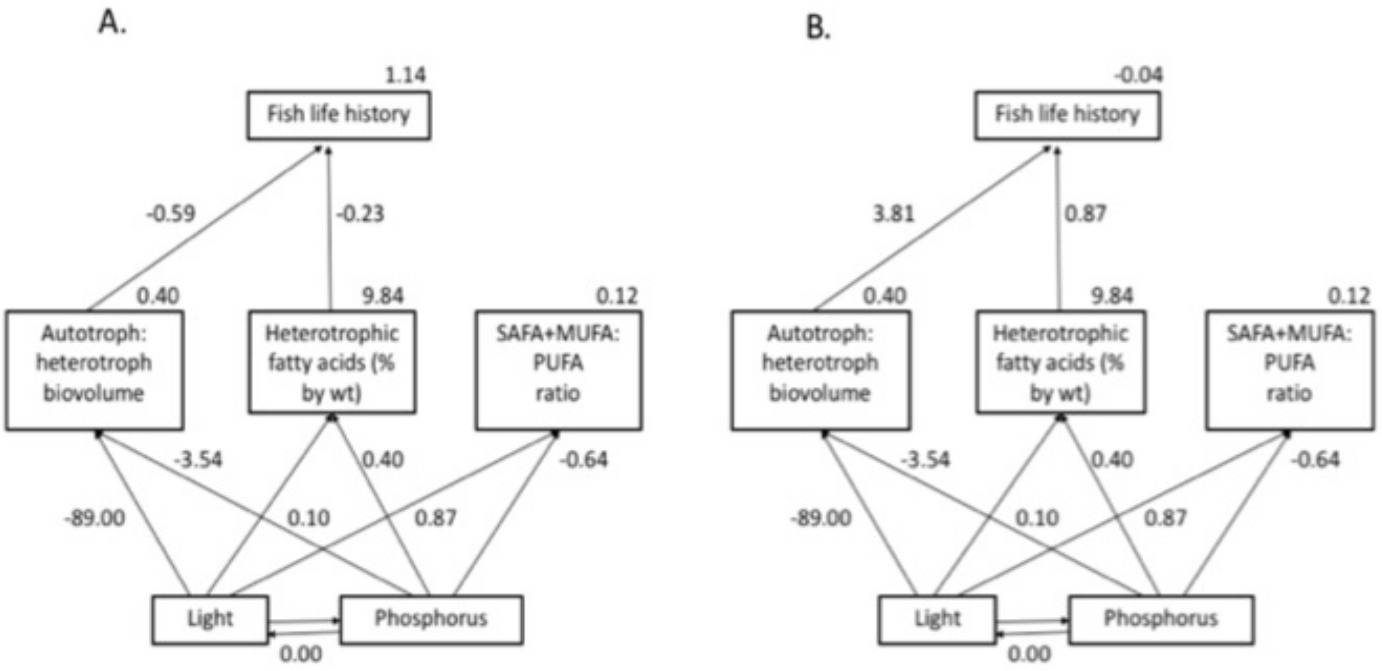

A.
Standard length (mm)
17
16
15
14
13
12
11
10
B.
Probability of survival (p')
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Light + P
Light only
Shade + P
Shade only
Carnivore
0
3
6
Week

FIG. 5.2.


A.
1.14
Fish life history
-0.59
-0.23
0.40
9.84
0.12
Autotroph: heterotroph biovolume
Heterotrophic fatty acids (% by wt)
SAFA+MUFA: PUFA ratio
-3.54
0.40
-0.64
-89.00
0.10
0.87
Light
Phosphorus
0.00
B.
-0.04
Fish life history
3.81
0.87
0.40
9.84
0.12
Autotroph: heterotroph biovolume
Heterotrophic fatty acids (% by wt)
SAFA+MUFA: PUFA ratio
-3.54
0.40
-0.64
-89.00
0.10
0.87
Light
Phosphorus
0.00

Supplementary Information

Table S.5.1. Average values ± 1 SD for all measured diet variables by treatment. NA= values could not be measured

Treatment	C:P	A:H biovolume	Het. FA (% by weight)	PUFA (% by weight)	SAFA (% by weight)	MUFA (% by weight)	EPA	DHA	ARA (% by weight)	Proportion of edible algal spp.
Ambient Periphyton	5229.33 ± 1470.86	16.26 ± 2.90	16.59 ± 2.62	11.37 ± 1.95	60.69 ± 3.56	27.94 ± 2.02	0.00 ± 0.00	0.00 ± 0.00	0.67 ± 0.12	0.16 ± 0.13
Bloodworms	78.06	NA	18.43	46.30	14.00	10.70	29.30	12.90	4.10	NA
Light+ P	648.24 ± 73.87	11.01 ± 5.86	7.29 ± 3.30	17.46 ± 8.54	54.48 ± 5.10	28.06 ± 6.76	0.21 ± 0.31	0.11 ± 0.25	1.03 ± 0.76	0.66 ± 0.25
Light only	1341.25 ± 630.36	13.80 ± 7.14	7.69 ± 2.54	12.77 ± 2.13	54.76 ± 3.94	32.48 ± 2.80	0.49 ± 0.32	0.18 ± 0.19	1.07 ± 0.57	0.34 ± 0.33
Shade + P	502.92 ± 35.57	23.81 ± 12.16	12.31 ± 1.43	22.43 ± 9.66	55.53 ± 5.64	22.04 ± 5.05	1.25 ± 0.34	0.04 ± 0.10	0.10 ± 0.23	0.61 ± 0.22
Shade only	1104.67 ± 358.47	18.98 ± 11.22	9.94 ± 2.17	15.08 ± 4.08	55.75 ± 5.44	29.17 ± 2.73	0.23 ± 0.22	0.18 ± 0.27	0.50 ± 0.36	0.25 ± 0.24

Table S.5.2. Average relative abundances ± 1 SD of autotrophs and heterotrophs comprising ambient periphyton and experimental epiphyton diets

TREATMENT	AUTOTROPHS					HETEROTROPHS	
	Diatoms	**Solitary green algae**	**Filamentous green algae**	**Coccoid cyanobacteria**	**Filamentous cyanobacteria**	**Heterotrophic bacteria**	**Fungi**
Ambient Periphyton	0.05 ± 0.03	0.05 ± 0.05	0.06 ± 0.07	0.77 ± 0.19	0.06 ± 0.07	0.03 ± 2 x 10^{-2}	6 x 10^{-5} ± 3 x 10^{-5}
Light+ P	0.03 ± 0.03	0.40 ± 0.37	0.07 ± 0.11	0.49 ± 0.41	0.01 ± 0.01	0.03 ± 0.01	1 x 10^{-3} ± 1 x 10^{-3}
Light only	0.15 ± 0.26	0.12 ± 0.08	0.07 ± 0.05	0.63 ± 0.37	0.63 ± 0.37	0.02 ± 0.01	1 x 10^{-3} ± 5 x 10^{-4}
Shade + P	0.12 ± 0.19	0.36 ± 0.23	0.12 ± 0.19	0.42 ± 0.30	0.03 ± 0.04	0.03 ± 0.02	2 x 10^{-3} ± 9 x 10^{-4}
Shade only	0.07 ± 0.11	0.14 ± 0.14	0.04 ± 0.03	0.74 ± 0.25	0.74 ± 0.25	0.02 ± 0.02	2 x 10^{-3} ± 7 x 10^{-4}

Table S.5.3. Average values ± 1 SD for all measured fish variables by treatment. There were no surviving individuals from 'Light + P' treatments, so fatty acid profiles were not available for these fish (represented by NA in the table).

Treatment	Survival score (*p'*) (3wk/6wk)	Size (mm) (3wk/6wk)	Het. FA (% by weight)	PUFA (% by weight)	SAFA (% by weight)	MUFA (% by weight)	EPA:DHA	ARA (% by weight)
Carnivore	0.63 ± 0.14/ 0.54 ± 0.07	14.19 ± 0.80/ 15.40 ± 0.70	9.53 ± 5.30	26.94 ± 3.55	46.41 ± 3.76	26.65 ± 7.31	0.15 ± 0.07	4.67 ± 2.16
Light+ P	0.29 ± 0.0.07/ 0.00 ± 0.00	14.26 ± 1.04/ 16.26 ± 0.53	NA	NA	NA	NA	NA	NA
Light only	0.42 ± 0.30/ 0.21 ± 0.14	15.95 ± 1.16/ 16.03 ± 0.58	5.51 ± 0.12	28.50 ± 0.48	49.23 ± 1.45	22.27 ± 0.97	0.13 ± 0.08	6.21 ± 0.12
Shade + P	0.33± 0.00/ 0.21 ± 0.07	13.59 ± 0.38/ 16.15 ± 0.48	6.23 ± 0.72	26.80 ± 1.15	50.57 ± 1.10	22.63 ± 0.58	0.14 ± 0.22	5.89 ± 0.25
Shade only	0.33 ± 0.12/ 0.21 ± 0.07	14.17 ± 1.09/ 16.30 ± 1.48	6.28 ± 0.35	28.72 ± 1.81	48.52 ± 0.72	22.76 ± 1.10	0.12 ± 0.06	6.33 ± 0.50

CHAPTER 6

NUTRITONAL LANDSCAPE OF THE EVERGLADES: MECHANISMS SHAPING CONSUMER NICHE DIVERSITY ALONG THE BROWN-GREEN FOOD WEB CONTINUUM

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Abstract

Niche-based models assume that resource quality and availability interact with the physical environment to drive community assembly based on consumer diets and food web function. Conversely, dispersal-based models predict that community assembly is driven by stochastic colonization, independent from species traits. It has been suggested that resource quality/quantity drives diet evolution, which can in turn, influence consumer function in a food web. Therefore, invoking models that ignore the role of species traits in shaping communities limits our ability to understand the evolutionary consequences of ecological processes.

To better understand the mechanisms shaping consumer niche diversity, I determined if niche- or dispersal-based predictions best described consumer dynamics in the Florida Everglades based on the nutritional landscape. I sampled periphyton and consumers from 22 sites across the ecosystem and measured variables describing food quality (macronutrients, stoichiometry, edibility, fatty acid profiles) and food availability (periphyton volume, herbivore density) in both the wet and dry seasons. I used Structural Equation modelling to examine these variables as potential drivers of consumer density and to identify the conditions where herbivory and omnivory may be favored. I interpreted the results in the context two hypotheses about the maintenance of herbivorous diets in food webs, the Heterotroph Facilitation Hypothesis (herbivory is adaptive because herbivores supplement their diets with heterotrophic microbes) and the Suboptimal Habitat Hypothesis (herbivory may be an adaptive strategy to allow organisms to invade habitats with decreased resource quality).

My data revealed that herbivores track food quality when habitats are stable, but they can persist in a multitude of habitat types and survive on resources of varying quality when habitats are variable. These results suggest that herbivore diets follow niche-based predictions in the wet season, but dispersal-based predictions in the dry season. In contrast, omnivores rely on high-quality resources in both seasons, consistent with niche-based predictions. Taken together, these results partially support the Suboptimal Habitat Hypothesis as an explanation for the evolution and maintenance of herbivory in this system. By identifying an evolutionary mechanism that promotes herbivory, we are able to more fully describe the complex role of these consumers in functional food webs. Future trophic studies may benefit by using a framework that incorporates both ecology and evolution to predict how food webs are organized in nature.

Keywords Adaptive evolution, Everglades, diet evolution, food web, herbivory, omnivory, trophic dynamics, niche-based models, dispersal-based models, community assembly

Introduction

Consumers inhabit landscapes that vary spatially and temporally in resource quality and quantity (Hunter 2016). Until recently, ecologists predicted that community assembly resulted from local processes such as habitat filtering and biotic interactions, and species' ability to invade established communities was determined by their traits (Chase and Leibold 2003; McPeek 2017). Niche-based models assume robust dispersal to describe how resource quality and availability interact with environmental stress to drive community assembly (Chase and Leibold 2003; Chase 2007).

When dispersal is assumed to be limited, neutral and patch dynamics models predict a role for stochastic colonization in community assembly, independent from species' traits (Hubbell 2001; Chase and Leibold 2003; Chase 2007). Some studies have shown that simplified dispersal-based models yield similar results to relatively complicated niche-based models (Condit et al. 2000; Bell 2001; Hubbell 2001, Volkov et al. 2003), challenging our ability to assign a single model to explain field data (Brown et al. 2017). There is no shortage of studies detailing the role of resource availability (e.g., Desilets and Houle 2005; Thompson and Townsend 2005) and environmental stress (e.g., Desilets and Houle 2005; Walters and Post 2011) in shaping communities via their effects on food webs. Therefore, inferring food web dynamics without considering the effects of both species' traits and environmental factors limits our ability to fully understand how communities are organized.

While these frameworks have allowed us to examine the underlying factors affecting food-web function, they do not consider the source of energy flow in their predictions. Determining the relative contribution of detrital and algal resources to aquatic food webs is an important goal in characterizing trophic structure (Moore et al. 2004; Belicka et al. 2012). Although studies on autotrophic food webs dominate the literature, it is becoming apparent that "brown" food webs play a key role in trophic structure, particularly in wetland ecosystems (Williams and Trexler 2006; Belicka et al. 2012; Sanchez and Trexler 2018). Models that link green and brown food webs have focused on nutrient cycling, where dead green matter transfers to a detrital pool that is mineralized. The mineralized nutrients then serve as sources of limiting nutrients for the primary producers comprising the algal pool (DeAngelis et al. 1989; DeAngelis 1992;

Wolkovich et al. 2014). In addition to nutrient cycling, consumers can connect detrital and algal food webs by accessing the mineralized detritus directly (by eating it), by eating lower consumers that are detritivores (Wolkovich et al. 2014), or by consuming closely associated primary producers (Sanchez and Trexler 2016). Typically, brown and green pathways are studied independently, but there is evidence that "true" herbivory is rare in nature and instead, detritivory facilitates herbivory (Sanchez and Trexler 2018).

Herbivores and omnivores mobilize the captured energy from detrital and algal pools, but it is believed to be constrained by the vast variation in food quality at the base of both food webs. In nature, resources are distributed across a heterogeneous landscape, resulting in natural variation in consumer life history that drives species relative abundances and distributions (Kareiva 1990; Tilman 1994; Polis et al. 1997; Power and Dietrich 2002; McIntosh et al. 2004; Torres-Ruiz et al. 2007; Doi 2009; Guo et al. 2016). Recent evolutionary studies have found that variation in resources can also drive diet evolution (Diehl 2003; Krivan and Diehl 2005; Namba et al. 2008; Sanchez and Trexler 2016, 2018), suggesting that adaptive evolution can influence consumer function in a food web and shape community structure. The evolution of herbivory or omnivory from carnivory has been documented in several lineages (Vermeij 1992; deMaintenon 1999; Van Damme 1999; Vermeij and Lindberg 2000; Bellwood 2003; Espinoza et al. 2004; Pauls et al. 2008, Bellwood et al. 2014; Reisz and Frobisch 2014), and indicates that there is some adaptive advantage to eating plants. A few modelling studies found that the evolution of omnivory is favored when basal resources are high in abundance, and when higher-quality animal prey are rare (Diehl 2003; Krivan and Diehl 2005), but the evolution of herbivory is relatively understudied.

Identifying the adaptive significance of herbivory may improve niche-based models by providing a mechanism that describes how resources and environments interact to drive community assembly. To better understand the mechanisms that shape consumer niche diversity, I evaluate the explanatory power of two hypotheses about the maintenance of herbivorous diets in food webs: 1) the Heterotroph Facilitation Hypothesis, which states that herbivory is adaptive because indirect detritivory supplements the herbivorous diet (i.e., assimilating nutrients from heterotrophs that were not the target diet item, but consumed as a consequence of their close association with primary producers), 2) and the Suboptimal Habitat hypothesis, which states that herbivory is adaptive because it allows organisms to invade and persist in 'suboptimal' habitats (Sanchez and Trexler 2016). I found evidence supporting both of these hypotheses in previous studies focusing on herbivorous members of the genus *Poecilia*. In both a lab and field study, I found that heterotroph-derived fatty acids supplement the diet of juvenile Sailfin Mollies (*P. latipinna*) and play an important role in their growth and survival (Sanchez and Trexler 2018), consistent with the Heterotroph Facilitation Hypothesis. An ancestral state reconstruction of diet and habitat across a phylogeny of the genus *Poecilia* revealed that herbivory evolved in response to habitat transitions across the freshwater-marine boundary (Sanchez et al., in review). This finding supports the Suboptimal Habitat Hypothesis as a mechanism behind the evolution of herbivory in the subgenus *Mollienesia* (includes *P. latipinna*).

Although these studies provide alternative explanations for the evolution of herbivory, the key distinction between these hypotheses is that heterotroph facilitation is a mechanism to overcome poor food quality, and invasion of suboptimal habitats allows passage into habitats with varying resource bases. The bases of these findings is the same, however; resource quality and/or availability is responsible for the evolution and/or maintenance of herbivory in nature and the relative green. Therefore, I predict that these adaptive hypotheses may be able to explain variation in consumer trophic dynamics using niche-based and dispersal-limited frameworks.

The Florida Everglades is an ideal system to study trophic dynamics because food quality varies greatly across the landscape. The Everglades ecosystem has been impacted by urban and agricultural activities that have resulted in water diversions and nutrient enrichment (Noe et al. 2001; Gaiser et al 2005). The wetlands in the northern region are managed by a series of water control structures that divert water from developed areas and maintain water in 'water conservation areas' (WCA) that serve as a supply for South Florida (Light and Dineen 1994). Because these marshes are heavily managed and in close proximity to agricultural lands, they are impacted by nutrient input. The marshes in the southern region are relatively oligotrophic and hydrology is driven by rainfall, unlike the WCAs (Noe et al. 2001). As a result, these areas experience a wet season (June-November), followed by drying events (December-May). Periphyton, the primary basal resource in Everglades (Browder et al. 1994; Radar and Richardson 1994; Williams and Trexler 2006), is composed of assemblages of autotrophs (green algae, diatoms and cyanobacteria) and heterotrophs (fungi and bacteria; Gaiser et al., 2004).

The complexity of these assemblages has resulted in oversimplifications about the relative contribution of green and brown energy channels to wetlands food webs (Taylor and Batzer 2010), but several studies have shown that both autotrophs and heterotrophs are sources of energy for consumers in the Everglades (Williams and Trexler 2006; Belicka et al. 2012; Sanchez and Trexler 2018). Both of these periphyton components respond rapidly to changes in hydrology and water chemistry (Pan et al. 2000; Noe et al. 2001; McCormick et al. 2002; Gottlieb et al. 2015), thus creating variation in the nutritional quality at the base of the Everglades food web. In addition to variation in food quality, the Everglades has an unusual Eltonian biomass pyramid, where there is an extremely high abundance of periphyton relative to consumers (Turner et al. 1999; Gaiser et al. 2005). The typical Everglades food web is dominated by omnivorous and herbivorous macroinvertebrates and small fishes (Chick et al. 2008), and there is significant heterogeneity in the distribution of consumers across the landscape. These food web characteristics combined with the longitudinal variation in resource quality suggests that bottom-up transfer of energy is not very efficient in the Everglades (Turner et al. 1999; Geddes and Trexler 2003).

The impacts of nutrients and hydrology on Everglades periphyton communities have been extensively studied as part of the Everglades restoration plan (e.g., Gaiser 2009; Gaiser et al, 2011), and researchers have gained interest in the resulting trophic dynamics in light of these studies (e.g., Williams and Trexler 2006; Belicka et al. 2012; Trexler et al. 2015).

In this study, I take advantage of the high producer biomass and natural variation in resources (brought about by hydrological disturbance and nutrient enrichment) in the Everglades to determine if hypotheses describing the evolution of herbivory can predict consumer dynamics across the landscape using niche-based and dispersal-limited frameworks.

Methods

Field Collection

I collected periphyton and fish samples from 22 sites across the Everglades (Fig. 6.1) landscape during the peak of the dry season (June-July 2016) and the wet season (January-February 2017). These sites span much of the freshwater Everglades ecosystem, thus capturing the longitudinal environmental gradient. In addition, these marshes vary in hydroperiod, where some experience annual dry-downs and others are constantly flooded (Table 6.1). I measured pH and conductivity from each site using a YSI meter and pH probe. Using a throw trap (Jordan et al. 1997), a random $1m^2$ plot was surveyed for periphyton cover (%), percentage cover of emergent macrophytes, total periphyton volume (mL), estimates of percentage of dominant submerged plant species (Table 6.1), and floating-mat and soil type (Table S.6.1). Hydrological data were obtained from the Everglades Depth Estimation Network (EDEN, United States Geological Survey; Table 6.1). I focused on days since dry (DSD; the number of days since the depth measured < 5 cm), and hydroperiod (the number of days in the 365 before sampling that the depth at the site measured > 5 cm) as hydrology metrics.

Because South Florida experiences a wet and dry season, hydroperiod was calculated by water year, which runs from the start of the wet season (May through October) through the end of the follow dry season (November through April).

Consumer density data were taken from July 2016 and February 2017 collections. I focused on six native fish and four macroinvertebrate species: Sailfin Molly (*Poecilia latipinna*), Flagfish (*Jordanella floridae*), Bluefin Killifish (*Lucania goodei*), Eastern Mosquitofish (*Gambusia holbrooki*), Golden Topminnow (*Fundulus chrysotus*), Least Killifish (*Heterandria formosa*), riverine grass shrimp (*Palaemonetes paludosus*), scuds (*Hyalella spp*), mayflies (Ephemeroptera), and midge larvae (chironomid *spp*). Consumer density was estimated by calculating the average number of consumers per 1-m^2 from 5-7 randomized throw-trap samples at each of the sites for each season. I estimated fish trophic groups using gut-content data from Everglades fishes collected in wet and dry seasons (Loftus 2000), and I used the trophic groups presented in Belicka et al. (2012) to categorize macroinvertebrate diets. There may be no "true" aquatic herbivores in the Everglades (Belicka et al. 2012, Sanchez and Trexler 2018), but rather herbivorous consumers that supplement their diet with microbes originating from detritus. Therefore, we grouped all consumers that subsist on the autotroph-detritus continuum into a group referred to as 'herbivores'. Trophic groups (herbivores and omnivores) were summed over each site to obtain consumer density (no. of consumers/m^2) for each season.

I collected periphyton from each site and characterized samples into 4 specific types: 1) *Floating mat*- aggregation of floating mature periphyton. Might be found in homogeneous floating aggregations, in small clumps in association with *Utricularia* spp., or in an epiphytic growth on emergent vascular plant stems; 2) *Benthic mat*- submerged periphyton adjacent to the exposed sediment. Might be found in homogeneous aggregations, or in smaller clumps in association with flocculent matter; 3) *Epiphyton*- newly colonizing epiphyton that is collected from the submerged stems of aquatic macrophytes; or 4) *Filamentous green algae*- filamentous green algal species usually occupying the water column in colonies visible to the naked eye; occasionally loosely attached to aquatic vegetation. Percentages of each periphyton type were assigned based on their abundance in the 1-m^2 sample plot (Table 6.2). These percentages were multiplied by the total periphyton volume (mL) in the sample plot to estimate the volume of each periphyton type in the 1-m^2 plot. For each periphyton type, 50 mL samples (N=3) were collected, placed in separate vials, and brought back to the lab on ice. Samples of 1-3 dominant fish consumers were also collected from each site by hand net or throw trap, euthanized using an overdose of MS-222, transported on ice and subsequently frozen. All collections were handled using gloves to prevent any nutrient input.

Food Quality Assays

Nutritional quality of periphyton for consumers was assessed by estimating edibility, macronutrient composition, stoichiometry, and fatty acid profiles. I use the definition of "high" food quality that is presented in my previous studies (Sanchez and Treler 2016 and 2018).

Specifically, food with high essential fatty acid content (PUFAs, EPA, DHA, ARA), increased protein or lipids relative to carbohydrates, increased TP, and/or increased edible algal content relative to food with the opposite metrics is considered "high-quality". In the lab, periphyton samples were homogenized directly in the sample vial using a hand-held biohomogenizer. Known volumes of each periphyton type from each site were placed onto a clean microscope slide and autotrophic species (algae, cyanobacteria, diatoms) were counted using standard light microscopy at 40x magnification. Counts were transformed into total cells/mL of material, which were then used to estimate the proportion of edible (green algae and diatoms) and inedible (cyanobacteria) components. These percentages were multiplied by the volume of each periphyton type (ml/m^2), summed by site, and converted to proportions to yield the proportion of edible and inedible species (%) for each site (for each season). A subsample of each periphyton type (from each site/season) was dried to constant weight at 60°C and placed in a muffle furnace at 500°C for 1 hour to estimate ash-free dry mass (AFDM). I then estimated the organic and mineral components of each periphyton type (mg/m^2).

The remaining samples (including fish) were freeze-dried and prepped for nutrient analyses. Total protein was measured using a modified Lowry technique (Markwell et al. 1978) by digesting 0.15 mg of dried sample in 50 µL NaOH for 1 hour at 60 C. I then followed the standard 96-well assay protocol listed in the Pierce BCA Protein Assay Kit (Thermo Scientific). This kit yields the colorimetric determination of total protein through reduction of copper in an alkaline medium. Samples were read on a microplate reader (Biotek Synergy HT multi-well) at 562 nm.

Total carbohydrates were quantified by digesting 0.4 mg of dried sample in Trichloroacetic acid for 3 hours at 90 C. Following sample digestion, I used the vanillin-sulfuric acid method (Masuko et al. 2005) in a 96-well microplate preparation and read results at 490 nm. Total lipid concentration was estimated by using the Folch method (Folch et al. 1956) to digest 0.25mg of dried sample in 0.2 ml of chloroform: methanol (2:1 v/v). I transferred 30µL of supernatant from each sample to microcentrifuge tubes and incubated uncovered for 30 minutes at 90 C. I then followed the methods of Cleveland and Montgomery (2003) to colorimetrically estimate total lipids and read these samples at 540 nm. All macronutrients were calculated as mg macronutrient/sample. I divided these values by the dry weight of the sample (e.g., 0.4 mg for carbohydrate estimation), and multiplied them by the dry weight of the organic portion of periphyton found in 1 m^2 (estimated from AFDM) to yield mg macronutrient/m^2 for each periphyton type. These values were summed over all periphyton types to yield total macronutrient/m^2 for each site, by season.

Freeze-dried periphyton and fish samples were sent to the Southeastern Research Center (SERC) located at Florida International University for stoichiometric (C:N:P) analyses (approx. 20mg per sample) and were sent to Microbial ID (Newark, DE) for lipid profile analyses (15 mg). Total nitrogen, carbon and phosphorus were converted from ug/g to moles in order to estimate stoichiometric ratios (C:P and N:P) for each periphyton type and for fish tissues. These ratios were averaged across all periphyton types and fish species for each site and reported by ratio and TP (ug/g).

Fatty acids were sorted by autotroph- and heterotroph-derived diet tracers (see Sanchez and Trexler 2018 for specific fatty acid tracers), by saturation (polyunsaturated, saturated, monounsaturated; PUFA, SAFA, MUFA, respectively) and by essential fatty acids (EPA, DHA, ARA). Values were returned as percentages of individual fatty acids in the total sample. For periphyton samples, saturation values were converted to a ratio (PUFA: (SAFA + MUFA), herein referred to as 'PUFA ratio'), and all others were converted to mg fatty acid/m^2 for each periphyton type by multiplying each percentage by periphyton volume (mL/m^2). These values were summed over all periphyton types to yield total fatty acid/m^2 for each site, by season. For fish tissues, I averaged percentages of individual fatty acids (autotrophic, heterotrophic, EPA, DHA and ARA) by species and by site to determine any intra- and interspecific differences in tissue composition.

Statistical analyses

The effects of hydroperiod and season on nutritional quality of food (periphyton) and consumer density were analyzed. To meet the assumptions of analyses, I (Log + 1)-transformed all non-normal data and converted hydroperiod (obtained from EDEN) to categorical variables using hierarchical cluster analysis with the Sorensen (Bray-Curtis) distance measure with flexible beta linkages (CLUSTER package in R; Maechler et al. 2017). Converting these values to categorical variables allowed us to group nutritional variables and consumer densities by hydroperiod, therefore increasing degrees of freedom.

I then calculated z-scores for environmental variables (pH, conductivity, hydroperiod, DSD, and percent emergent plants), variables describing periphyton availability (periphyton cover %, floating mat volume, benthic mat volume, epiphyton volume, and filamentous algae volume), and variables describing periphyton food quality (protein, carbohydrate, lipid, heterotrophic fatty acid %, PUFA ratio, EPA %, TP, and edible algae). I reduced these variables using Principal Components Analyses (PC; using devtools package in R) with hydroperiod categories as grouping variables. I assessed variables for collinearity and those with Tolerance levels < 0.20 and Variance Inflation Factors > 5.00 were considered overlapping and were removed from the analyses. Variables that were highly collinear (mineral content, DHA, ARA, autotrophic fatty acid %, C:P, and N:P) were excluded from the analyses.

I compared algal composition of different periphyton types among hydroperiod categories using Analysis of Similarity (ANOSIM) and Similarity Percentage Analysis (SIMPER) using Primer v7. I used one-way Analysis of Variance (ANOVA) and Tukey Post-hoc tests to compare PC scores for periphyton quality/availability and consumer density across hydroperiod categories for each season, and to compare fish tissue composition (fatty acids, macronutrients, stoichiometry) among species by hydroperiod and season.

I estimated trophic groups of the common Everglades fish species using hierarchical cluster analysis (Sorensen distance measure) with flexible beta linkages (CLUSTER package in R; Maechler et al. 2017). This analysis was performed using gut content data collected from consumers in the Everglades sampled during the wet and dry seasons (taken from Loftus 2000).

The resulting diet clusters were used to categorize fish consumers in this study. The PC scores calculated from the environmental data (taken from the first two axes) were regressed with scores representing periphyton availability/quality and consumer density (omnivores and herbivores).

I used the residuals from these analyses as input variables for Structural Equation Models (SEM; Grace 2006), which were fit using the AMOS package in SPSS (Arbuckle 2014). Performing these analyses using residuals allowed us to determine patterns in consumer density that were uniquely attributed to periphyton availability and quality, independent of environment (pH, conductivity, hydroperiod, DSD, and percent emergent plants). Paths were varied between basal resource variables (periphyton quality and periphyton availability) and consumer density (herbivore and omnivore density) in each model. For herbivore models, the linkage between omnivores and herbivores represents the direct effects of competition with omnivores and/or predation by omnivores. Conversely, the linkage between these trophic groups in the omnivore models represents the direct effects of herbivore predation by omnivores.

Models were compared using Akaike's Information Criterion (AIC) by calculating ΔAICc (ΔAICc = AIC_i –min AICc, where i=model i), Akaike weight ($AIC_w = (e^{(-0.5 *\Delta AIC_i)}/\sum(e^{(-0.5 *\Delta AIC_r)}$), Relative likelihood ($L_r$), and Evidence Ratios ($w_{min}/w_j$, where w_{min} = AIC_w for the model with the smallest ΔAICc and w_j = AIC_w for the current model; Anderson and Burnham 2002). Path coefficients (regression weights) were assessed to determine which variables best predicted life history. Following Anderson & Burnham (2002), models with ΔAICc < 2.0 were considered equally explanatory.

Hypotheses Tests

The Suboptimal Habitat hypothesis predicts that herbivore density will vary in proportion to food quantity (independent of other ecological interactions). The Heterotroph Facilitation hypothesis predicts that heterotrophic microbes (e.g., bacteria, fungi) supplement and compliment the herbivorous diet, therefore, herbivore density should increase in proportion to the percentage of heterotrophic fatty acids found in the basal resource (periphyton). I used linear regression to assess the relationship between periphyton quality (residuals of PC1 and PC2), periphyton availability (residuals of PC1 and PC2) and herbivore density (residuals). Furthermore, I determined the relationship between periphyton availability (residuals of PC1 and PC2) and periphyton quality (residuals of PC1 and PC2) for each season. I interpreted the results of the regression analyses in the context of the Suboptimal Habitat and Heterotroph Facilitation predictions to determine if herbivore density could be explained by either of these mechanisms.

Results

Hierarchical cluster analysis of hydroperiod data produced four hydroperiod categories (Cophenetic correlation = 0.827): 1) water depth is greater than 5 cm for less than 300 days per year, 2) water depth is greater than 5 cm for 300-324 days per year, 3) water depth is greater than 5 cm for 325-350 days per year, and 4) water depth is greater than 5 cm for greater than 350 days per year. See Table 6.1 for list of sites and hydroperiod classifications.

Wet Season

There were few differences in environmental variables across hydroperiods in the wet season. Sites with < 300 days of inundation had average depths of 18.75 $\pm$ 4.65 cm, sites with water 300-324 days were 43.6 $\pm$ 7.83 cm, sites with water 325-350 days were 49.86 $\pm$ 7.47 cm, and sites with > 350 days of inundation were 53.5 $\pm$ 8.94 cm in the wet season. There were no differences in pH and conductivity among hydroperiods, but the shorter hydroperiods had more emergent vascular plants per m^2 than the longer hydroperiods. Sites inundated < 300 days had 82.4% greater emergent plant stem density than sites inundated with water > 350 days ($F_{3,20} = 5.71$, $P = 0.007$). There were no differences in periphyton C:P and N:P ratios across sites in the wet season (CP: $F_{3,18} = 1.61$, $P = 0.228$; NP: $F_{3,18} = 0.722$, $P = 0.554$). I was unable to statistically compare stoichiometric ratios of the different periphyton types due to variation in the types of periphyton available among sites, but filamentous green forms had the lowest C:P and C:N ratios, which were 49% lower than benthic mats (the highest ratios). Similarly, periphyton TP could not be statistically compared by periphyton type among sites, but filamentous green algae had the highest concentration of TP, which was 62% higher than floating mats, 45% higher than epiphyton, and 22% higher than benthic mats. Periphyton TP was not different among hydroperiods ($F_{3,21} = 1.17$, $P = 0.347$) during the wet season, and there were no differences in algal community composition of periphyton by hydroperiod ($R = -0.059$, $P = 0.902$) at that time.

Environmental and periphyton quality/availability variables were reduced using Principal Components Analysis using correlation matrices. All variables loaded strongly on the first two axes (loading $\geq$ 0.30; Table 6.3) and these data were used in further

analyses. Environmental PC axis 1 and 2 explained 49.9% and 27.8% of the variance in the data, respectively. Hydroperiod (+), DSD (+), and plant cover (-) loaded strongly ($\geq$ 0.30) on axis 1, whereas pH (+) and conductivity (-) loaded strongly on axis 2. Multivariate Analysis of Variance corroborated these findings and suggested that sites with < 300 days of inundation had greater plant density than sites with > 350 days in the wet season (γ= 0.054, $F_{15,45} = 4.53$, $P < 0.0001$). The first PC axis describing periphyton quality contained strong loadings (all +) for macronutrients (protein, carb, lipid), heterotrophic fatty acid % and edible algae (55.3% explained variation). Principal Components food quality axis 2 contained strong loadings for PUFA ratio (-), EPA % (+) and TP (-), and explained 15.4% of the total variation. Periphyton cover % (-), benthic mat (-), epiphyton (-) and floating mat volume (-) loaded on periphyton availability PC axis 1 (35.4% explained variation), and floating mat (+), epiphyton volume (-) and filamentous algae volume (+) loaded on PC axis 2 (23.5% explained variation).

Periphyton quality varied among hydroperiods in the wet season. Sites that were inundated for < 300 days had higher periphyton quality in terms of increased macronutrients, heterotrophic fatty acid %, and edible algae (PC1: $F_{3,21} = 5.53$, $P = 0.008$). Furthermore, short-hydroperiod sites had floating mats with 99% more protein (mg/m^2), 86% more carbohydrates (mg/m^2), and 94% more lipids (mg/m^2) than floating mats growing in long-hydroperiod sites (Protein: $F_{3,19} = 3.86$, $P = 0.03$; Carb: $F_{3,19} = 4.00$, $P = 0.027$; Lipid: $F_{3,19} = 4.15$, $P = 0.024$; Figs. 6.3 a&b). Short hydroperiods also had epiphyton with 96% greater lipid composition (mg/m^2) than long hydroperiod sites ($F_{3,11} = 4.27$, $P = 0.045$).

Sites did not vary in availability of floating mat or benthic mat (FM: $F_{3,19} = 1.31$, $P = 0.305$; BM: $F_{1,4} = 0.64$, $P = 0.48$), but long-hydroperiod sites had 91% less epiphyton than sites with < 300 water inundation ($F_{3,11} = 4.04$, $P = 0.05$).

The hierarchical cluster analysis of consumer gut-content data collected in the wet season produced two diet categories: 1) herbivore-detritivore (referred to as "herbivore"); 2) and omnivore (Cophenetic correlation = 0.996). In the wet season, Sailfin mollies and Flagfish were classified as herbivores, and the remaining four fish species were classified as omnivores (Fig. 6.2a). During this time, there were more 53% more herbivores than omnivores ($F_{1,43} = 7.66$, $P = 0.008$). However, there were no differences in herbivore and omnivore density by hydroperiod (Herb: $F_{3,21} = 0.45$, $P = 0.722$; Omni: $F_{3,21} = 0.69$, $P = 0.571$; Fig. 6.3a).

Based on ΔAICc values and evidence ratios, SEMs suggested that herbivore density best explained density of omnivores in the wet season (Fig 6.5a; Table 6.4). There were several equally supported Structural Equation Models (on ΔAICc < 2.00), but path coefficients for the linkages between herbivore and omnivore density were positive in all models, suggesting that increased herbivore density resulted in increased omnivore density. In addition, Akaike weights suggest that the best-fit model (ΔAICc = 0) is 6x more likely than the least supported model (ΔAICc = 3.579). Because linkages between periphyton variables and omnivore density were not statistically significant and dropping these linkages from the model did not improve the ΔAICc value, I inferred that omnivores are not directly influenced by periphyton quality and/or availability in the wet season.

Similar to omnivore models, there were several equally supported herbivore SEMs (on ΔAICc < 2.00), but they all suggest that herbivore density was best explained by increased PUFA ratio, increased TP, decreased EPA % (negative PC2 axis values for periphyton quality), and increased omnivore density (Fig 6.6a; Table 6.5). Akaike weights suggest that the best-fit model (ΔAICc = 0) is 80x more likely than the model with the highest ΔAICc value (ΔAICc = 8.779). Although linkages between herbivore and omnivore density were statistically significant, they occurred in the positive direction, suggesting that omnivore competition and/or predation did not directly affect herbivore density.

Consumer tissues showed spatial variation in nutrient composition in the wet season. Omnivore tissues contained 18% higher TP than herbivore tissues, but these values were also driven by hydroperiod. The mid-range hydroperiod sites (300-350 days inundation) had consumers with decreased tissue TP (Diet x Hydroperiod: $F_{2,49} = 7.96$, $P = 0.003$). Similarly, omnivore tissues had 83% more autotrophic fatty acids and 92% more heterotrophic fatty acids than herbivore tissues (Autotroph: $F_{1,50} = 7.41$, $P = 0.009$; Heterotroph: $F_{1,44} = 4.81$, $P = 0.034$). However, herbivores had tissues with the highest proportion of protein, which was 40% greater than omnivores ($F_{1,50} = 8.70$, $P = 0.005$). Lipids varied among trophic group and hydroperiod, with herbivore tissues having 60% more total lipids than omnivores ($F_{1,50} = 38.15$, $P < 0.0001$). In addition, sites with < 300 days of inundation had fish with a 32% decrease in tissue lipid concentration ($F_{3,50} = 5.91$, $P = 0.002$). Carbohydrates did not vary between herbivore and omnivore tissues ($F_{6,50} = 1.38$, $P = 0.246$).

Tissue EPA was on average 96% higher in omnivores; however, in sites with > 325 days inundation, EPA was 40% higher in herbivore tissues (Trophic group: $F_{1,50} = 10.46$, $P = 0.002$; Hydroperiod: $F_{3,50} = 3.25$, $P = 0.031$). Omnivores had tissues with 51% higher DHA and 71% higher ARA than herbivores (DHA: $F_{1,44} = 42.84$, $P < 0.0001$; ARA: $F_{1,44} = 50.08$, $P < 0.0001$). Similarly, omnivore tissues had 40% higher PUFAs than herbivores ($F_{1,50} = 56.15$, $P < 0.0001$), but herbivore tissues had 19% higher MUFAs ($F_{1,44} = 19.78$, $P < 0.0001$; Figs. 6.4a&b).

I did not find evidence supporting the Heterotroph Facilitation hypothesis, as increased heterotrophic fatty acid (%) was not a main predictor of herbivore density in the wet season. Herbivore density was found to decrease with increasing periphyton quality as predicted by the Suboptimal Habitat hypothesis (macronutrients, heterotrophic fatty acid %, edible algae, and EPA; Fig. 6.7a). But, herbivore density increased with increasing PUFA ratio and TP, which is inconsistent with predictions of the Suboptimal Habitat hypothesis. Furthermore, I found that periphyton availability and quality were not inversely related (Avail. PC1 x Quality PC2: $R^2 = 0.14$, $t = 2.07$, $P = 0.050$; Avail. PC2 x Quality PC1:$R^2 = 0.46$, $t = 4.15$, $P = 0.001$), which violates a main prediction of the Suboptimal Habitat hypothesis. Similar to herbivores, omnivore density was not driven by heterotrophic fatty acids. Furthermore, omnivore density was not driven by quality or availability of periphyton, as direct and indirect paths between these variables were not significant in the SEMs. Instead, I found that omnivore density was driven only by density of herbivores, suggesting that omnivore dynamics are not explained by either adaptive hypothesis in the wet season.

Dry Season

There were several differences in environmental variables across hydroperiods in the dry season. Sites with < 300 days of inundation had average depths of 11.75 $\pm$ 8.62 cm, sites with water 300-324 days were 19.8 $\pm$ 6.72 cm, sites with water 325-350 days were 35.00 $\pm$ 10.02 cm, and sites with > 350 days of inundation were 48.67 $\pm$ 9.09 cm. There were no differences in pH among hydroperiods, but short hydroperiods (< 300 days inundation) had 53% higher conductivity than sites with > 350 days of water ($F_{3,20}$ = 4.04, P = 0.026). There were no differences in periphyton C:P and N:P ratios across sites in the dry season (CP: $F_{3,18}$ = 1.68, P = 0.214; NP: $F_{3,18}$ = 1.614, P = 0.228). Similar to the wet season, I was unable to statistically compare stoichiometric ratios of the different periphyton types as a result of the variation in the types of periphyton available among sites, but filamentous green forms had the lowest C:P and C:N ratios, which were 68% and 61% lower than floating mats (the highest ratios), respectively. Similarly, periphyton TP could not be statistically compared by periphyton type among sites, but benthic mats had the highest concentration of TP, which was 51% higher than filamentous green algae, 49% higher than floating mat, and 14% higher than epiphyton. Periphyton TP was not different among hydroperiods ($F_{3,19}$ = 0.45, P = 0.722), but wet-season periphyton had 41% higher TP than dry-season periphyton ($F_{1,41}$ = 4.68, P = 0.037). Therefore, dry-season periphyton had 58% and 73% greater C:P and N:P ratios than wet-season periphyton, respectively (CP: $F_{1,36}$ = 12.41, P = 0.001; NP: $F_{1,36}$ = 23.07, P < 0.0001).

Emergent vascular-plant stem density was not different among hydroperiods in the dry season ($F_{3,20} = 0.594$, $P = 0.628$), but long-hydroperiod sites (> 350 days) had periphyton with 61% lower filamentous cyanobacteria density and 48% higher filamentous green algal density than short-hydroperiod sites (41.95% dissimilarity, $R= 0.072$, $P = 0.043$). There were no significant differences between wet and dry season algal composition (34.91% dissimilarity, $R= 0.005$, $P = 0.317$).

Similar to wet-season variables, all dry-season environmental and periphyton quality/availability variables loaded strongly on the first two PC axes and those data were used in further analyses. Environmental PC axis 1 and 2 explained 73.6% and 18.0% of the variance in the data, respectively (Table 6.3). Axis 1 contained loadings highly correlated (loading ≥ 0.30) to pH (-), conductivity (-), and DSD (-), whereas axis 2 was highly correlated with variation in hydroperiod (-) and emergent plant cover (+). Macronutrients (protein, carbohydrate, lipid), heterotrophic fatty acid %, EPA % and edible algae (all +) loaded strongly on periphyton quality PC axis 1 (52.6% explained variation), whereas PUFA ratio (-) and TP (-) loaded on axis 2 (16.6% explained variation). For the periphyton availability variables, periphyton cover, floating mat volume, benthic mat volume and epiphyton volume (all -) loaded strongly on PC axis 1 (37.8% explained variation). Benthic mat (-), epiphyton (-) and filamentous algae (+) volume, and periphyton cover (+) loaded on PC axis 2 (23.7% explained variation). Multivariate Analysis of Variance did not reveal any differences in these variables by hydroperiod ($\gamma= 0.445$, $F_{15,39} = 0.89$, $P = 0.582$).

Periphyton quality varied across hydroperiods in the dry season. The sites with the longest hydroperiod (inundated for > 350 days) had higher periphyton quality in terms of increased PUFA ratio and TP (PC2: $F_{3,22} = 3.56$, $P = 0.035$). Mid-range hydroperiod sites (inundated for 300-324 days or 325-350 days) had higher periphyton quality (PUFA ratio and TP) than the sites with the shortest hydroperiod (< 300 days), but lower periphyton quality than long-hydroperiod sites (> 350 days). But, short-hydroperiod sites (inundated for < 300 days) had floating mats with 94% higher lipid composition than the sites with water for 300-324 days, and 86% higher than sites with water for > 350 days ($F_{3,19} = 5.07$, $P = 0.012$). Furthermore, long-hydroperiod sites (> 350 days) had epiphyton with the lowest proportion of protein and carbohydrates (99% less than 300-324 days of inundation; Protein: $F_{2,8} = 95.95$, $P < 0.0001$; Carb: $F_{2,8} = 26.26$, $P = 0.001$; Figs. 6.3c&d). Sites did not vary in availability of floating mat or benthic mat (FM: $F_{3,19} = 1.55$, $P = 0.240$; BM: $F_{3,19} = 1.48$, $P = 0.404$), but long-hydroperiod sites had 99% less epiphyton availability than sites with water inundation for 300-350 days ($F_{2,8} = 10.06$, $P = 0.012$). A summary of changes in food quality and availability from the wet season to the dry season can be found in Table 6.6.

Hierarchical cluster analysis of consumer gut content data collected in the dry season produced two diet categories: 1) herbivore; and 2) omnivore (Cophenetic correlation = 0.87). Similar to the wet season, Sailfin Mollies and Flagfish had diets comprised of basal resources, although Flagfish consumed more periphyton in the dry season and more detritus in the wet season. Least Killifish were primarily consuming invertebrates in the wet season but switched to periphyton in the dry season.

Therefore, I classified this species as omnivorous (referred to as "diet-switching omnivores" in Fig. 6.2b) since they were not obligate herbivores. Eastern Mosquitofish were classified as omnivores, but consumed approximately 41% periphyton in the dry season compared to only 7% consumed in the wet season (Loftus 2000). Golden Topminnows and Bluefin Killifish were classified as omnivores in both seasons as their diets did not change (Fig. 6.2b). There were 93% more herbivores and 47% more omnivores in the wet season than the dry season (Herb: $F_{1,41} = 35.86$, $P < 0.0001$; Omni: $F_{1,41} = 3.90$, $P = 0.013$). In addition, there were 69% more omnivores than herbivores in the dry season ($F_{1,41} = 13.45$, $P = 0.001$); however, there were 44% more herbivores than omnivores in sites with < 300 days of water. There were no statistical differences in herbivore density across hydroperiods ($F_{3,21} = 2.25$, $P = 0.118$), but there were 82% more omnivores in sites that were inundated for 325-350 days than in sites with water for < 300 days ($F_{3,17} = 4.49$, $P = 0.017$; Fig. 6.4b).

SEMs suggested that increased periphyton quality best explained density of omnivores in the dry season (Fig 6.5b; Table 6.5). There were 4 equally supported Structural Equation Models (on ΔAICc < 2.00), but path coefficients for the linkages between periphyton PC 2 and omnivore density were negative in all models, indicating that increased PUFA ratio and TP resulted in increased omnivore density. Akaike weights suggest that the best-fit model (ΔAICc = 0) was 66x more likely to be the best model than the model with the highest ΔAICc value (ΔAICc = 8.382). Linkages between omnivore density and other periphyton variables, or between omnivore density and herbivore density, were not statistically significant and dropping these linkages from the model did not improve the ΔAICc value.

There were several equally supported herbivore SEMs (on ΔAICc < 2.00), but there were no statistically significant path coefficients between periphyton variables and herbivore density, suggesting that herbivore density was not explained by food quality or availability in the dry season. Similar to wet season results, increased herbivore density was explained by increased omnivore density, suggesting that competition with omnivores and/or predation by omnivores also did not influence herbivore density in the dry season (Fig 6.6b; Table 6.5).

Consumer tissues varied in molecular composition in the dry season. Omnivore tissues contained 30% greater TP and 21% more heterotrophic fatty acid markers than herbivore tissues (TP: $F_{1,48} = 11.13$, $P = 0.002$; Heterotrophic FA: $F_{1,52} = 5.99$, $P = 0.018$). However, herbivore tissues had 15% more autotrophic-derived fatty acid markers than omnivore tissues ($F_{1,52} = 7.69$, $P = 0.008$). Omnivores had 42% more DHA and 55% more ARA in their tissues than herbivores (DHA:$F_{1,52} = 4.33$, $P = 0.043$; ARA:$F_{1,52} = 8.61$, $P = 0.005$). Furthermore, omnivore tissues had 18% more PUFAs than herbivore tissues ($F_{1,52} = 4.70$, $P = 0.035$), but herbivore tissues had 12% more MUFAs ($F_{1,52} = 4.08$, $P = 0.049$). There were no differences in EPA or SAFA concentrations between omnivore and herbivore tissues (EPA: $F_{1,52} = 0.12$, $P = 0.733$; SAFA: $F_{1,52} = 0.00$, $P = 0.999$). Both herbivore and omnivore tissues had similar macronutrient composition during the dry season (Protein: $F_{1,51} = 0.08$, $P = 0.776$; Carb: $F_{1,51} = 0.17$, $P = 0.682$; Lipid: $F_{1,51} = 0.13$, $P = 0.725$); but, tissue lipids were highest in fish collected from sites with 324-350 days of inundation ($F_{3,51} = 4.11$, $P = 0.012$).

Body condition of both omnivores and herbivores were less robust in the dry season than in the wet season. Compared to omnivore tissues in the wet season, omnivores had 91% lower protein concentration, 71% higher carbohydrate concentration, and 51% lower lipid concentration (Protein: $F_{1,84} = 272.75$, $P < 0.0001$; Carb: $F_{1,84} = 123.19$, $P < 0.0001$; Lipid: $F_{1,84} = 19.05$, $P < 0.0001$). Herbivore tissues had 92% lower protein concentration and 65% higher lipid concentration than herbivore tissues in the wet season (Protein: $F_{1,17} = 6.58$, $P = 0.02$; Lipid: $F_{1,17} = 15.57$, $P = 0.001$). Herbivore tissues contained similar amounts of carbohydrates in both seasons ($F_{1,17} = 0.31$, $P = 0.583$). A summary of changes in consumer diet and tissue composition from the wet season to the dry season can be found in Table 6.7.

Similar to the wet-season results, increased % heterotrophic fatty acid was not a main predictor of herbivore density in the dry season ($R^2 = 0.013$, $t = 1.131$, $P = 0.271$) and thus, the results did not support the Heterotroph Facilitation hypothesis. Periphyton availability (periphyton cover %, floating mat volume, benthic mat volume and epiphyton volume) was inversely related to periphyton quality (macronutrients, heterotrophic fatty acid %, EPA % and edible algae), supporting one prediction of the Suboptimal Habitat hypothesis ($R^2 = 0.45$, $t = 3.73$, $P = 0.001$; Fig. 6.7b). However, herbivore density was not found to increase in proportion to periphyton availability or decrease in proportion to periphyton quality. Instead, I found that herbivore density did not change with changing periphyton quality. Omnivores became more herbivorous in the dry season, but similar to herbivores, their density was not dependent on heterotrophic fatty acids.

Furthermore, I found that omnivores increased with increasing periphyton quality (PUFA ratio and TP), suggesting that omnivore dynamics are not explained by either adaptive hypothesis in the dry season.

Discussion

Results revealed that herbivores do not track resource quality or availability during environmental stress, such as the conditions experienced by consumers during the Everglades dry season. During this time, short-hydroperiod sites were either dry or receding, thus concentrating consumers in these shrinking habitats.

As a result, consumers were vulnerable to predators unless they migrated to longer hydroperiod refuges (DeAngelis et al. 2010). In addition, longer hydroperiod sites had higher quality periphyton than sites with < 300 days of inundation, better to support larger consumer populations in the dry season. Omnivore density was largely driven by the higher quality basal resources offered by the longer hydroperiod sites (300-350 days of inundation), consistent with niche-based predictions. Higher periphyton quality in dry-season long-hydroperiods sites may result from nutrient regeneration (Geddes and Trexler 2003; Dorn et al. 2006) and/or consumer transport and deposition (Stevenson and Childers 2004) as they move into these refuge sites. Though the study sites varied in levels of periphyton availability and quality, herbivore density was consistent across all hydroperiods, suggesting that herbivore fitness is not hampered by variation in resources. These results are consistent with dispersal-limited predictions.

In the wet season, newly inundated habitats (< 300 days of water) had the highest quality basal resources. At these sites, herbivores outnumbered omnivores by 83%. However, herbivore density was predicted by decreased EPA, and by increased PUFA ratio and TP, which were all characteristic of long-hydroperiod sites in the wet season. Omnivore density was also a strong predictor of herbivore density. I thereby inferred that predation or competition by omnivores was not a strong driver of herbivore dynamics in the wet season. Similar to the dry season, herbivore density did not vary consistently across hydroperiods. However, herbivore density was influenced by food quality and availability, suggesting that herbivore diets are consistent with niche-based predictions in the wet season. Omnivore density was best predicted by herbivore density (i.e., prey) and by periphyton quality, suggesting that omnivores more closely track the availability of higher quality resources than herbivores. Similar to herbivores, omnivore diets were consistent with niche-based predictions in the wet season.

Taken together, these results partially support the Suboptimal Habitat Hypothesis, which suggests that herbivory is adaptive because it allows organisms to invade and persist in 'suboptimal' habitats. However, these results do not clearly support each prediction of this hypothesis as outlined in Sanchez and Trexler (2016). Specifically, herbivore density was not inversely related with periphyton quality or density of omnivores in the dry season. Furthermore, in the wet season, I failed to obtain evidence that periphyton availability was inversely related to periphyton quality, or that herbivore density was inversely related to omnivore density. However, I found that herbivore density was similar across habitats with varying levels of disturbance, and when habitats were inundated, herbivore density was predicted by periphyton quality.

I did not find evidence for the Heterotroph Facilitation Hypothesis in this study as heterotrophic fatty acids did not drive herbivore density; however, previous studies suggest that heterotrophic bacteria are important for consumers in this system (Belicka et al. 2012; Sanchez and Trexler 2018), so the explanatory power of this hypothesis remains unclear. Unlike herbivores, omnivore populations were driven by availability of high-quality food items. In the dry season, when high quality prey items were rare, omnivores sought high-quality basal resources, but in the wet season when prey items were abundant, omnivores exploited this food source. This finding supports the current hypotheses on the adaptive evolution of omnivory (e.g., Diehl 2003; Krivan and Diehl 2005).

These results suggest that herbivory may have evolved as an adaptive strategy to deal with fluctuating conditions. In this study, herbivores track food quality when habitats are stable (wet season) but can survive in a multitude of habitat types during disturbance events (dry season). These results imply that herbivore density is driven by both species traits and dispersal. Conversely, omnivores were limited to quasi-permanent habitats (e.g., long-hydroperiod sites) and were outnumbered by herbivores at all sites in the wet season, and in short-hydroperiod sites in the dry season. Furthermore, omnivores rely on high quality resources in both wet and dry seasons, suggesting that they are less flexible than herbivores in their diet and habitat requirements. Previous studies of Everglades consumers have found that omnivore density increases with time since flooding, while herbivore density tends to decrease with time following drought (Sargeant et al. 2011). Other studies have found negative correlations between omnivore densities and measures of disturbance (Trexler et al. 2002, 2005; Liston 2006).

These findings support food quality as a mechanism driving omnivore dynamics, and infer that herbivores are better adapted than omnivores to conditions with variable hydrology, food supply, and food quality.

Studying trophic dynamics in an evolutionary context allows researchers to better understand the forces driving species organization. Many studies have laid the groundwork for this type of research by describing the role of ecological influences on food webs. However, the current findings offer oversimplified predictions for how food webs are organized. For example, previous ecological studies have established that herbivores have such an integral role in food webs that their removal reveals a trophic cascade (Power 1992). In this study, I concluded that herbivores are better adapted to fluctuating resources than higher level consumers, suggesting that herbivory may "buffer" food webs from stressful environmental factors. By identifying an evolutionary mechanism that promotes herbivory, we are able to more fully describe the complex role of these consumers in food webs. Future trophic studies may benefit by using a framework that incorporates both ecology and evolution to predict how food webs are organized in nature.

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Table 6.1. Environmental data from the 22 sampled sites across the Everglades landscape in the wet season (July 2016) and the dry season (February 2017).

Site Name, Location	Hydroperiod Category	DSD	pH	Conductivity	Emergent Plant %	Periphyton cover %	Total periphyton volume (mL/ m^2)	Total phosphorus (ug/g)
A55, Panhandle	< 300 days	307/ --	6.85/ --	520/ --	20/ --	85/ --	5940/ --	126.97/ --
A60, Panhandle	< 300 days	307 /528	6.73/ 6.83	471/ 613	15/ 15	85/ 65	5940/ 3960	100.63/ 61.83
A62, Panhandle	< 300 days	92/ --	6.78/ --	435/ --	10/ --	95/ --	4950/ --	306.92/ --
A59, Panhandle	< 300 days	339/ 559	6.81/ 6.46	456/ 1280	5/ 10	50/ 70	1760/ 1188	424.97/ 233.21
MDD, Taylor Slough	300-324 days	402/ 616	7.36/ 7.73	366.2/ 503	5/ 5	80/ 50	5700/ 890	271.70/ 530.91
37, Shark River Slough	300-324 days	378/ 581	6.75/ 7.46	531/ 566	15/ 20	60/ 15	360/ 250	145.77/ 181.82
3, WCA 3	300-324 days	363/ 580	6.57/ 7.14	290.7/ 320.4	5/ 7	40/ 35	4900/ 375	189.11/ 388.70
10, WCA 3	300-324 days	326/ 529	6.01/ 6.88	490/ 631	10/ 7	80/ 35	6080/ 2250	211.06/ 379.86
8, Shark River Slough	300-324 days	379/ 581	7.01/ 7.64	527/ 587	5/ 3	80/ 40	1980/ 1062	104.12/ 369.92
TSD, Taylor Slough	325-349 days	443/ 658	7.55/ 7.03	316.6/ 510	5/ 5	70/ 25	5890/ 720	270.50/ 351.92
9, WCA 3	325-349 days	1532/ 1736	5.84/ 6.80	520/ 559	10/ 30	65/ 25	3250/ 1170	491.98/ 146.26
TSA, Taylor Slough	325-349 days	436/ 662	7.5/ 7.19	324.5/ 414	5/ 2	70/ 35	1600/ 2190	378.38/ 431.88
MDA, Taylor Slough	325-349 days	436/ 658	6.61/ 6.60	351.8/ 512	5/ 5	60/ 40	2400/ 1225	283.31/ 235.31
7, Shark River Slough	325-349 days	451/ 654	6.82/ 7.59	502/ 512	5/ 5	80/ 25	2250/ 1100	518.28/ 204.63
7, WCA 3	325-349 days	379/ 582	6.65/ 7.16	540/ 687	5/ 10	70/ 75	4440/ 2400	133.18/ 320.13
CP, Taylor Slough	325-349 days	1531/ 1756	6.89/ 7.67	353.8/ 620	1/ 1	85/ 10	7650/ 800	139.57/ 200.53
2, WCA 3	> 350 days	412/ 620	6.59/ 6.93	307.6/ 363.5	3/ 5	80/ 25	420/ 375	204.57/ 351.07
1, WCA 3	> 350 days	1843/ 2052	6.79/ 6.80	403.6/ 374.1	3/ 5	40/ 15	957/ 126	--/ 439.15
8, WCA 3	> 350 days	351/ 554	6.69/ 6.93	603/ 694	5/ 7	80/ 80	2520/ 1400	197.02/ 211.24
6, WCA 3	> 350 days	1859/ 2062	6.51/ 7.21	475/ 478	1/ 2	60/ 10	540/ 117	158.61/ 229.87
4, WCA 3	> 350 days	9193/ 9404	6.28/ 7.18	389/ 367.4	1/ 2	75/ 5	700/ 15	458.22/ 374.96
5, WCA 3	> 350 days	1863/ 2067	6.52/ 6.79	365/ 385.1	1/ 25	60/ 1	704/ 1820	242.15/ 628.82

Table 6.2. Percentage of each periphyton type at each site in the wet season (July 2016) and the dry season (February 2017). Sites A55 and A60 were not able to be sampled in the dry season. Sorted in order from shortest to longest hydroperiod.

	WET SEASON				DRY SEASON			
Site Name, Location	**Floating Mat**	**Benthic Mat**	**Epiphyton**	**Fil. Green Algae**	**Floating Mat**	**Benthic Mat**	**Epiphyton**	**Fil. Green Algae**
A55, Panhandle	95	5	--	--	--	--	--	--
A60, Panhandle	98	2	--	--	100	--	--	--
A62, Panhandle	73	2	25	--	--	--	--	--
A59, Panhandle	95	--	--	5	100			
MDD, Taylor Slough	100	--	--	--	98	--	--	2
37, Shark River Slough	100	--	--	--	100	--	--	--
3, WCA 3	80	--	20	--	100	--	--	--
10, WCA 3	95	--	5	--	75	25	--	--
8, Shark River Slough	95	--	5	--	50	--	50	--
TSD, Taylor Slough	98	--	--	2	90	--	10	--
9, WCA 3	90	5	5	--	100	--	--	--
TSA, Taylor Slough	70	20	10	--	60	10	30	--
MDA, Taylor Slough	65	--	35	--	80	--	20	--
7, Shark River Slough	90	--	10	--	100	--	--	--
7, WCA 3	98	--	2	--	90	5	5	--
CP, Taylor Slough	95	--	5	--	50	30	20	--
2, WCA 3	100	--	--	--	98	--	2	--
1, WCA 3	100	--	--	--	99	--	1	--
8, WCA 3	98	--	2	--	99	1	--	--
6, WCA 3	100	--	--	--	100	--	--	--
4, WCA 3	95	--	5	--	95	--	5	--
5, WCA 3	100	--	--	--	50	--	50	--

Table 6.3. Principal components loadings for environmental, food availability, and food quality variables for the wet season (July 2016) and the dry season (February 2017). Loadings ≥ 0.30 (abs. value) are highlighted in grey. DSD = Days since last dry-down, TP = Total phosphorus.

	WET SEASON		DRY SEASON	
Variables	**PC 1**	**PC 2**	**PC 1**	**PC 2**
ENVIRONMENTAL				
Hydroperiod	0.53	-0.01	-0.39	-0.55
DSD	0.53	-0.23	-0.50	-0.14
Emergent plant %	-0.59	-0.12	-0.29	0.82
pH	-0.14	0.73	-0.51	-0.01
Conductivity	-0.26	-0.63	-0.50	0.09
FOOD AVAILABILITY				
Periphyton cover %	-0.51	0.18	-0.55	0.41
Floating mat volume	-0.42	0.55	-0.67	0.13
Benthic mat volume	-0.46	0.24	-0.39	-0.43
Epiphyton volume	-0.41	-0.38	-0.31	-0.56
Fil. algae volume	0.41	0.68	-0.05	0.56
FOOD QUALITY				
Protein	0.41	-0.24	0.30	-0.03
Carbohydrate	0.41	-0.14	0.41	-0.07
Lipid	0.47	-0.06	0.37	-0.13
Bacterial fatty acid %	0.45	0.07	0.38	-0.14
Edible algae (mg)	0.39	0.29	0.41	0.27
EPA %	-0.09	0.81	0.39	0.25
PUFA ratio	-0.19	-0.37	0.27	-0.64
TP	-0.19	-0.30	0.25	-0.50

Table 6.4. Comparison of structural equation models used to predict omnivore density in the wet and dry seasons. Total model includes paths between Periphyton Quality PC 1 & 2 (Q1 & Q2), Periphyton Availability PC 1 & 2 (A1 & A2), herbivores and omnivores. Paths between consumers were not varied. AIC_w = Akaike weights, w_{min}/w_j = Evidence ratios. ΔAICc values $\leq$ 2 are highlighted in bold.

		WET			DRY		
Model	**Removed from model**	**ΔAICc**	**AIC_w**	**w_{min}/w_j**	**ΔAICc**	**AIC_w**	**w_{min}/w_j**
1	None	3.27	0.03	5.13	5.87	0.02	18.81
2	Q1 + Q2 + A1	**1.27**	**0.09**	**1.88**	8.57	0.005	72.49
3	Q2 + A1 + A2	**1.93**	**0.06**	**2.62**	7.12	0.01	35.16
4	Q1 + Q2 + A2	**1.32**	**0.09**	**1.94**	6.91	0.01	31058
5	Q1 + A1 + A2	**0.16**	**0.13**	**1.08**	**0.00**	**0.36**	**1.00**
6	Q1 + Q2	**1.97**	**0.06**	**2.68**	8.66	0.005	75.75
7	A1 + A2	**1.99**	**0.06**	**2.71**	**1.98**	**0.14**	**2.70**
8	Q2 + A2	3.23	0.03	5.02	8.66	0.005	75.98
9	Q2 + A1	3.13	0.04	4.77	8.88	0.005	84.56
10	Q1 + A2	**1.88**	**0.07**	**2.57**	**1.93**	**0.14**	**2.62**
11	Q1 + A1	**0.58**	**0.13**	**1.34**	**1.91**	**0.14**	**2.60**
12	Q1	**1.81**	**0.07**	**2.47**	**3.89**	**0.05**	**6.99**
13	Q2	3.58	0.03	5.99	10.30	0.002	172.09
14	A1	2.28	0.05	3.13	**3.91**	**0.05**	**7.07**
15	A2	3.71	0.03	6.39	**3.92**	**0.05**	**7.10**
16	All	**0.00**	**0.17**	**1.00**	6.57	0.01	26.70

Table 6.5. Comparison of structural equation models used to predict herbivore density in the wet and dry seasons. Total model includes paths between Periphyton Quality PC 1 & 2 (Q1 & Q2), Periphyton Availability PC 1 & 2 (A1 & A2), herbivores and omnivores. Paths between consumers were not varied. AIC_w = Akaike weights, w_{min}/w_j = Evidence ratios. ΔAICc values $\leq$ 2 are highlighted in bold.

		WET			DRY		
Model	**Removed from model**	**ΔAICc**	**AIC_w**	**w_{min}/w_j**	**ΔAICc**	**AIC_w**	**w_{min}/w_j**
1	None	**1.49**	**0.10**	**2.10**	4.63	0.02	45.77
2	Q1 + Q2 + A1	8.78	0.00	80.60	**1.30**	**0.12**	**8.65**
3	Q2 + A1 + A2	5.57	0.01	16.21	**1.59**	**0.10**	**9.97**
4	Q1 + Q2 + A2	4.14	0.03	7.92	**0.00**	**0.22**	**4.51**
5	Q1 + A1 + A2	2.84	0.05	4.15	**0.62**	**0.14**	**7.14**
6	Q1 + Q2	3.19	0.04	4.93	2.68	0.06	17.22
7	A1 + A2	**0.78**	**0.14**	**1.48**	2.50	0.06	15.77
8	Q2 + A2	2.37	0.06	3.27	3.07	0.05	20.97
9	Q2 + A1	7.41	0.01	40.57	3.25	0.04	22.97
10	Q1 + A2	2.16	0.07	2.95	**1.37**	**0.11**	**8.94**
11	Q1 + A1	2.07	0.07	2.81	2.49	0.06	15.64
12	Q1	**0.00**	**0.21**	**1.00**	2.64	0.06	16.86
13	Q2	4.11	0.03	7.82	4.60	0.02	44.93
14	A1	2.62	0.06	3.70	4.11	0.03	35.30
15	A2	**0.39**	**0.17**	**1.22**	3.31	0.04	23.62

Table 6.6. Summary of the changes (by hydroperiod) in food quality and availability from the wet season to the dry season. Upward facing triangles indicate relatively high values, whereas downward facing triangles indicate relatively low values. FA= fatty acid, NC= no change.

	FOOD			
	< 300 days	**300-324 days**	**325-350 days**	**> 350 days**
Quality				
Protein	▼	▼	▼	▼
Carbohydrate	▼	▲	▲	▲
Lipid	▼	▼	▼	▼
Algal FAs	▼	▼	▼	▼
Bact. FAs	▼	▼	▼	▼
Unsaturated FAs (PUFA, SAFA, MUFA)	▼	▼	▼	▼
Omega-3 FAs (EPA, DHA)	▲	▲	▲	▲
ARA	▲	▼	▲	▼
Total phosphorus	▼	▲	▼	▲
Stoichiometry (C:P & N:P)	▲	▲	▲	▲
Availability				
Edible algae %	▼	▼	▼	▼
Total Periphyton volume	▼	▼	▼	▲
Floating mat %	▼	▼	▼	▼
Herbivore density	▼	▼	▼	▼

Table 6.7. Summary of the changes in consumer diet and tissue composition (herbivores and omnivores) from the wet season to the dry season. Values are averaged across all hydroperiods. Upward facing triangles indicate relatively high values, whereas downward facing triangles indicate relatively low values. FA= fatty acid, NC= no change.

	HERBIVORES	OMNIVORES
Diet		
Algae	▼	▲
Detritus	▲	▲
Macroinverts.	NC	▼
Tissues		
Protein	▼	▼
Carbohydrate	▼	▼
Lipid	▲	▲
Algal FAs	▲	▲
Bact. FAs	▲	▲
Unsaturated FAs (PUFA, SAFA, MUFA)	▲	▲
EPA	▲	▼
DHA	▲	▲
ARA	▲	▲
Total phosphorus	▲	▲
Stoichiometry (C:P & N:P)	▼	▼

Figure Legends

Fig. 6.1. Map showing location of 22 sampled locations across the Everglades landscape

Fig. 6.2. Classification of diets by gut contents using Sorensen (Bray-Curtis) distance measures with flexible beta linkage. Although some species showed seasonal diet shifts, Hierarchical Cluster analysis identified the same 2 diet categories in the wet season (A) and in the dry season (B). Pie charts represent amount of each food type present in the gut (estimated from Loftus 2000). White= periphyton, grey= detritus, black= animal material

Fig. 6.3. Seasonal variation of periphyton quality by hydroperiod. (A) Periphyton Quality PC1 represents macronutrients (protein, carb, lipid), edibility and % of heterotrophic fatty acids. These food quality variables decrease with increasing hydroperiod in the wet season. (B) Periphyton Quality PC2 represents PUFA Ratio (-), EPA and TP (-). Periphyton Quality PC2 increased with increasing hydroperiod, suggesting that long hydroperiod sites have decreased PUFA ratios, increased EPA and decreased TP in the wet season. (C) Periphyton Quality PC1 represents macronutrients (protein, carb, lipid), edibility, EPA and % of heterotrophic fatty acids in the dry season. These variables increase with increasing hydroperiod in the dry season, contrary to the pattern in the wet season. (D) Periphyton Quality PC2 represents PUFA ratio (-) and total phosphorus (-) in the dry season. Similar to the wet season, Periphyton Quality PC2 increased with increasing hydroperiod in the dry season, suggesting that long hydroperiod sites have decreased PUFA ratios and TP

Fig. 6.4. Number of consumers per m^2 found in sites with different hydroperiods in the wet season (A) and dry season (B)

Fig. 6.5. The structural equation models with the best fit (ΔAICc = 0.00) showing (A) herbivore density as the best predictor of omnivore density in the wet season, (B) and PUFA ratio and TP as the best predictors of omnivore density in the dry season. Solid lines indicate statistically significant relationships and dashed lines indicate non-significant relationships. Numbers indicate regression (path) coefficients for each path analyzed

Fig. 6.6. The structural equation models with the best fit (ΔAICc = 0.00) showing (A) PUFA ratio, TP, EPA %, and omnivore density as the best predictors of herbivore density in the wet season, (B) and no statistically significant relationships between periphyton variables and herbivore density in the dry season. Solid lines indicate statistically significant relationships and dashed lines indicate non-significant relationships. Numbers indicate regression (path) coefficients for each path analyzed

Fig. 6.7. Verified predictions of the Suboptimal Habitat Hypothesis. (A) In the wet season, herbivore density decreases with periphyton quality (PC1: macronutrients, edibility and % of heterotrophic fatty acids). Herbivore residuals were taken from a regression with environmental variables and herbivore density to obtain the unique pattern attributable to periphyton quality.

(B) In the dry season, Periphyton Availability PC1 represents periphyton cover % (-) and floating mat abundance (-) and Periphyton Quality PC1 represents macronutrients (protein, carb, lipid), edibility, EPA and % of heterotrophic fatty acids. The relationship between these PC scores suggests that periphyton quality decreases with increasing periphyton cover estimations and floating mat abundance

FIG. 6.1.

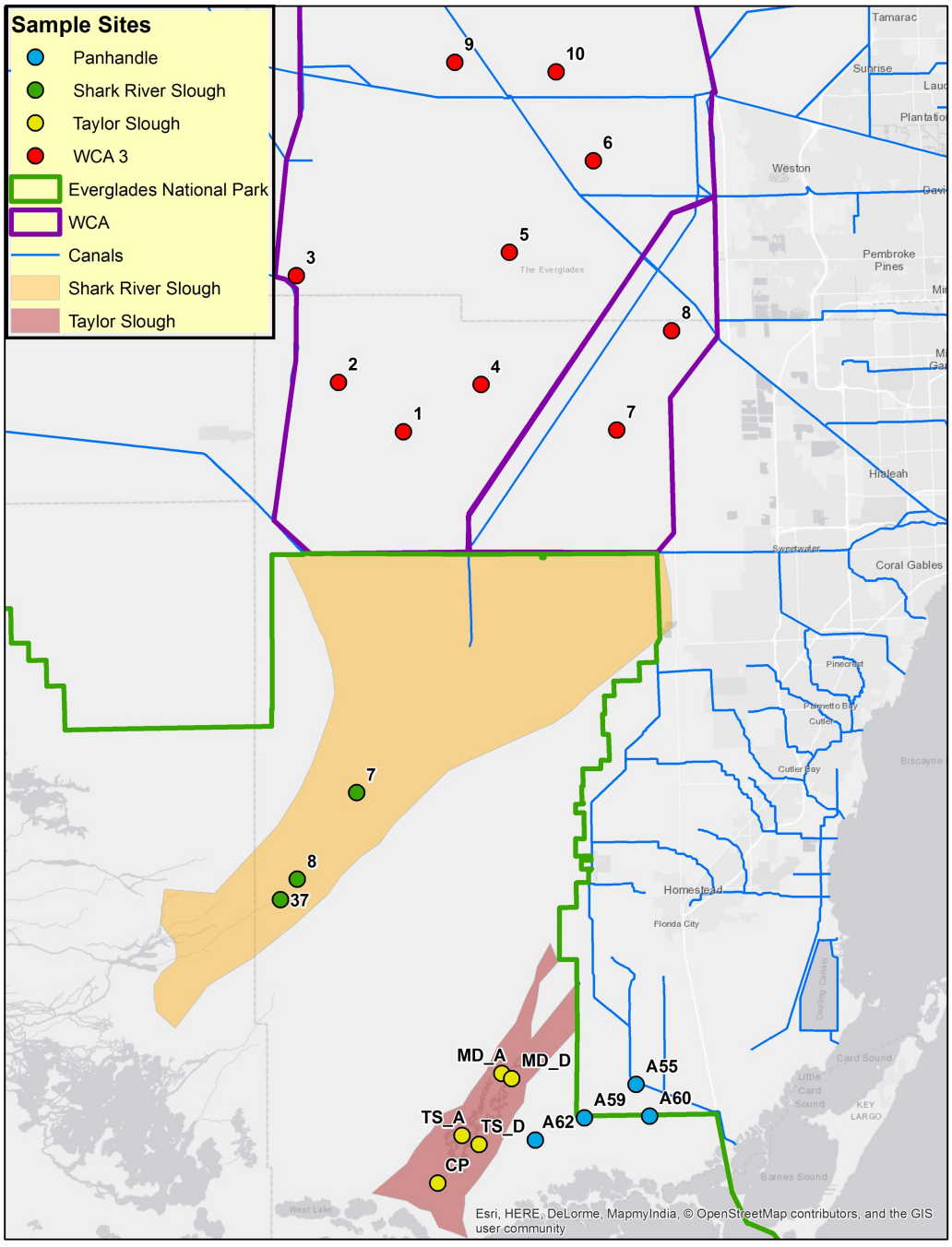

Sample Sites
Panhandle
Shark River Slough
Taylor Slough
WCA 3
Everglades National Park
WCA
Canals
Shark River Slough
Taylor Slough
9
10
6
5
3
8
2
4
1
7
7
8
37
MD_A
MD_D
A55
A59
A60
TS_A
TS_D
A62
CP
Tamarac
Sunrise
Weston
Pembroke Pines
The Everglades
Hialeah
Coral Gables
Homestead
Florida City
Cutler Bay
Card Sound
Barnes Sound
Esri, HERE, DeLorme, MapmyIndia, © OpenStreetMap contributors, and the GIS user community

FIG. 6.2.

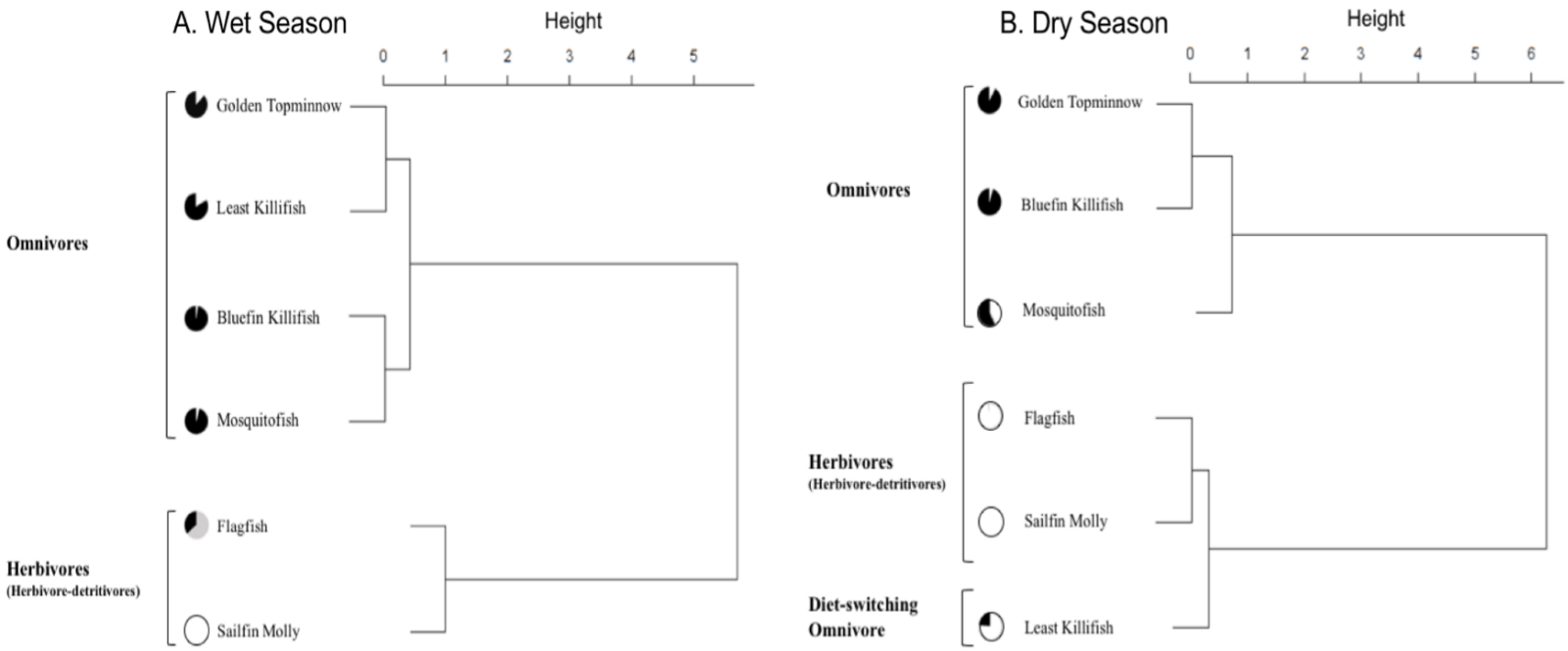

A. Wet Season
Height
0 1 2 3 4 5
Golden Topminnow
Least Killifish
Bluefin Killifish
Mosquitofish
Flagfish
Sailfin Molly
Omnivores
Herbivores
(Herbivore-detritivores)
B. Dry Season
Height
0 1 2 3 4 5 6
Golden Topminnow
Bluefin Killifish
Mosquitofish
Flagfish
Sailfin Molly
Least Killifish
Omnivores
Herbivores
(Herbivore-detritivores)
Diet-switching
Omnivore

FIG. 6.3

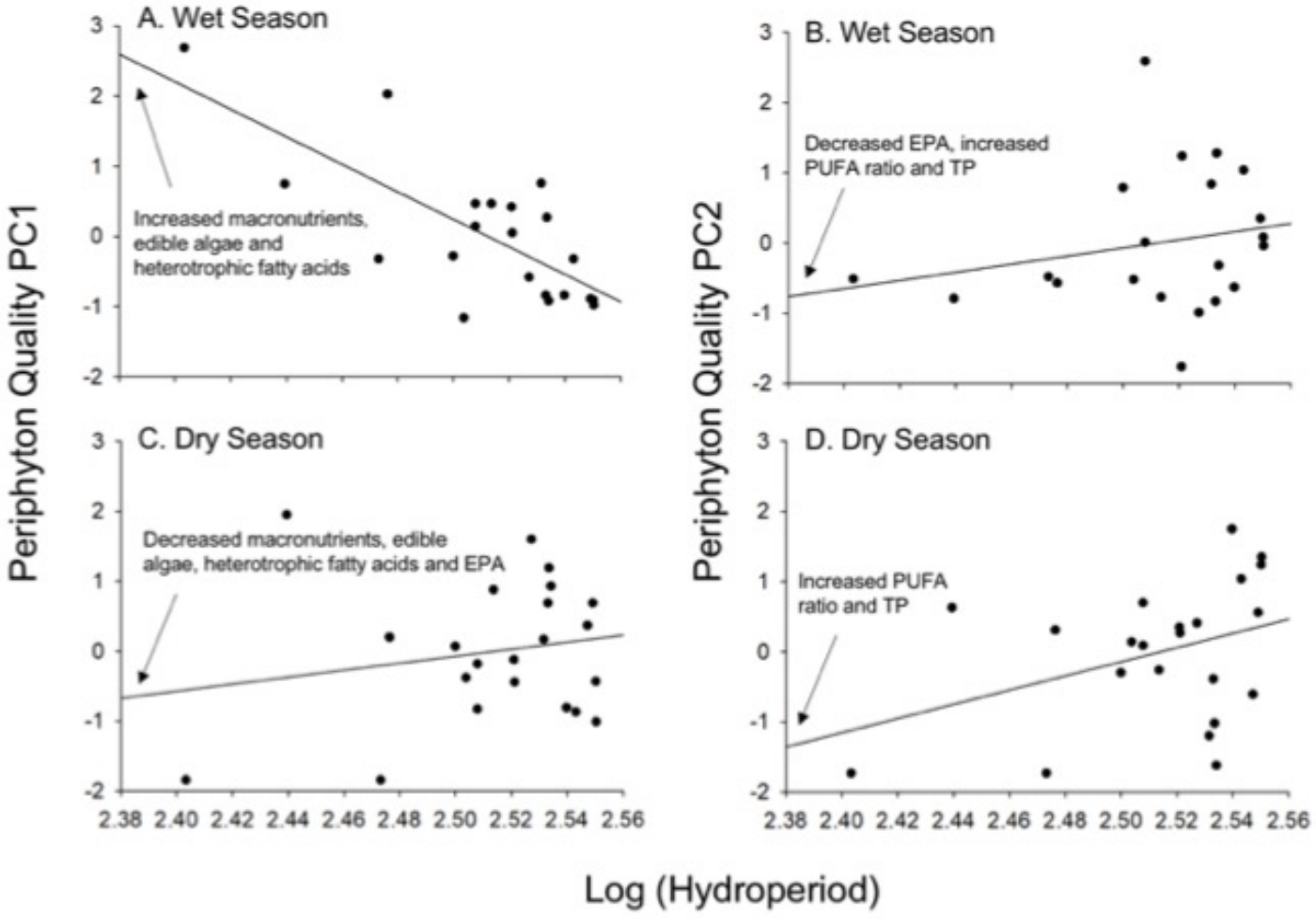

A. Wet Season
Increased macronutrients, edible algae and heterotrophic fatty acids
B. Wet Season
Decreased EPA, increased PUFA ratio and TP
C. Dry Season
Decreased macronutrients, edible algae, heterotrophic fatty acids and EPA
D. Dry Season
Increased PUFA ratio and TP
Periphyton Quality PC1
Periphyton Quality PC2
Log (Hydroperiod)
3
2
1
0
-1
-2
2.38 2.40 2.42 2.44 2.46 2.48 2.50 2.52 2.54 2.56

FIG. 6.4.

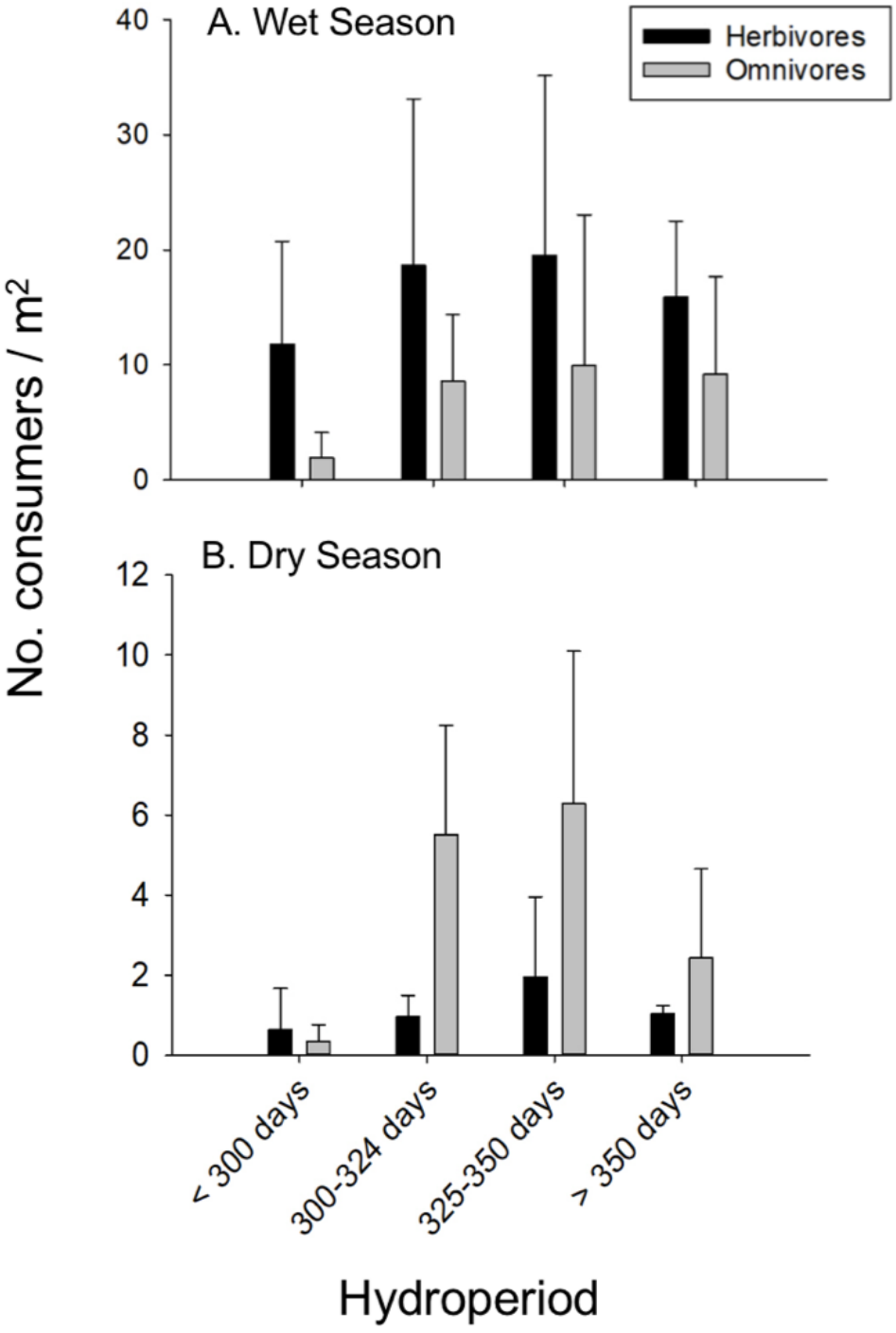

A. Wet Season
Herbivores
Omnivores
B. Dry Season
No. consumers / m2
0
10
20
30
40
0
2
4
6
8
10
12
< 300 days
300-324 days
325-350 days
> 350 days
Hydroperiod

FIG. 6.5.

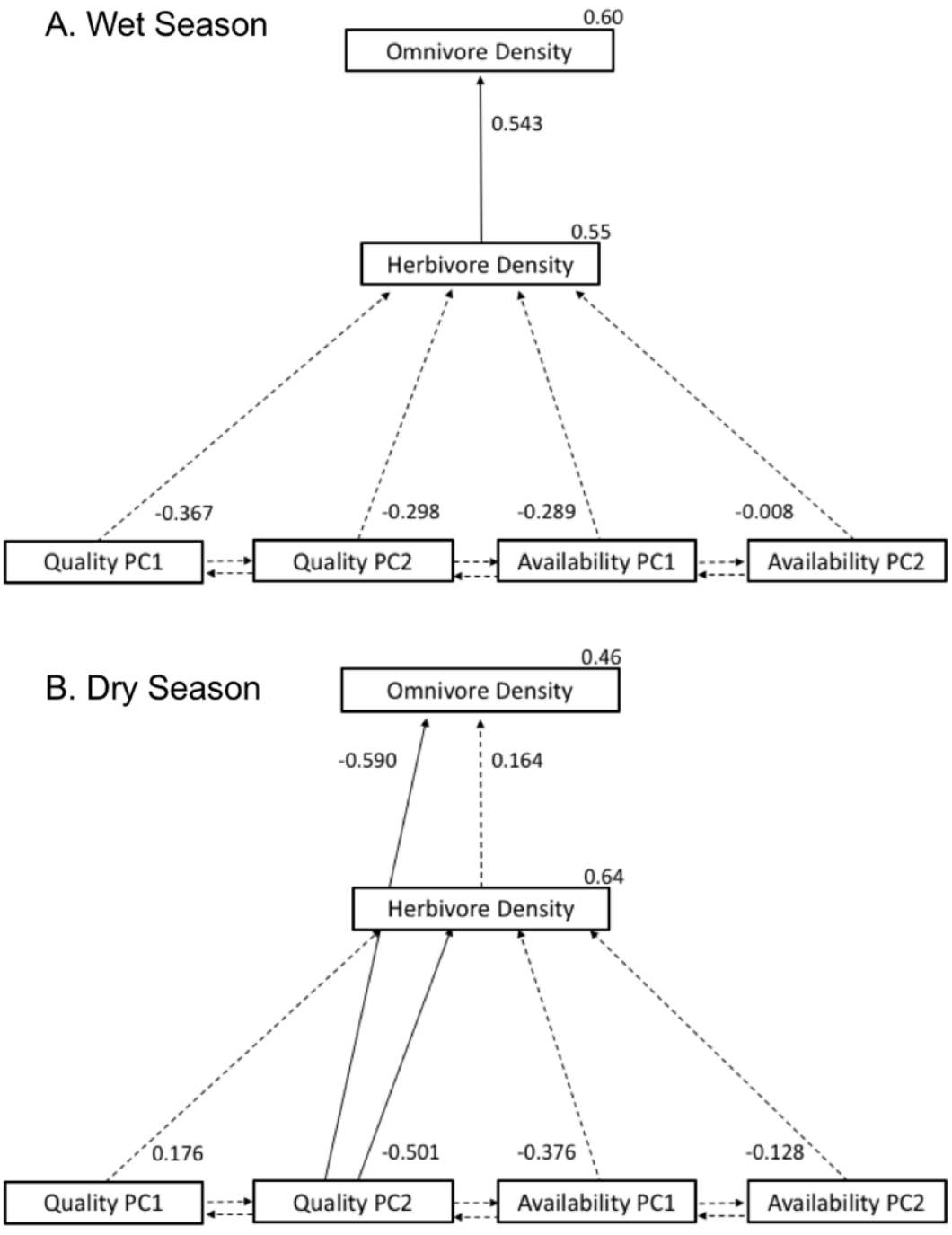

A. Wet Season
0.60
Omnivore Density
0.543
0.55
Herbivore Density
-0.367
-0.298
-0.289
-0.008
Quality PC1
Quality PC2
Availability PC1
Availability PC2
B. Dry Season
0.46
Omnivore Density
-0.590
0.164
0.64
Herbivore Density
0.176
-0.501
-0.376
-0.128
Quality PC1
Quality PC2
Availability PC1
Availability PC2

FIG. 6.6.

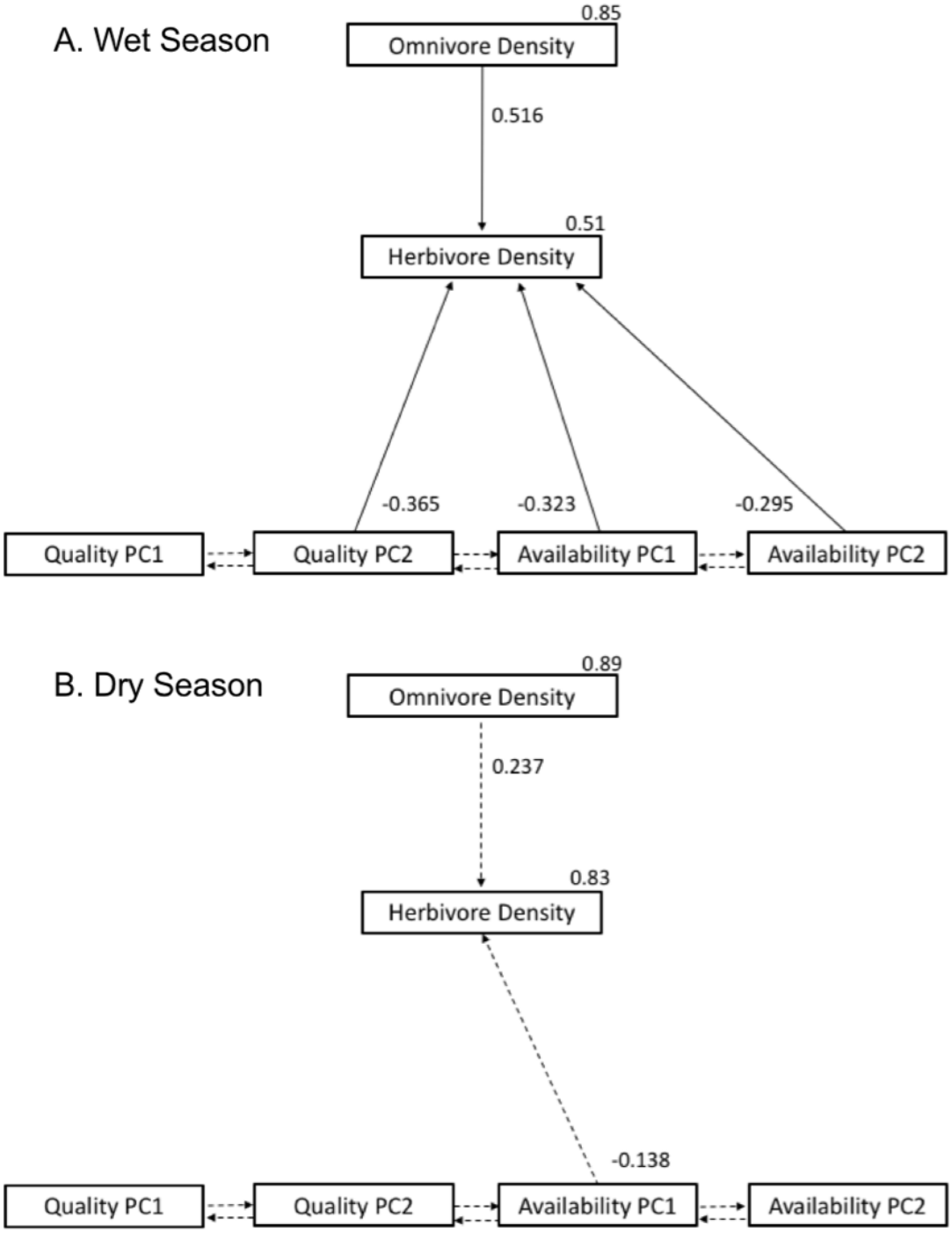
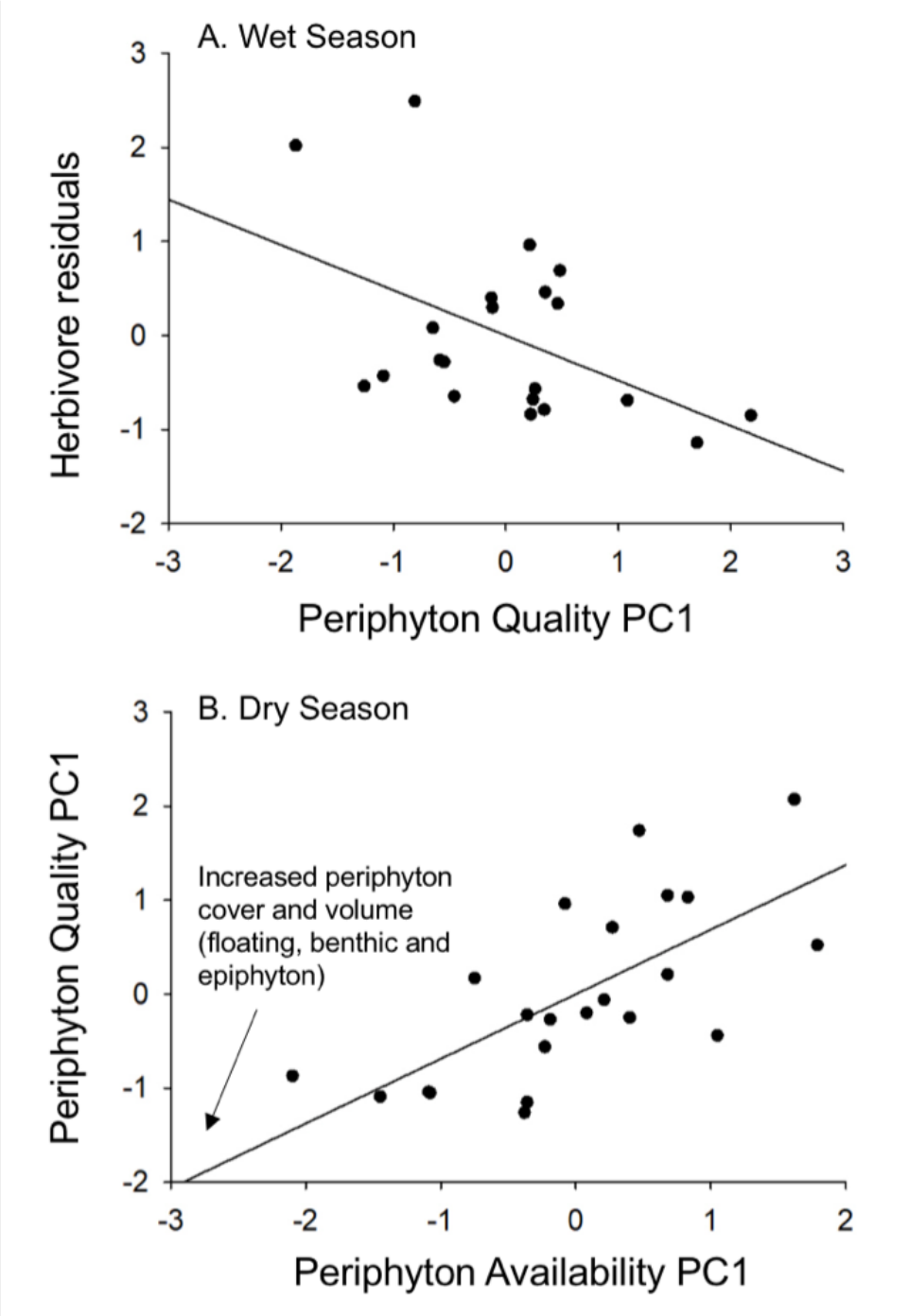

A. Wet Season
0.85
Omnivore Density
0.516
0.51
Herbivore Density
-0.365
-0.323
-0.295
Quality PC1
Quality PC2
Availability PC1
Availability PC2
B. Dry Season
0.89
Omnivore Density
0.237
0.83
Herbivore Density
-0.138
Quality PC1
Quality PC2
Availability PC1
Availability PC2

FIG. 6.7.


A. Wet Season
Herbivore residuals
Periphyton Quality PC1
B. Dry Season
Periphyton Quality PC1
Increased periphyton cover and volume (floating, benthic and epiphyton)
Periphyton Availability PC1

Supplementary Information

Table S.6.1. Additional environmental characteristics of the 22 sampled sites across the Everglades landscape in the wet season (July 2016) and the dry season (February 2017). WCA 1 periphyton was not processed for nutrients in the wet season due to sample contamination, and sites A55 and A62 were inaccessible by boat in the dry season and thus not able to be sampled. Sorted in order from shortest to longest hydroperiod.

Site Name, Location	UTM	Soil Type	Mat Type	Dominant plant genera
A55, Panhandle	17R 0548370, 2799637	Marl	Calcareous	Cladium
A60, Panhandle	17 R 0549633, 2796673	Marl	Calcareous	Cladium
A62, Panhandle	17R 0538937, 2794437	Peat + Marl	Calcareous	Eleocharis
A59, Panhandle	17R 543522, 2796517	Peat + Marl	Calcareous	Eleocharis
MDD, Taylor Slough	17R 0536563, 2800183	Peat	Calcareous	Eleocharis
37, Shark River Slough	17R 0515124, 2816895	Peat	Organic	Eleocharis
3, WCA 3	17R 0516614, 2875215	Peat	Organic	Eleocharis
10, WCA 3	17R 0540853, 2894243	Peat	Calcareous + Organic	Eleocharis
8, Shark River Slough	17R 0516610, 2818861	Peat + Marl	Organic	Eleocharis
TSD, Taylor Slough	17R 0533471, 2794060	Peat	Calcareous + Organic	Eleocharis
9, WCA 3	17R 0531411, 2895129	Peat	Organic	Eleocharis
TSA, Taylor Slough	17R 0532105, 2794861	Peat	Calcareous	Eleocharis
MDA, Taylor Slough	17R 05355703, 2800594	Marl	Calcareous	Eleocharis
7, Shark River Slough	17 R 0522246, 2826891	Peat	Organic	Eleocharis
7, WCA 3	17R 0546523, 2860779	Marl	Organic	Eleocharis
CP, Taylor Slough	17R 0529841, 2790479	Marl	Calcareous	Eleocharis
2, WCA 3	17R 0520550, 2865235	Sand	Organic	Nymphaea
1, WCA 3	17R 0526618, 2860613	Sand	Organic	Nymphaea
8, WCA 3	17R 0551663, 2870064	Marl	Organic	Eleocharis
6, WCA 3	17R 0544333, 2885929	Peat	Organic	Nymphaea
4, WCA 3	17R 0533854, 2865022	Peat	Organic	Nymphaea
5, WCA 3	17R 0536494, 2877413	Peat	Organic	Nymphaea

Table S.6.2. Detailed periphyton quality metrics of the 22 sampled sites across the Everglades landscape in the wet season (July 2016) and the dry season (February 2017).

Site Name, Location	C:P	N:P	Protein (mg/ m²)	Carb (mg/ m²)	Lipid (mg/ m²)	Het. FAs	PUFA Ratio	EPA%	Edible Algae %
A55, Panhandle	9580.91/--	211.02/--	4.65/ --	1.77/ --	4.15/ --	9.85/ --	0.13/ --	0.00/ --	18.17/ --
A60, Panhandle	15988.72/ 9458.06	429.38/ 409.10	6.04/ 0.22	2.32/ 1.48	6.81/ 1.41	23.59/ 0.24	0.12/ 0.33	0.00/ 0.03	16.07/ 14.13
A62, Panhandle	6871.58/--	157.23/--	0.25/ --	0.24/ --	0.49/ --	1.28/ --	0.14/ --	0.00/ --	13.70/ --
A59, Panhandle	4082.64/ 14046.57	112.57/ 621.09	0.59/ 0.05	1.59/ 0.60	1.49/ 0.22	7.24/ 0.29	0.14/ 0.19	0.00/ 0.001	13.00/ 8.98
MDD, Taylor Slough	5981.73/ 15184.13	124.51/ 665.19	1.63/ 0.10	0.43/ 0.0001	1.92/ 0.08	2.03/ 0.26	0.15/ 0.16	0.00/ 0.02	13.64/ 15.40
37, Shark River Slough	5564.27/ 4116.38	160.69/ 196.16	0.17/ 0.01	0.01/ 0.10	0.07/ 0.05	0.05/ 0.07	0.20/ 0.17	0.005/ 0.008	18.33/ 77.42
3, WCA 3	8462.29/ 10994.97	331.44/ 590.67	0.80/ 0.02	0.73/ 0.24	0.88/ 0.06	0.96/ 0.02	0.20/ 0.12	0.004/ 0.001	12.59/ 5.03
10, WCA 3	9368.17/ 17515.24	269.89/ 925.04	1.07/ 0.04	0.15/ 0.50	1.51/ 0.15	5.16/ 0.09	0.13/ 0.11	0.02/ 0.006	23.70/ 18.35
8, Shark River Slough	4964.35/ 8636.87	158.78/ 511.34	0.72/ 0.18	0.01/ 0.67	0.54/ 0.19	1.39/ 0.23	0.13/ 0.20	0.01/ 0.04	16.90/ 15.58
TSD, Taylor Slough	6123.88/ 23692.3	162.28/ 1090.94	2.92/ 0.08	0.84/ 0.33	1.76/ 0.11	2.27/ 0.13	0.17/ 0.17	0.004/ 0.01	15.27/ 11.08
9, WCA 3	5135.69/ 5962.24	201.25/ 294.07	0.05/ 0.04	0.88/ 0.37	1.12/ 0.09	3.30/ 0.09	0.33/ 0.15	0.02/ 0.01	22.87/ 4.46
TSA, Taylor Slough	7049.61/ 31110.77	166.38/ 1414.61	0.34/ 1.27	0.13/ 1.11	0.43/ 0.69	1.46/ 0.25	0.16/ 0.18	0.00/ 0.02	8.07/ 8.62
MDA, Taylor Slough	5749.19/ 15250.43	157.98/ 736.48	0.17/ 0.06	0.09/ 0.33	0.19/ 0.13	0.43/ 0.33	0.18/ 0.23	0.00/ 0.04	8.11/ 15.30
7, Shark River Slough	5181.92/ 13537.77	205.98/ 735.31	0.40/ 0.06	0.01/ 0.74	0.16/ 0.14	0.21/ 0.42	0.17/ 0.11	0.006/0.04	26.08/ 37.21
7, WCA 3	9233.22/ 35902.72	217.95/ 1852.43	0.02/ 0.22	0.88/ 0.99	0.76/ 0.23	2.01/ 0.33	0.19/ 0.14	0.01/ 0.03	29.54/ 35.22
CP, Taylor Slough	11483.59/ 42009.44	304.42/ 1745.62	1.71/ 0.06	0.47/ 0.00005	1.39/ 0.02	3.03/ 0.40	0.15/ 0.14	0.009/ 0.03	31.49/ 13002
2, WCA 3	4393.56/ 8485.61	198.05/ 442.62	0.36/ 0.02	0.24/ 0.60	1.39/ 0.06	0.21/ 0.40	0.14/ 0.21	0.004/ 0.002	16.66/ 12.13
1, WCA 3	--/ 23563.12	--/ 1068.86	--/ 0.006	--/ 0.05	--/ 0.03	--/ 0.01	--/ 0.26	--/ 0.0004	--/ 9.60

8, WCA 3	6908.87/ 26118.89	230.40/ 1374.89	0.02/ 0.08	0.39/ 0.56	0.35/ 0.13	1.33/ 0.14	0.17/ 0.13	0.01/ 0.02	21.47/ 48.59
6, WCA 3	4572.22/ 2844.16	176.47/ 113.17	0.01/ 0.006	0.10/ 0.13	0.17/ 0.02	0.18/ 0.01	0.18/ 0.19	0.009/ 0.001	30.45/ 21.89
4, WCA 3	5350.26/ 13412.59	191.63/ 654.54	0.01/ 0.0003	0.23/ 0.007	0.25/ 0.003	0.67/ 0.00	0.16/ 0.20	0.009/ 0.00009	13.91/ 32.73
5, WCA 3	3630.15/ 6020.10	158.75/ 310.27	0.02/ 0.11	0.20/ 0.86	0.18/ 0.43	0.12/ 0.23	0.20/ 0.20	0.008/ 0.02	22.47/ 6.99

Fig. S.6.1

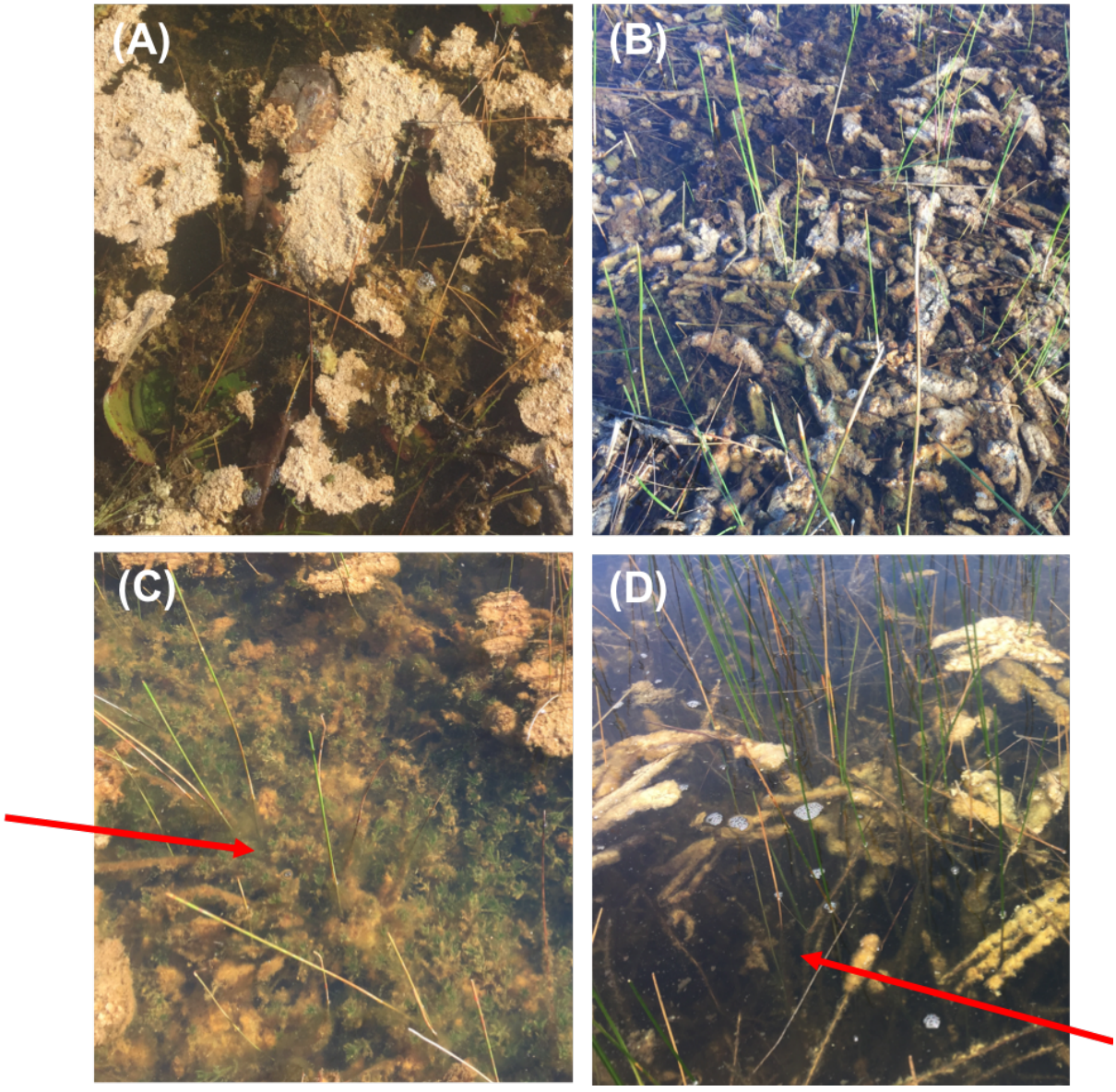


Fig. S.6.1. Examples of various periphyton types sampled in this study. (A) Floating mat aggregation (WCA 8, Dry season). (B) Alternative floating mat form: epiphytic growth on emergent vascular plant stems (WCA 3, dry season). (C) Filamentous green algae mass occupying the water column (see red arrow; PHD-A59, wet season). (D) Epiphyton collected from submerged stems of aquatic macrophytes (see red arrow; TSL-MDA, wet season).

CHAPTER 7

CONCLUSION

The objectives of my dissertation research were: 1) to propose testable hypotheses for the adaptive evolution of herbivory; 2) and to test several of these proposed hypotheses to understand the evolutionary and ecological consequences of adopting an herbivorous diet. I reviewed the herbivory literature and identified existing studies that could be interpreted in the context of adaptive evolution of diet. I used these studies to build a framework of five testable hypotheses and tested three of these hypotheses (Heterotroph Facilitation, Lipid Allocation, and Suboptimal Habitat) using a series of phylogenetic, experimental (lab and field), and community assembly studies. I found evidence supporting the Heterotroph Facilitation Hypothesis in my experimental work, but the Suboptimal Habitat Hypothesis had greater explanatory power when assessing the evolution of herbivory from a phylogenetic and community assembly perspective. Although these studies provide alternative explanations for the evolution of herbivory, the key distinction between these hypotheses is that heterotroph facilitation is a mechanism to overcome poor food quality, and invasion of suboptimal habitats allows passage into habitats with varying resource bases. The basis of these findings is the same, however; resource quality and/or availability is responsible for the evolution and/or maintenance of herbivory in nature. There were no explicit studies examining the adaptive significance of herbivory before I began this research, so I was interested in identifying the "gaps" in the current research that limit our knowledge of the evolution of diet (Ch. 2).

Our previous understanding of herbivory was defined by studies focusing on herbivory from the perspective of the primary producers (e.g., Power 1992; Strong 1992; Pare and Tumlinson 1999; Howe and Jander 2008; and others), or by studies examining herbivore responses to diet (e.g., Sinclair et al. 1982; Targett and Targett 1990; Simpson and Simpson 1990; Pennings et al. 1993; Stachowicz and Hay 1996; Cruz-Rivera and Hay 2000b;Van der Wal et al. 2000; Fink and von Elert 2006, and others). A recent body of work has begun to identify patterns of diet evolution in related species using comparative analyses (e.g., Van Damme 1999; Espinoza et al. 2004; deMaintenon 1999; Eubanks et al. 2003; Pauls et al. 2008; Bellwood 2003; Bellwood et al. 2014, and others), which has brought us closer to understanding the adaptive significance of herbivory. In reviewing these works, I found evidence that eating plants is favored when higher quality food is limiting (e.g., Chubaty et al. 2014). Furthermore, I found that freshwater herbivore diets are not always inadequate as they can provide a different suite of important dietary elements such as plant-derived lipids and sterols (e.g., Martin-Creuzburg et al. 2011), or heterotroph-derived nutrients (e.g., Bowen 1984, Smoot and Findlay 2010, Belicka et al. 2012) that are deficient in carnivorous diets. Exploring these already established ideas from an adaptive perspective has provided us with a better understanding of the conditions that promote the evolution of herbivory in nature.

In Chapter 3, I evaluated the Suboptimal Habitat Hypothesis by reconstructing ancestral states of habitat and diet across a phylogeny of the genus *Poecilia* (comprised of 7 subgenera). Species comprising the genus *Poecilia* exhibit a variety of diet preferences, with obligate herbivory concentrated in the subgenus *Mollienesia*.

Extant species belonging to the subgenus *Mollienesia* inhabit both fresh and euryhaline habitat types (Meffe and Snelson 1989). I found that the most recent common ancestors (MRCA) of subgenera *Acanthophacelus*, *Micropoecilia*, *Psychropoecilia* and *Allopoecilia* had low salinity affiliations and were either omnivorous or carnivorous. Furthermore, the divergence of the subgenera *Poecilia* and *Mollienesia* resulted in MRCAs with euryhaline roots, and the transition from low to high salinity affiliation drove diet diversification favoring the appearance of obligate herbivory in these groups. Salinity affiliation explained 26% of the total variation in the diet of *Poecilia* species, and jaw morphology was associated with percent animal material in the gut, but not with percent of species occupying saline habitats. These findings suggest that in this genus, herbivory evolved in response to habitat transitions between fresh and euryhaline habitats, and jaw morphology evolved in response to the appearance of herbivory. These results are consistent with the Suboptimal Habitat Hypothesis.

I experimentally evaluated the Heterotroph Facilitation and Lipid Allocation hypotheses using field (Ch. 4) and lab (Ch. 5) studies. I found that herbivorous Sailfin Mollies (*Poecilia latipinna*) benefit from a diet supplemented with heterotrophic microbes, supporting the suggestion that "true" herbivory is rare in nature (White 1985). In my field cage experiment, increased autotroph biovolume, increased proportion of monounsaturated fatty acids, and decreased percentage of heterotrophic fatty acids in the diet best predicted early Sailfin Molly life history (6-9 weeks of age). However, later in development (9-12 weeks of age), cages with high heterotroph fatty acid production yielded the highest juvenile survival.

Because autotroph-derived lipids were not the main influencer of herbivore success, the Lipid Allocation Hypothesis was not supported. Rather, I found that heterotrophs supplement herbivorous diets, and the quality (e.g. fatty acid abundance) of these microbes strongly influences herbivore life history by increasing survival by up to 53%. My lab study yielded comparable results to those found in my field experiment and confirmed that Sailfin Mollies benefit from a diet that incorporates heterotrophic food sources. Both studies suggest that heterotrophs supplement the herbivorous diet in ways that affect consumer life history, thereby supporting the Heterotroph Facilitation Hypothesis.

In my final chapter (Chapter 6), I measured food quality and availability across the Everglades landscape and examined these variables as potential drivers of consumer density. I interpreted these results in the context of traditional niche theory (niche-based and dispersal-based models) and hypotheses about the maintenance of herbivorous diets in food webs (Heterotroph Facilitation and Suboptimal Habitat). I found that herbivores track food quality when habitats are stable (e.g., in the wet season), but they can persist in a multitude of habitat types and survive on resources of varying quality when habitats are variable (e.g., in the dry season). These results suggest that herbivore diets follow niche-based predictions in the wet season, but dispersal-based predictions in the dry season. In contrast, omnivores rely on high-quality resources in both seasons, consistent with niche-based predictions. Taken together, these results partially support the Suboptimal Habitat Hypothesis as an explanation for the evolution of herbivory in this system.

My goal for this research was to explore the conditions that would favor the evolution of an herbivorous diet from a carnivorous or omnivorous diet. Overall, these studies suggest that herbivory may have evolved as an adaptive strategy to deal with variable/unproductive habitats, and is maintained in natural systems by supplemental detritivory. My results show that invading 'suboptimal' habitats has significant evolutionary consequences by enhancing the possibility for novel phenotypes to spread (i.e., herbivory), thereby promoting new ecological interactions between species. Once the diet strategy has appeared in a lineage, it is maintained as an alternative to carnivory or omnivory, as long as it provides the necessary nutrients to sustain herbivore life processes. Exploring these adaptive hypotheses has established a much-needed research framework, allowing us to more fully understand the evolution of diet in freshwater and other systems.

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VITA

JESSICA SANCHEZ MONTELONGO

Born, Ancon, Panama

2005-2009	B.S., Biological Sciences Sam Houston State University Huntsville, TX
2009-2010	Teaching Assistant Ecology and General Biology Sam Houston State University Huntsville, TX
2010-2012	M.S., Biological Sciences Sam Houston State University Huntsville, TX
2010-2012	Teaching Assistant General Biology, Vertebrate Embryology and Zoology Sam Houston State University Huntsville, TX
2010-2012	Graduate Scholarship Fellow Sam Houston State University Huntsville, TX
2012-2013	Teaching Assistant Human Biology Florida International University Miami, FL
2013-2016	Editorial Assistant, Oecologia Florida International University Miami, FL
2016-2018	Head Editorial Assistant, Oecologia Florida International University Miami, FL

PUBLICATIONS AND PRESENTATIONS

Sanchez, J. L., and J. C. Trexler. 2018. When is an herbivore not an herbivore? Detritivory facilitates herbivory in a freshwater system. Ecology and Evolution. doi 10.1002/ece3.4133

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