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ECOLOGICAL DRIVERS OF SPECIES RADIATIONS IN THE GENUS *LIMIA* (TELEOSTEI, POECILIIDAE) IN THE WEST INDIES

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ABSTRACT

The archipelagoes of the West Indies in the Caribbean represent geographically composite regions that provide ideal settings to study the evolution of a very distinctive biota. The combination of complex geological events and tropical climatic conditions has driven radiation processes that are not homogeneous for all groups in the Caribbean. In the course of my dissertation work, I examined the case of livebearing fishes of the genus *Limia* (Teleostei: Poeciliidae) that show some deviations from the positive species-area relationship predicted by the theory of island biogeography. I particularly analyzed the potential role of some ecological drivers in explaining the lopsided distribution of *Limia* fishes in the Greater Antilles. Chapter one is an extensive review about the West Indian biogeography that highlights divergent diversification patterns observed in terrestrial versus aquatic groups. This review also sheds light on the unbalanced number of studies covering the biogeography of these groups of organisms in the West Indies. Chapter two is an assessment of the variation of tolerance to extreme temperatures (measured as critical thermal minimum (CTmin) and maximum (CTmax)) and also the thermal breadth for multiple species of *Limia* occurring in the West Indies and that occupy different altitudinal distributions. This study analyzes the role of temperature gradients across elevations in determining species distribution in the genus. Chapter three evaluates the diet and feeding specializations of *Limia* through a comparative analysis based on data of the gut content of eight species. This chapter explores the potential role of the feeding ecology in the radiation of the genus *Limia* in the Caribbean. Finally, chapter four uses laboratory experiments and also fieldwork data to test whether sexual selection or natural selection may explain the presence of color polymorphism in *Limia vittata*, an endemic species to Cuba.

Overall, my results show that species richness in the genus *Limia* is positively correlated with island size but also with the presence of elevations showing that not only island area but also mountainous relief may be an important factor determining the number of freshwater species in

the Greater Antilles. In addition, we found no association between thermal tolerance and the altitudinal distribution in *Limia* species, suggesting that biotic factors such as species interactions, diet specializations, and others should be taken into account when interpreting current distribution patterns. Even though our study showed that the majority of *Limia* species tend to be feeding generalists, some degree of diet specialization occurs in certain species from Hispaniola where the diversity of the genus *Limia* is remarkably higher compared to the rest of the Greater Antilles. Lastly, we revealed that natural selection determined by habitat variation in terms of salinity levels, is the most important factor in maintaining color polymorphism in *L. vittata*. Salinity levels could be indirectly responsible for maintaining different color morphs in this species, likely due to the regulatory effect of saline gradients on predation regimes.

CHAPTER 1: BIOGEOGRAPHY OF THE WEST INDIES: A COMPLEX SCENARIO FOR SPECIES RADIATIONS IN TERRESTRIAL AND AQUATIC HABITATS

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INTRODUCTION

The Caribbean region stands out as a leading biodiversity hotspot in the world exhibiting high levels of diversity and endemism both in flora and fauna (Myers, Mittermeier, Mittermeier, da Fonseca & Kent, 2000). Particularly in the islands of the West Indies, which include the archipelagoes of the Bahamas, the Greater Antilles, and the Lesser Antilles (Berman, 2008; Hofmann, 2008), the presence of a very distinctive biota has resulted from a unique and complex combination of geological events and tropical climatic conditions. These two elements have been shown to be a major cause of diversification and speciation in reptiles (Glor et al., 2004; Gifford & Larson, 2008), amphibians (Rodríguez, Vences, Nevado, Machordom & Verheyen, 2010; Alonso, Crawford & Bermingham, 2012), and freshwater fishes (Rivas, 1958; Rosen & Bailey, 1963; Doadrio, Perea, Alcaraz, & Hernández, 2009; Ponce de León et al., 2014). Furthermore, it is responsible for an extraordinary diversity and endemism in several genera of plants (Santiago-Valentin & Olmstead, 2004).

The West Indies represent a geographically complex region that provides a suitable setting to study the colonization and diversification processes that resulted in a plethora of animal and plant species currently occurring in the insular Caribbean. Although several taxa exhibit radiations mainly in the Greater Antilles, the speciation process has not been homogeneous for all groups in the Caribbean. Even though the majority of terrestrial vertebrates show higher diversification in larger islands (Hedges, 1996a; Losos, 1996, 2009), thereby confirming the positive species-area relationship predicted by the theory of island biogeography (MacArthur & Wilson, 1967), some deviations from theory are observed in aquatic groups such as some genera of livebearing fishes (Poeciliidae). According to Ricklefs & Bermingham (2008), the West Indies are ideal natural laboratories for biogeographic and evolutionary analyses since a series of combined factors such as distance to sources of colonists as well as age and different sizes of the islands allow us to explore the evolution of several groups under diverse scenarios.

Indeed, one of the most dynamic debates in biogeography is related to the different hypotheses of colonization of the West Indies, and particularly the Greater and Lesser Antilles. It has been a contentious topic for more than a century with hypotheses that emphasize the important role of land connections and vicariance (Babour & Matthew, 1916; Schuchert, 1935; Rosen, 1975) to studies suggesting that transoceanic dispersal has been a significant component in the origin and establishment of the insular Caribbean biota (Simpson, 1956; Darlington, 1957; Williams, 1989; Hedges, Hass & Maxson, 1992) (Table 1). Certainly, the study of species composition and phylogenetic relationships in extant terrestrial and freshwater organisms of the West Indies can shed light on the history of the extant Caribbean biota. One reason for this is because these species depend on passive dispersal due to their limited ability to cross transoceanic barriers. Even though this review provides some context of both geological and biogeographic elements that may explain the origin and diversification of noticeable groups that have radiated mostly in the Greater Antilles, this paper does not intend to accomplish an exhaustive analysis about the biogeography of the West Indies. Actually, there are numerous works including research articles, book chapters and entire books that examine in detail several aspects of the biogeography of the West Indies including the paleogeography of this complex geographic region, causes of diversification of several groups of organisms and also conservation problems of the biodiversity in the Caribbean islands (Crother & Guyer, 1996; Iturralde-Vinent & MacPhee, 1999; Hedges, 2001; Santiago-Valentin & Olmstead, 2004; Ricklefs & Bermingham, 2008; Crews & Esposito, 2020). Instead, our review paper aims to highlight contrasting biogeographical trends seen in different taxonomic groups distributed in the West Indies (specifically in the Greater Antilles), and also shed light on the unbalanced number of studies covering the biogeography of terrestrial versus aquatic organisms. For this, we use an objective method to gather existing information in the field and produce a rigorous literature review (Haddaway et al. 2020). This paper is particularly relevant in highlighting divergent diversification patters that might occur in terrestrial versus aquatic groups of the West Indies biota. Here we analyze both classic and scarcely studied examples of species radiations in the West Indies. Finally, this study sheds light on the importance of this region for the conservation of biodiversity.

METHODS FOR LITERATURE SEARCH

In order to provide an objective and reproducible scientific compilation that meets the goals of this review and also guarantees the reproducibility of our results, we conducted a literature search on the Web of Knowledge (Web of Science Core Collection database) through the University of Oklahoma Library website on December 17th, 2020. We obtained a total of 890 article records published between 1900 and 2020 using the following key word combinations for the search: "West Indies biogeography" (238 records), "Caribbean biota" (153 records), "Caribbean islands colonization" (234 records), "adaptive radiations Caribbean" (128 records) and "Antilles biodiversity" (131 records). After each search using a specific key word combination, all available outcomes (publications) were assessed and scrutinized based on the topic of the each study (Figure 1).

Here we only considered publications in which the central theme was related to aspects of the Caribbean biogeography (i.e. historical biogeography, species radiations, phylogenetic and phylogeographic studies). In addition, we classified the publications according to the specific area coverage for each study, studied group(s) and the analysis of biogeographical trends or biogeographical theories supported by each study (if any).

Geology and geological history of the West Indies

The three main archipelagoes that form the West Indies (the Bahamas, the Greater Antilles and the Lesser Antilles) lay in the Caribbean Sea between the continental masses of North and South America (James, 2005). Despite of the geographic proximity of these three archipelagoes, the geological origin of their present-day land territories is totally different from each other and also quite complex (Figure 2). The Bahamas, which are geographically more related to North America, were formed by the accumulation of carbonate marine sediments during lower sea levels in the Pleistocene glacial periods. The origin of the Bahamas platform is completely independent of the Caribbean plate and also unrelated to the origin of the Greater and Lesser Antilles (Meyerhoff & Hatten, 1974). Consequently, the biota of the Bahamas show relatively low endemism with a flora and fauna mostly derived from North America and the Greater Antilles (Ricklefs & Bermingham, 2008).

Conversely, the Greater Antilles are old islands formed by fragments of continental crust that have been carried to their current position by plate movements (Pindell, 1994). Several parts of the landmasses that currently form Cuba, Hispaniola (Haiti and the Dominican Republic) and Puerto Rico, are thought that have stayed above sea level since the Middle Eocene (Donnelly, 1989; Iturralde-Vinent & MacPhee, 1999). However, in the formation of the contemporary Greater Antilles periods of extensive inundations caused by sea level changes coupled with movements of the Caribbean plate have resulted in the creation of isolated landmasses that have fragmented and rejoined multiple times resulting in very complex geographical histories (Iturralde-Vinent & MacPhee, 1999). In fact, Hedges (1996b) stated that the study of the Caribbean biota faces particular challenges due to the difficulty in determining which areas were above or under sea level during the history of the islands.

Although information about the historical relationships among landmasses and geological composition of the islands of the Greater Antilles are yet incomplete, it is broadly established that Hispaniola and Cuba are created by compound regions (Pindell & Dewey, 1982). The geological differences between the western and eastern portions of Cuba represent a clear example of the complex origin of the islands in the Greater Antilles. The eastern part of Cuba, north-central Hispaniola, and Puerto Rico were probably connected as a single magmatic arc during the Paleocene-Eocene (Draper & Barros, 1994) and until the Oligocene (Iturralde-Vinent, 1994). By contrast, the western part of Cuba is exclusive in the Antilles in terms of geological origin (Draper & Barros, 1994) and it is thought to be probably related with the North American Plate (Graham, Cozadd, Areces-Mallea & Frederiksen, 2000).

The Lesser Antilles are composed of several volcanic islands located at the margin of the

Caribbean plate and forming an arc that extends northwest from north of South America to Puerto Rico. This arc of small islands is separated from the Greater Antilles by the Anegada Passage (Santiago-Valentin & Olmstead, 2004), which is a set of marine basins and deep valleys located across the volcanic arc at the transition between the Greater and the Lesser Antilles (Laurencin et al., 2017). The Lesser Antilles most likely originated about 20 million years ago emerging from above a subduction zone (Wadge, 1994). These small islands which form the Lesser Antilles originated independently from the Greater Antilles, never had a direct connection with the continent (Ricklefs & Bermingham, 2008). Despite this, they have been basically colonized by the biota from South America (Hedges, 1996b).

Even though the West Indies are geographically located in the neotropics which is relatively stable in terms of temperature, differences if relief among islands lead to some climate heterogeneity that can generate temperature and also precipitation gradients along different elevations (Borhidi, 1996; Rumney & Oliver, 1998). Hispaniola, for instance, contains several mountains with more than 2000 meters in elevation. For example, the highest peak, Pico Duarte in the Dominican Republic, reaches 3098 meters. To some extent this topographic heterogeneity has led to the evolution of high elevation specialists (mainly amphibians and reptiles) on Hispaniola (Wollenberg, Wang, Glor & Losos, 2013; Muñoz, Wegener & Algar, 2014). Although less high in elevation, other highland areas reaching over 1500 meters can be also found in other parts of Hispaniola, eastern Cuba and Jamaica. High elevations have been shown to be an important property in determining the distribution and endemism of island biodiversity (Kallimanis et al., 2010). Such habitat heterogeneity coupled with the relatively large size of islands like Cuba and Hispaniola has been thought to provide niche opportunities for speciation and endemism in several terrestrial groups (Gentry, 1982; Losos & Schluter, 2000; Algar & Mahler, 2016).

Hypotheses that explain the origin of the West Indies biota

Despite an intensive debate about models and hypotheses to explain the origin of biodiversity across the Caribbean islands, much of the origin of the biota is still poorly understood and remains an important topics for biogeographers and evolutionary biologists (Dávalos, 2004; Santiago-Valentin & Olmstead, 2004; Vázquez-Miranda, Navarro-Siguenza & Morrone, 2007; Rodríguez et al., 2010; Reznick, Furness, Meredith & Springer, 2017; Tucker, Hedges, Colli, Pyron & Sites, 2017; Čandek, Agnarsson, Binford & Kuntner, 2019). A better understanding of the Caribbean biogeography would also aid investigations of other archipelagoes such as the Philippines, which has an equally complex geological origin (Mitchel, Hernandez & de la Cruz, 1986). Equally, the multifaceted distinctiveness of the archipelagoes of the West Indies in terms of geology and geological history, varying sizes of the emerged landmasses and their distances to sources of colonists, make them ideal settings to explore the theory of the island biogeography equilibrium and also the influence of *in situ* speciation processes in the origin of the biodiversity (Ricklefs & Bermingham, 2008).

Three main hypotheses have been historically proposed to explain the origin of biodiversity in the Caribbean islands (Table 1). First, the vicariance model proposed by Rosen (1975), based on terrestrial, freshwater and marine taxa, suggested that the Proto-Antilles had a direct connection to mainland South and North America approximately 100-70 million years ago. While this hypothesis has been supported by other authors (Page & Lydeard, 1994; Crother & Guyer, 1996; Van Ee, Berry, Riina & Gutiérrez-Amaro, 2008; Crews & Esposito, 2020), the lack of strong geological evidence has led to some controversy around the vicariance model (Williams, 1989; Hedges, 1996a, 2001; Iturralde-Vinent, 2006). Second, overwater dispersal is another hypothesis that has been proposed to explain the origin of the West Indies biota. This model has received support from several studies that have tested comprehensively many groups of organisms contrasting the fossil records and extant species with precise estimation times of origin of different lineages (Williams, 1989; Hedges et al., 1992, Hedges, 2001). For some terrestrial organisms such as amphibians and reptiles, for instance, transoceanic dispersal likely from South America seems to be the most probable origin of many living groups of these vertebrates in the

Caribbean islands (Heinicke, Duellman & Hedges, 2007; Hedges & Conn, 2012). Both theories, vicariance and overwater dispersal hypotheses, have disagreed with each other with no consensus over many decades. However, some authors have suggested that both models could have played important roles in biogeography for different groups in the Caribbean (Newton, 2003; Hrbek, Seckinger & Meyer, 2007).

The third model that explains the biogeography of the Caribbean basin was published by Iturralde-Vinent & MacPhee (1999). They hypothesized a recent continuous land bridge, GAARlandia (Greater Antilles and Aves Ridge), between north of South America and the Greater Antilles. Such land connection supposedly existed 35-33 million years ago which coincides with intervals of low sea levels that exposed the Aves Ridge allowing the continental South American biota to reach the Caribbean islands. Although there is some support for the GAARlandia hypothesis (Rican et al., 2013; Matos-Maraví et al., 2014; Weaver et al., 2016), there is still not enough geological evidence to support the land bridge postulate of a continuous dry connection (Ali, 2012). In addition, the absence of many groups of terrestrial mammals in the West Indies do not offer support for a land bridge connection either (Dávalos, 2004; Ricklefs & Bermingham, 2008). Recent molecular evidence has also suggested that GAARlandia did not act as a colonization route for plants from South America to the Antilles (Nieto-Blazquez, Antonelli & Roncal, 2017).

Certainly, the debate about the colonization of the West Indies biota has moved back and forth between vicariance, land connections and dispersal in order to explain the origin of the biodiversity in these archipelagoes. In most cases the momentary establishment of a governing hypothesis has been followed by the publication of influential works that offer at least partial evidence supporting a particular theory. Nevertheless, more intricate biogeographical scenarios that include both dispersal and vicariance models might explain the formation of the biotas on this region (Heaney, 2007).

The West Indies as special scenarios for species radiations and endemism

Islands represent ideal settings to study macroevolutionary processes. Most of the time islands offer novel ecological opportunity for colonists such as abundant food sources and heterogeneous habitats to proliferate (Simpson, 1953). Other advantages that these new environments offer to the initial colonizers are the absence of predators and competitors (Losos & Ricklefs, 2009). Often immigrant lineages radiate filling unoccupied ecological niches in the islands and new species arise through ecological diversification (Schluter, 2000) often in the form of an adaptive radiation (Schluter, 2000; Losos, Glor, Kolbe & Nicholson, 2006).

Adaptive radiations are extraordinarily important in the origin of biodiversity. Some authors consider this as one of the most important evolutionary processes and likely responsible of a significant part of the ecological and phenotypic diversity of life (Simpson, 1953; Schluter, 2000). There are many classic examples of adaptive radiation that have occurred in islands including Darwin's finches of the Galápagos islands (Grant & Grant, 2002), Hawaiian silverswords (Raven, Evert & Eichhorn, 1992) and Hawaiian honeycreepers (Lovette, Bermingham & Ricklefs, 2002). In the West Indies adaptive radiations have been an important cause of diversification in several groups of organisms. In large and topographically heterogeneous islands like Cuba and Hispaniola a burst of species formation can be seen in some groups (Ricklefs & Bermingham, 2008). Probably the most recognized and well-studied is the case of *Anolis* lizards with more than 150 species in the Caribbean (Losos & Schluter, 2000; Glor, Kolbe, Powell, Larson & Losos, 2003; Glor et al., 2004). Anoles have diversified extraordinarily in the Greater Antilles to produce the same set of habitat specialists or ecomorphs in each island (Losos et al., 2006; Gavrilets & Losos, 2009). In the case of the massive adaptive radiation of *Anolis* lizards in the Caribbean, species richness on islands is related to area (Losos, 1996), which is actually a general prediction of the theory of island biogeography (Darlington, 1957; MacArthur & Wilson, 1967) that can also be observed in several other components of the island biota such as plants (Gentry, 1982; Santiago-Valentin & Olmstead, 2004), spiders (Gao & Perry, 2016; Čandek et al., 2019), butterflies (Matos-Maraví et al., 2014), amphibians (Rodríguez et al., 2010; Alonso et al., 2012). Other groups of extant vertebrates in the Greater Antilles have also experienced high rates of speciation leading to significant endemic biodiversity. Frogs of the genus *Eleutherodactylus* (Eleutherodactylidae), for instance, have been able to radiate in the Greater Antilles making them the dominant group of amphibians in the West Indies (Hedges, Duellman & Heinicke, 2008). In Cuba, for example, 90% (50 species) of the native amphibians are members of this genus (Hedges et al., 2008; Díaz & Cádiz, 2008). The fossil record also offers evidence of multiple radiation events occurred in the past in several lineages of mammals (i.e. primates, sloths and rodents) of the West Indies (Hedges, 2006). Although extant groups of mammals are not as diverse as the Caribbean herpetofauna, rodents are the richest terrestrial mammal group of the West Indies. Particularly, capromyid rodents (Capromyidae), commonly called hutias, have experienced the largest radiation in the Caribbean islands with eight genera and 32 species that display several ecomorphological adaptations (Fabre et al., 2014). Furthermore, some groups of freshwater fishes also show a significant radiation in the Greater Antilles. Examples of the very interesting patterns of species distribution mainly in the family Poeciliidae are being described later in this review. Although the majority of the studies on biogeography of the Caribbean islands are primarily focused on animal groups, plant diversity is also outstanding in this region to the point that the Caribbean is considered a distinctive phytogeographic unit within the Neotropics (Gentry, 1982). There are approximately 13 000 seed plants of which about 8000 are endemic in the West Indies (Acevedo-Rodríguez & Strong, 2008). The alpha-diversity of plant species in the Caribbean region is similar to that of Madagascar, and much larger than that of New Caledonia. Over 50% of the vascular plants are endemic to the West Indies, which makes the Caribbean islands a leading hotspot in species-level endemism (Myers et al., 2000). Particularly in the Greater Antilles, Cuba and Hispaniola the two largest islands in the Caribbean have the richest flora and highest endemism at the specific and generic level (Gentry, 1982; Santiago-Valentin & Olmstead, 2004). The smaller islands, Jamaica and Puerto Rico, have less plant diversity, and endemism is mostly at the species level (Santiago-Valentin & Olmstead, 2004).

Some plant groups show remarkable species radiations in the West Indies such as lineages within the families Melastomataceae (Michelangeli et al., 2008) and Asteraceae (Francisco-Ortega et al., 2008). Palms (Arecaceae), for example, are also well represented with 135 species of which 121 are endemic (Zona, Verdecia, Leiva-Sánchez, Lewis, & Maunder, 2007) with most of the endemism concentrated to the Greater Antilles (Roncal, Zona & Lewis, 2008). Substrates of serpentine rocks are a very distinctive feature of some islands like Cuba and Puerto Rico (Cedeño-Maldonado & Breckon, 1996). These environments host an extraordinary biodiversity of unique plants with around 35% of all endemic genera known in Cuba confined to serpentine (Berazaín-Iturralde, 1976; Brooks, 1987).

Unfortunately, there is an unbalanced number of biogeographical studies creating taxonomic bias in the study of the West Indian biota. After reviewing the available literature in the Web of Science Core Collection database using different key word combinations, we found that most of the studies that examine the biogeography of the West Indies and the Caribbean islands in general involve terrestrial groups. Of the 890 publications in the field that resulted from our initial search and after a critical appraisal, we identified 177 to be relevant to the study of the biogeography of the West Indies. The scientific work in this field has been growing notably. For example, during the last five years alone 62 studies have been published, representing 35% of all the literature compiled in our review. Most of the publications we found (162 studies, 91.5%) were related in the study of terrestrial organisms, only a small portion covered aquatic groups (13 studies, 7.3%) and only a couple review contributions (2 studies, 1.2%) covered the study of both terrestrial and aquatic organisms. We identified vertebrates (mainly terrestrial vertebrates, which included birds and bats) as the most studied group (102 studies, 57.6%), followed by invertebrates (46 studies, 23.7%) and plants (28 studies, 15.8%). In studies that tested biogeographical models of the origin of the West Indian biota, we found that several of them included more than one single model to explain the biodiversity found on the islands. However, over-water dispersal emerged as the most relevant mechanism of colonization of the West Indies not only for organisms with good dispersal abilities (flying insects, birds, bats, etc.), but also for species with poor dispersal abilities, mainly through rafting flotsam moved by large storms and marine currents.

Our results show that studies on groups such as plants, terrestrial or aquatic arthropods and freshwater fishes are vastly underrepresented relative to their taxonomic diversity. One of the most important steps to fill the information gap on these groups (mainly in aquatic organisms) requires the preparation of specialists with the necessary knowledge and skills to document biodiversity in these scarcely studied groups. It is also imperative to perform integrative analyses that uncover the possible origins of these groups in the Caribbean islands and also evolutionary mechanisms leading to speciation.

Species radiation in aquatic environments of the West Indies: the case of livebearing fishes in the Greater Antilles

Certainly, the classic example of adaptive radiation in aquatic vertebrates is the case of cichlid fishes (Cichlidae) from the East African Great Lakes (Turner, Seehausen, Knight, Allender & Robinson, 2001). The explosive speciation rate in this group is known to be responsible for generating an outstanding diversity in behavior, coloration, body shapes and a huge diversity of trophic and other ecological specializations (Fryer & Iles, 1972; Meyer, 1993; Kornfield & Smith, 2000; Turner, 2007; Seehausen, 2015; Meier et al., 2017; Malinsky et al., 2018).

Inland water bodies in the West Indies are relatively small even on the larger islands (Vergara, 1992). This size limitation imposes some constrains for species diversification in aquatic groups. However, although not quite as rampant as the case of the cichlid radiation in the African rift valley lakes, the West Indies exhibit some examples of young adaptive radiations in fishes. For instance, three species of *Cyprinodon* (Cyprinodontidae) that coexist sympatrically in hypersaline lagoons within the San Salvador Island, Bahamas have been described to have trophic partitioning (Martin & Wainwright, 2013; Martin, 2016). *C. variegatus*, a species with broad geographic distribution and with detritivorous feeding habits, is thought to have given origin to this small Bahamian radiation (Hernandez et al., 2018). The other two species are diet specialists: *C. brontotheroides* specializes in consuming hard prey (i.e. ostracods, gasteropods) and *C. desquamator* a specialized scale-eater (Martin & Wainwright, 2013).

Nonetheless, livebearing fishes (Poeciliidae) is the group of freshwater vertebrates that has experience the highest radiation in aquatic environments of the West Indies with three endemic genera (*Girardinus*, *Quintana* and *Limia*) distributed in the Antilles (Rosen & Bailey, 1963; Hamilton, 2001; Doadrio et al., 2009; Reznick et al., 2017). The genus *Gambusia* is also represented in the archipelagoes of the Greater Antilles and the Bahamas where ecological speciation has been shown to be associated with divergent predation regimes (Langerhans, Gifford & Joseph, 2007).

Poeciliids represent an ideal model to study the origin of the Caribbean biota (García-Machado et al., 2020). They are the dominant group of freshwater fishes in the insular Caribbean and also exhibit an extraordinary diversity in continental Middle America (Rosen & Bailey, 1963; Vergara, 1992). Furthermore, this group of fishes offers a complex scenario when analyzing colonization events in the Caribbean as compared to terrestrial or other freshwater groups. Several poeciliid species show varying levels of tolerance to water salinity, which could made possible overseas dispersal from the mainland (Myers, 1938; Darlington, 1938; Rosen & Bailey, 1963; Briggs, 1984). In fact, much of the controversy between dispersal and vicariance advocates in relation to the origin of the Antillean freshwater fish fauna revolves around the fact that poeciliids are a group that ecologically lies between terrestrial and purely freshwater species so that both models can explain the biogeographic distribution observed in this group (Rivas, 1958; Rosen & Bailey, 1963; Hrbek et al., 2007; Palacios et al., 2016; Weaver et al., 2016; Reznick et al., 2017).

Even though the general prediction from theory that relates species richness to island size is valid for livebearing fishes in the West Indies, where larger islands have higher number of species mainly due to *in situ* speciation (Furness, Reznick & Avise, 2016), the origin of the different the lineages and the species composition among genera show a very interesting pattern in the Greater Antilles (Figure 3). The oldest radiation event documented for poeciliids in the West Indies was related with the ancestors of today's genus *Girardinus* and its sister genus *Quintana*, both endemic to Cuba (Rivas, 1958; Hrbek et al., 2007; Doadrio et al., 2009; Reznick et al., 2017). *Girardinus* shows a higher diversity in western Cuba and it has eight described species (Lucinda, 2003): *G. metallicus* Poey, 1854; *G. uninotatus* Poey, 1860; *G. creolus* Garman, 1895; *G. denticulatus* Garman, 1895; *G. cubensis* (Eigenmann, 1903); *G. falcatus* (Eigenmann, 1903); *G. microdactylus* Rivas, 1944 and *G. rivasi Barus and Wohlgemuth, 1994. Quintana is a monotypic genus represented by the species Quintana atrizona Hubbs, 1834, which is only distributed in southwestern Cuba including Isla de la Juventud.* Rivas (1958) proposed that the ancestor of *Girardinus* colonized Cuba from Yucatan during upper Miocene-Pliocene via a land bridge that connected western Cuba to the Yucatan peninsula. Rosen & Bailey (1963) also supported a Yucatan-Cuba land connection and considered *Quintana* and the genus *Carlhubbsia* from eastern Yucatan sister genera of *Girardinus*. Recent studies have questioned the Yucatan-Cuba land connection and other alternative models (i.e GAARlandia model) have been proposed to explain the origin this lineage and poeciliids in general in the Greater Antilles. Molecular evidences suggest that common ancestors of these taxa dispersed from South America approximately 45-40 million years ago (Hrbek et al., 2007; Reznick et al., 2017).

Another dispersal event that is relevant to the origin of the Caribbean ichthyofauna occurred also from South America about 22-16 million years ago and originated the radiation of the genus *Limia* (genus *Poecilia* subgenus *Limia* sensu Reznick et al. (2017)) (Palacios et al., 2016; Weaver et al., 2016; Reznick et al., 2017). Palacios et al. (2016) also showed that three endemic *Poecilia* species to Hispaniola: *P. dominicensis, P. elegans* and *P. hispaniolae*, were closely related and basal to the *Limia* clade which might suggest that this triad of species are the result of a second colonization event from South America (Reznick et al., 2017). Certainly, the speciation process in the genus *Limia* has resulted in the largest radiation of the family Poeciliidae in the West Indies. So far 22 species of this genus have been described from Cuba, Hispaniola, Jamaica and Grand Cayman. The center of radiation of *Limia* is located on Hispaniola with 19 species while only one endemic species from Cuba, Jamaica and Grand Cayman each are currently known (Burgess & Franz, 1989; Hamilton, 2001; Weaver et al., 2016; Rodriguez-Silva & Weaver 2020; Rodriguez-Silva, Torres-Pineda, & Josaphat, 2020). This divergent species composition, mainly in the cases of Cuba and Hispaniola, is not predicted by the theory of island biogeography as the number of available ecological niches and island size is likely the same in both islands. A brief analysis of probable causes of the deviation from the species-area relationship in this genus and in livebearing fishes in general is provided in the next section of this paper.

Finally, the most recent dispersal event that is known for poeciliids of the West Indies occurred between 1-11 million years ago and involved the genus *Gambusia* (Reznick et al., 2017). Both classic revisions of the genus (Rivas, 1963; Rauchenberger, 1989) and recent studies suggest that the ancestor of *Gambusia* arrived in the Caribbean islands from Central America likely via overwater dispersal (Lydeard, Wooten & Meyer, 1995; Hrbek et al., 2007; Palacios et al., 2016; Reznick et al., 2017).

The debate over the complex origin of the livebearing fishes in the West Indies as well as its implication in the disjunctive distribution patterns observed in the area has been broadly discussed for a long time (Darlington, 1938; Myers, 1938; Rivas, 1958; Rosen & Bailey, 1963). Yet today, several questions still exist on how members of the family Poeciliidae have radiated, colonizing almost every available fresh and brackish water environment in the Caribbean islands.

Species-area relationship (SAR): the trends of terrestrial versus aquatic groups in the Antilles

The trend of larger islands to containing more species and smaller islands containing fewer species emerges as a rule in general terms in the Caribbean islands (Darlington, 1957; Losos, 1996; Losos & Schluter, 2000; Ricklefs & Bermingham, 2004), and this has been a core principle of the classical island biogeography theory in general (MacArthur & Wilson, 1967). While a one-dimensional interpretation of the SAR essentially focused on the correlation between island area and species richness has been widely adopted, it has been shown that ignoring functional differences among species may be a simplistic approach as it considers all species ecologically similar (Hubbell, 2001). Thus, the niche theory, which focuses mostly in the importance environmental heterogeneity and niche partitioning as key drivers of species richness, has emerged as another major hypothesis for our understanding of the SAR (Hortal et al., 2009). Since elements of the two theories likely act together, an integrated view of both has been lately proposed to explain diversity patterns observed on islands (Kadmon & Allouche, 2007; Lomolino & Brown, 2009; Franzén, Schweiger & Betzholtz, 2012).

To this day, integrative approaches addressing the influence of the two SAR components on species richness in the Antilles are limited, and most of them have been concerning terrestrial organisms (Losos, 1996, 2009; Rodríguez-Durán & Kunz, 2001; Weerd, Robinson & Rosenberg, 2016). On the other hand, studies of aquatic groups (mainly freshwater species) are very rare in the literature. These two factors, the scarce number of studies that offer an integrated perspective of the SAR in the insular Caribbean, as well as the unbalanced taxonomic treatment for the analysis of terrestrial groups over aquatic species, has led to generalizations about the SAR without deeper examinations of how island area together with environmental heterogeneity and niche partitioning may affect species richness in terrestrial (excluding some studies in Caribbean anoles e.g. Losos (2009)), and aquatic environments differently.

Probably, the most contrasting example about diverging speciation patterns relative to island size in aquatic versus terrestrial organisms in the Greater Antilles is the radiation process of livebearing fishes of the genus *Limia*. It has been very different from what is currently observed in anole lizards (Figure 4) in which larger islands display similar species richness (Hedges 1996a;

Losos, 2009). Hence, it is intriguing to ask which mechanisms have been influencing the speciation of *Limia* in such extreme different ways and what could explain this lopsided distribution.

Other contrasting trends between island size and species richness in terrestrial versus aquatic habitats have been observed in freshwater macroinvertebrates of the Lesser Antilles. In a study of the biodiversity of freshwater macroinvertebrates in 14 small Caribbean islands, Bass (2003) found that the patterns of species richness showed some divergence with those observed in the case of the Caribbean herpetofauna (Darlington, 1957; Hedges 1996a; Losos, 2009). According to the results of his study, Bass (2003) proposed that number and height of elevations in each island might also be considered an important factor influencing the number of macroinvertebrate species present because of the direct, positive relationship between elevation, rainfall and number of freshwater habitats. These findings show that the classical island biogeography theory alone may not completely explain the observed species richness patterns and also suggest that ecological opportunity could outweigh the importance of island size in speciation.

In our study we ran a similar comparison to determine the relationship between island size, the presence of elevations and species richness of livebearing fishes in the Greater Antilles. Our analysis reveals a similar pattern observed in aquatic invertebrates of the Lesser Antilles (Table 2) showing that not only the island size but also mountainous reliefs may be an important factors that determine the number of livebearing fishes species in the Greater Antilles

A Spearman correlation showed a positive, yet non - significant relationship between island size and the number of livebearing fish species (r_s= 0.80, n=4, p=0.20). However, when accounting for the effect of island area combined with elevation there was a positive, significant relationship for both island size x maximum elevation and island size x average elevation and the number of livebearing fish species in the Greater Antilles (r_s=1, n=4, p<0.001).

Human impact and threats to the biodiversity of the West Indies

The growth rate of the human population has exponentially increased worldwide becoming an evolutionary force of extraordinary pressure on global biodiversity (Palumbi, 2001). The level of stress and exploitation that human activities exert on natural ecosystems is so high that they can cause evolutionary changes sometimes within a few hundred years (Hendry & Kinnison, 1999; Reznick & Galambor, 2001). Factors such as global climate change (Omann, Stocker & Jager, 2009) and its direct effect on sea-level rise (Courchamp, Hoffmann, Russell, Leclerc & Bellard, 2014), habitat degradation and fragmentation (Brooks et al., 2002; Hanski, 2011) and exotic species (Sala et al., 1999; Groom, Meffe & Carroll, 2005; Tye, Key & Copsey, 2018) have been shown to lead to new selection pressures on biodiversity causing an increase in the risk of extinction of several groups. Although all these threats affect the biodiversity worldwide, there some intrinsic features of islands make them more fragile and vulnerable than the continental biota (Loope, Sanchez, Tarf, Loope, & Anderson,1988; Vitousek, 1998; Vermeij, 1991; Paulay, 1994). According to MacArthur & Wilson (1967), natural dynamics of colonization and extinction have occurred for million years on islands where an equilibrium is established once rates of colonization and extinction are equal. In the West Indies the existing biodiversity that has evolved surviving major, sometimes catastrophic, geological events is now facing an extinction rate without precedent due to anthropogenic activities mainly after the human occupation of the West Indies (Ricklefs & Bermingham, 2008).

For example, the percent annual rates of deforestation in the Caribbean are the highest among all biodiversity hotspots in the world, which threatens most endemic plant and animal species (Food and Agriculture Organization, 1997). Haiti, for instance, is one of the most deforested countries in the world with less than 1% of its original primary forest (Hedges, Cohen, Timyan & Yang, 2018). Certainly, the effect of habitat destruction has been a key factor in the extinction of Caribbean terrestrial vertebrates and particularly endemic and specialized mammals (i.e. cave-dwelling bats species) that are either threatened or already extinct (Woods, 1989; Morgan, 2001; Brooks et al., 2002). Habitat loss has been also reported as one of the major causes of species decline and extinction in amphibians (Hedges, 1993; Hedges et al., 2018), reptiles (Hedges et al.,

2018) and birds (Devenish-Nelson, Weidemann, Townsend & Nelson, 2019).

Human-mediated species introduction, intentionally or accidentally, is also the foremost cause of biodiversity loss in the West Indian archipelagoes (Ricklefs & Bermingham, 2008). Frequently these exotic species share ecological niches with the native ones causing negative effects on the biodiversity. Although not always well documented in all groups, the impacts of introduced species include predation of endemic species, disease transmission and competition for resources (Courchamp, Chapuis & Pascal, 2003; Kelly, Paterson, Townsend, Poulin & Tompkins, 2009; Rasambainarivo & Goodman, 2019). Even though introduced species represent a threat to the biodiversity worldwide, recent studies have shown that the magnitude of the contemporary effects of species translocations on island biogeography is determined by the economic isolation of human populations (Helmus, Mahler, & Losos, 2014; Furness et al., 2016).

There are countless examples of introduced species in the archipelagoes of the West Indies. Nevertheless, empirical approaches that assess distribution ranges and the damaging effects of exotic species are not very abundant. One of these is the case of the mongoose (*Herpestes javanicus*), which is widespread in most Caribbean islands and is known to drastically affect native species of birds (Horst, Hoagland & Kilpatrick, 2001). Another example is the introduction of the common green iguana (*Iguana iguana*) in the Lesser Antilles that has displaced other native iguana species through competition and hybridization (Vuillaume, Valette, Lepais, Grandjean & Breuil, 2015). Freshwater environments in the West Indies are also under pressure of introduced species as several alien fishes are being introduced as source of human protein (Ponce de León, Rodríguez & Núñez, 2013; Rodríguez-Barreras & Zapata-Arroyo, 2019), as biological control of mosquito larvae (Deacon, Ramnarine & Magurran, 2011) and also as result of the pet trade (Bunkley-Williams et al., 1994; Rodríguez-Barreras, Zapata-Arroyo, Falcón & Olmeda, 2020). A coordinated conservation effort in the Caribbean region is a challenging task because of the lack of financial resources and the presence of many islands administrated by independent governments with their own conservation strategies and priorities (Ricklefs &

Bermingham, 2008), as well as language barriers. However, the conservation of the biota in the West Indies requires immediate action in view of the increasing threats to biodiversity. Collaborative work that involves both local and foreign researchers will definitely help in the design of urgent management plans to ameliorate the negative effects of human actions on native species and ecosystems.

Definitely, studies of biogeographic patterns of the Caribbean biota have been one of the most fertile topics in investigations of island biogeography. However, still limited, inconclusive information exist regarding the origins and determinants of posterior diversification for many groups in the West Indies. The characteristic archipelagoes that are found in this geographic region offer countless possibilities to develop empirical microevoloutionary studies to understand the origin and posterior diversification of the insular Caribbean biota.

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Author contributions

Rodet Rodriguez-Silva: conceptualization (equal); data curation (lead); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); writing—original draft (lead); writing-review & editing (equal). Ingo Schlupp: conceptualization (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); writing-review

& editing (equal).

Conflict of interest

The authors have declared that no competing interests exist.

Data accessibility statement

Data curated from the Web of Knowledge (Web of Science Core Collection database) that was analyzed in this study is available in the Dryad Digital Repository (available at https://doi:10.5061/dryad.0k6djh9zv)

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TABLES

Table 1: Main biogeographic models that explain the origin of the biota of the West Indies. Some lines of evidence and studies that support each model are also included.

Biogeographic Model	**Explanation**	**Study**	**Supporting evidence**
Vicariance	Mechanism based on the plate-tectonics theory. It states the presence of a direct land connection between the Proto-Antilles and continental North and South America.	(Rosen, 1975) (Page & Lydeard, 1994) (Crother & Guyer, 1996)	Freshwater fishes (i.e. *Ophisternon*, *Gambusia*). Also some genera of amphibians and reptiles which argues Hedges et al.'s (1992) study (i.e., *Eleutherodactylus, Anolis*, *Spherodactylus*)
Overwater dispersal	Transoceanic dispersal following the Caribbean current, which has been observed by means of natural rafts. The current moves northwestward through the Caribbean Sea from the equatorial Atlantic Ocean via the North Equatorial, North Brazil, and Guiana currents.	(Schuchert, 1935) (Hedges et al., 1992) (Hedges, 1996c) (de Queiroz, 2005) (Heinicke et al. 2007) (Palacios, Voelker, Rodriguez, Mateos & Tobler, 2016) (Reznick et al. 2017)	Several groups of Caribbean vertebrates (i.e. small mammals, *Anolis* lizards, *Eleutherodactylus* frogs, *Limia* and *Gambusia* fishes
GAARlandia	Vicariance hypothesis that explains the colonization of the Caribbean by means of a land bridge connection that supposedly connected the Greater Antilles with South America.	(Iturralde-Vinent & MacPhee, 1999) (Heinicke et al. 2009) (Crews & Gillespie, 2010) (Rican, Pialek, Zardoya, Doadrio &	Tested in individual lineages of different groups (i.e. freshwater fishes of Poeciliidae and Cichlidae, butterflies of Nymphalidae, spiders of Araneae)

		Zrzavy, 2013) (Matos-Maraví et al. 2014) (Weaver, Cruz, Johnson, Dupin & Weaver, 2016)	*Eleutherodactylus* frogs

Table 2: Livebearing fish species richness in the Greater Antilles in relation to island size, maximum elevation and average elevation.

Island	**Number of species**	**Area (km$^{2)}$**	**Maximum elevation (m)**	**Area x maximum elevation**	**Average elevation (m)**	**Area x average elevation**
Cuba	16	109,884	1,974	216,911,016	137	15,054,108
Hispaniola	24	76,192	3,175	241,909,600	424	35,305,408
Jamaica	4	11,420	2,256	25,763,520	340	3,882,800
Puerto Rico	0	9,104	1,338	12,181,152	261	2,376,144

FIGURES AND CAPTIONS

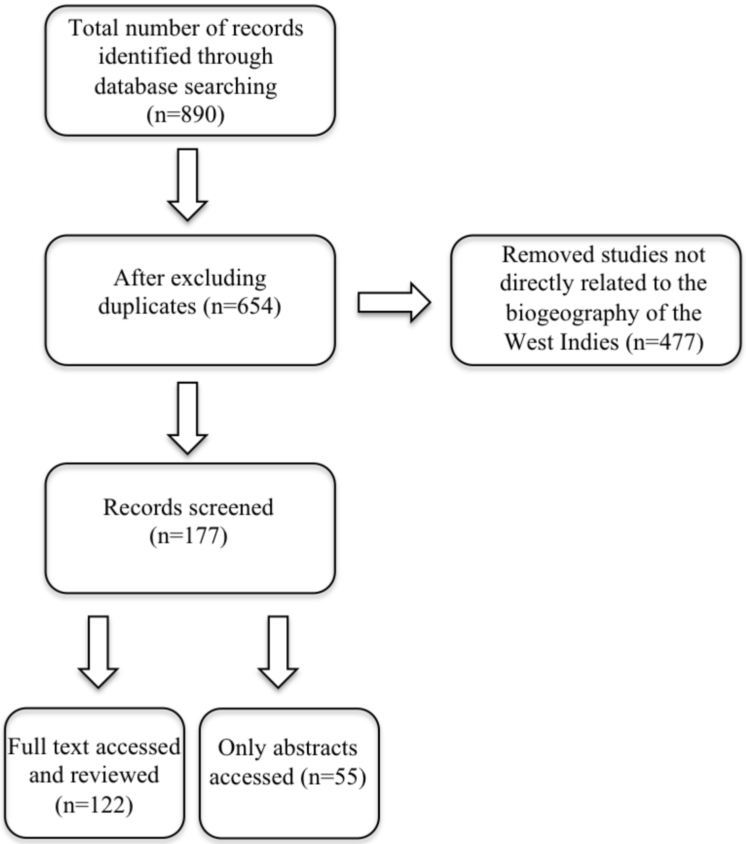

Figure 1: Flow chart of search results and selection process following Dougherty & Shuker (2015). Summary table of studies of the biogeography of the West Indies is available in the Dryad Digital Repository (available at https://doi:10.5061/dryad.0k6djh9zv).

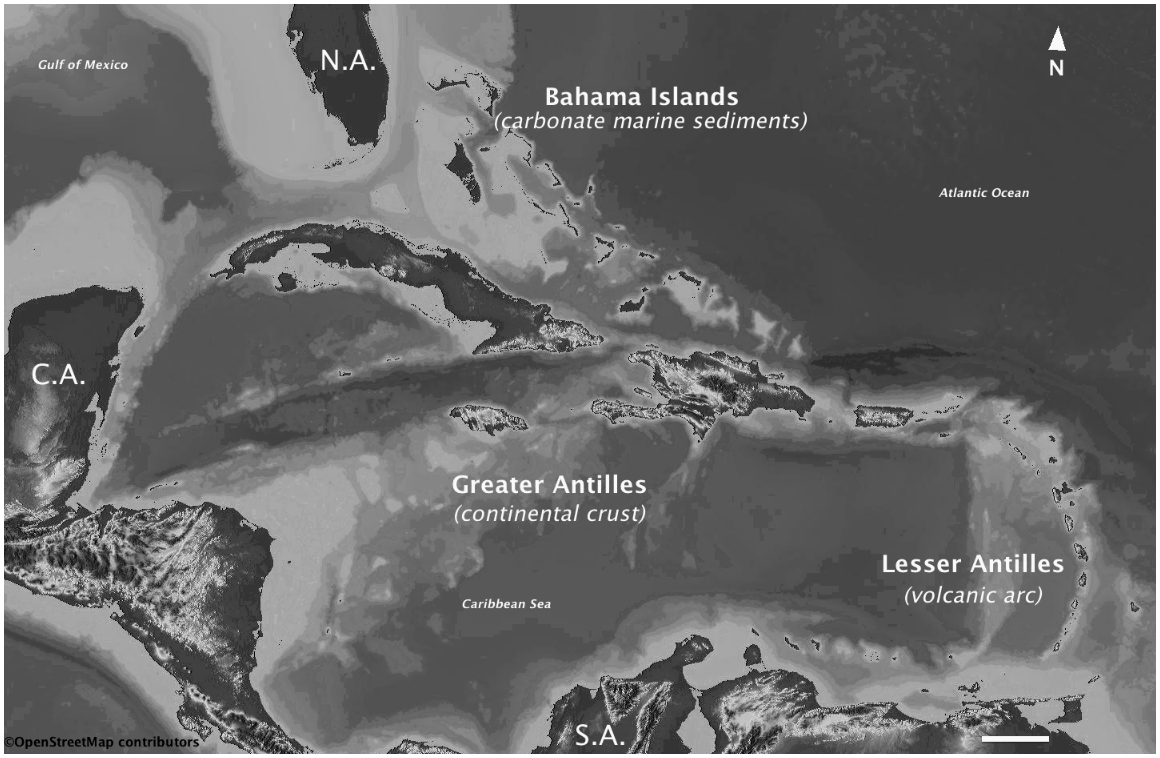

Figure 2: Map of the Caribbean basin showing the three main archipelagoes with corresponding geologic origins that form the West Indies. N.A: North America, C.A: Continental Central America, S.A.: South America (scale bar at the bottom represents 200 kilometers).

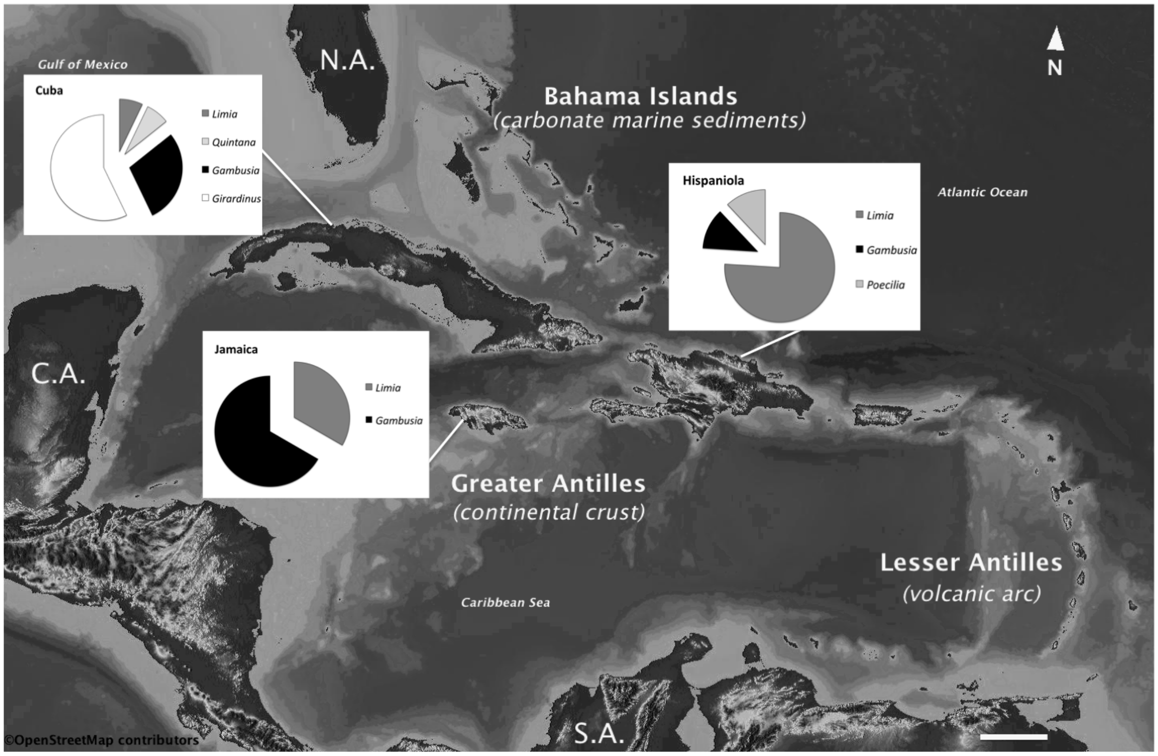

Figure 3: Map of the West Indies showing the number of endemic species by genera in the Greater Antilles. Hispaniola shows the largest radiation since a single lineage (*Limia*) has radiated in 19 known species. Puerto Rico has a very limited freshwater faun with no endemic species of poeciliids (scale bar at the bottom represents 200 kilometers).

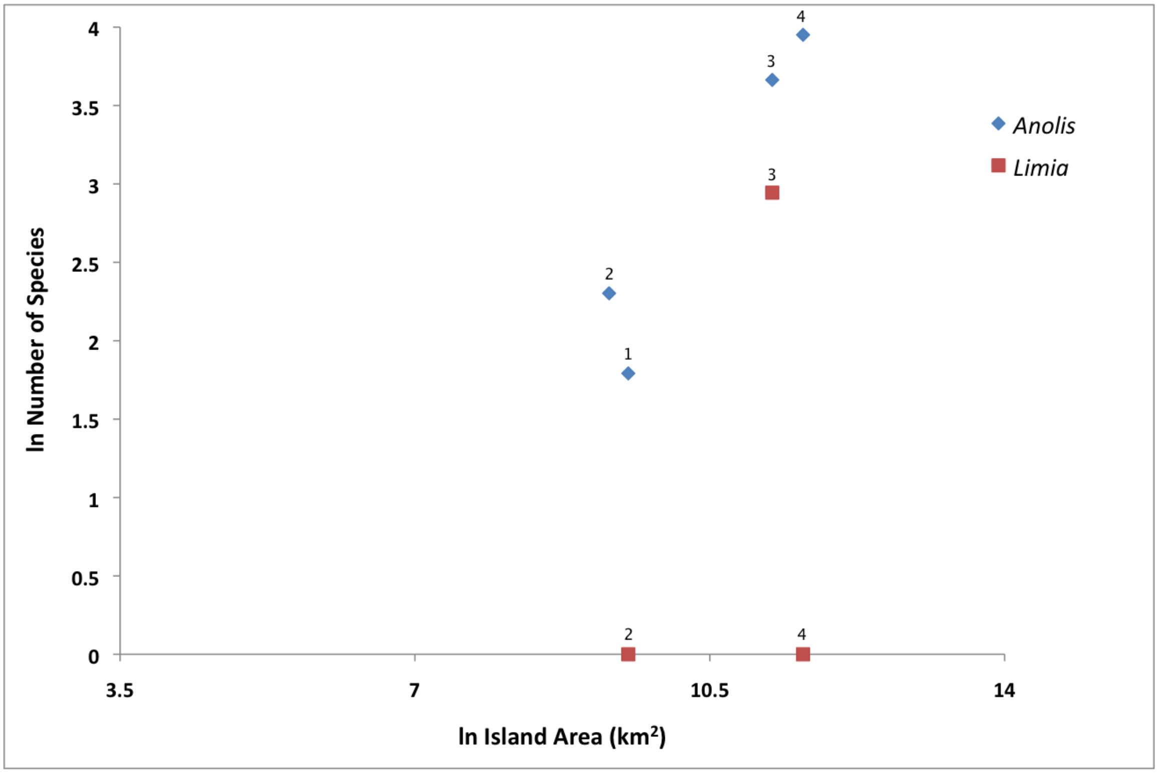

Figure 4. Diversification of *Anolis* lizards and livebearing fishes of the genus *Limia* in the Greater Antilles. The ln of the number of species of *Anolis* is strongly correlated with ln island area (r = 0.96), but not the ln of the number of species of *Limia* with ln island area (r = 0.35). 1: Puerto Rico, 2: Jamaica, 3: Hispaniola, 4: Cuba. Data from Algar & Mahler (2016) and Furness et al. (2016).

CHAPTER 2: INFLUENCE OF ELEVATIONAL GRADIENTS AT LOCAL SCALE IN POPULATIONS OF LIVEBEARING FISHES OF THE GENUS *LIMIA* FROM THE GREATER ANTILLES

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Keywords:

Caribbean, elevation, species distribution, temperature

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INTRODUCTION

Species distributions in natural systems are strongly modulated by climate, which ultimately affects both the ecology and physiology of organisms. This is particularly evident in ectothermic animals. Janzen (1967) published one of the most prominent papers in ecology that connected climatic variation across latitude and elevation, physiological adaptation and species distribution in a synthetic theory commonly referred as "Janzen's hypothesis" (Ghalambor *et al.*, 2006; Muñoz & Bodensteiner, 2019). One of the main predictions of this hypothesis is that due to the decrease in mean annual temperature with elevation, the seasonal temperature overlap is lower in the tropics than in temperate regions. Hence, mountain passes in the tropics may represent more effective physiological barriers to dispersal than the topographical component of change in altitude (Ghalambor *et al.*, 2006). Therefore, the low overlap in temperature regimes between low and high elevations in the tropics should select for organisms with relatively narrow thermal tolerances. Janzen's hypothesis also predicts that species develop physiological adaptations mirroring the range of ecological variation present in their surrounding area with populations living in high altitude evolving narrow tolerance for colder temperatures while low altitude populations developing narrow tolerance for warmer temperatures. Janzen's hypothesis has been widely adopted and some studies have provided at least partial evidence at both local and global scale supporting his predictions and assumptions in both terrestrial and aquatic ectothermic organisms. For example, Pintanel *et al.* (2019) found that frog species occurring in open habitats, such as in valleys and lowland environments in general, had higher tolerance to high temperatures (CTmax) than species restricted to forest habitats, showing small climatic overlap across an elevation gradient. Moreover, Polato *et al.* (2018) provided strong evidence in support of Janzen's hypothesis showing that tropical stream insects had noticeably narrower thermal tolerances and a lower dispersal ability than temperate species, which result in higher tropical speciation rates. However, despite of general support of the theory, several components have never been thoroughly tested and critically evaluated across multiple taxa, potentially questioning the generality of Janzen's theory (Ghalambor *et al.*, 2006). In addition, under Janzen's hypothesis is unclear whether the predictions refer to individual thermal niches or species thermal niches, which in fact are determined by different factors (Hua, 2016). In fact, some studies have not found support for Janzen's theory: in amphibians (Valdivieso & Tamsitt, 1974) and in *Anolis* lizards from Hispaniola (Muñoz & Bodensteiner, 2019) factors such as daily variation in temperature and behavioral mechanisms might cause deviations from Janzen's predictions. Furthermore, Navas *et al.* (2013) demonstrated that the effect of different microclimates within a specific biome is more relevant for species distributions than just the elevation at which certain species of amphibians may be found. McCain (2009) provided additional evidence for the effects of thermoregulation, daily temperature variability, and other climate variables such as precipitation as potential variables that could explain distribution ranges across multiple groups of vertebrates; including mammals, birds, reptiles and amphibians. In other words, Janzen's theory may have to be amended by including more complexity.

Several key features related to the geographic distribution of *Limia* make these fishes an excellent system to explore how temperature fluctuations associated to elevational gradients might be linked to dispersal. *Limia* fishes are one of the most dominant groups in freshwater ecosystems in the Caribbean with at least 19 endemic species on Hispaniola and one endemic species each occurring in Cuba, Jamaica, and Grand Cayman (Burgess & Franz, 1989; Rodriguez, 1997; Hamilton, 2001; Rodriguez-Silva *et al*., 2020). These freshwater fishes occur in a wide distribution range occupying diverse aquatic habitats on these islands (Weaver *et al.*, 2016 a). Although the altitudinal distribution of freshwater fish species in general is known to be considerably more constrained than in terrestrial species by several factors including for example productivity, physicochemical characteristics of the water and others (Jaramillo-Villa *et al*., 2010; Graham *et al*., 2014; Carvajal-Quintero *et al*., 2015), differences in altitudinal distribution in species of the genus *Limia* can be observed in natural habitats. In the present study, we tested some predictions of the Janzen's hypothesis at the local scale through the analysis of the individual thermal niche breadth in several populations of livebearing fishes of the genus *Limia* and its relationship with their altitudinal distribution in some islands of the Greater Antilles in the Caribbean.

According to theory, we hypothesize that populations of species distributed in lowland habitats have evolved to resist higher extreme temperatures, which may be a factor limiting their dispersal into higher elevations. Conversely, populations occurring at higher elevations in mountain streams should have evolved to cope with lower temperatures, which reduce dispersal abilities into warmer habitats. Specifically, we predict that low elevation populations will be more tolerant to higher temperatures than mid and high elevation populations showing higher critical thermal maximum (CTmax) and critical thermal minimum (CTmin). In contrast, high elevation populations are expected to be more tolerant to lower temperatures showing lower CTmax and CTmin values. We also predict the thermal breadth (the range of temperatures they can tolerate) to be smaller for higher altitude fishes as result of little variability in CTmax.

MATERIALS AND METHODS

The care and use of experimental animals complied with the University of Oklahoma animal welfare laws, guidelines and policies as approved by Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare under the assurance number A3240-01. Experiments were performed under the approved IACUC protocol R17-011 and specimens were collected in the field as part of surveys of the native livebearing fishes of the Greater Antilles (protocol R18-005). No fishes were euthanized nor surgical procedures were performed.

Study area and species: Even though thermal regimes of Greater Antillean streams are relatively stable, geological differences among islands lead to some climate heterogeneity that can generate environmental barriers. Hispaniola, for example, has several mountain ranges of more than 2000 meters in altitude. For instance, Pico Duarte in the Dominican Republic reaches 3098 meters and is the highest peak in the Caribbean. Previous studies have shown that high elevation specialists (mainly amphibians and reptiles) have evolved on Hispaniola as a consequence of this climate heterogeneity (Wollenberg *et al*., 2013; Muñoz *et al*., 2014). Mountains reaching around 2000 meters can be also found in eastern Cuba (Pico Turquino) and Jamaica (Blue Mountains) with significant levels of biodiversity associated.

In this study we analyzed populations of some species such as *L. perugiae*, *L. vittata*, *L. yaguajali* and *L. sulphurophila* that were reported to live in low elevation, warm environments including saline coastal lagoons. We also included in the analysis other species such as *L. zonata* and *L. melanogaster* that were obtained from low to intermediate elevations and often associated to relatively cool springs. Finally, one population of *L. dominicensis* and another of *L. versicolor* collected in mountain streams at relatively high elevations were analyzed too (Table 1). Specifically, we compared thermal breadth for the eight populations of the *Limia* species abovementioned that naturally occur in Cuba, Hispaniola, and Jamaica (Fig. 1). For a more fine-grained picture, we analyzed three additional populations of *L. perugiae*, the most widely distributed species of *Limia* on Hispaniola in order to test for local adaptation to environmental variation in temperature. All populations analyzed but *L. vittata* came from wild caught stocks that were transported into the United States and then kept in common garden conditions at the University of Oklahoma for variable periods of time (Table 1). *L. vittata* specimens were obtained from aquarium stocks and have been kept in common garden conditions at a greenhouse in the Aquatic Research Facility at the University of Oklahoma for more than 10 years.

Laboratory methods: We used the critical thermal method (Cowles & Bogert, 1944) to describe variation in temperature tolerance in 113 adult fish of eight *Limia* species representing a total of 11 different populations (Fig. 1). Prior to testing all fishes were acclimated to laboratory conditions with temperatures ranging between 25^0C-27 ^{0}C for 45 days. This was the most common temperature range for the species at their origins. We tested 10 reproductively mature adult fish of both sexes per population.

Fishes were individually tested under a constant increase (to determine critical thermal maximum, CTmax) or decrease (to determine critical thermal minimum, CTmin) in temperature until reaching an appropriate endpoint. The endpoint we used to determine CTmax was the pre-death thermal point at which signals of sudden onset of muscular spasms appeared (Lutterschmidt & Hutchison, 1997; Beitinger *et al*., 2000). In the case of the CTmin the endpoint used was the absence of motion of the pectoral fins in which the fish did not start to move again even when the experimenter disturbed the fish (Fischer & Schlupp, 2009). Using this data, temperature tolerance was calculated as the arithmetic means of high and low temperatures (CTmax or CTmin) at which the endpoint was reached by individuals in the sample (Lowe & Vance, 1955). We also calculated the thermal breadth as CTmax minus CTmin for each individual. The three measures, although connected, reflect different physiological properties of the species.

Before the actual experiment fishes were not fed for 24 hours. Each fish was individually tested in a spherical 2-liter glass container. After a 10-minutes acclimation period before each trial, the container was heated using a concave heating plate at a constant rate of 1^0C/min while temperature was constantly monitored with a thermometer in order to record CTmax. Each trial was immediately stopped, and the final temperature was measured once the fish showed symptoms of sudden muscular spasms, which were characterized by disorganized and high frequency muscular movements. All fish were weighed after each trial, placed in individual tanks and allowed to rest for at least 72 hours until application of the other extreme temperature to the same individual. Temperature exposure (CTmax or CTmin) that a fish experienced first was randomized to avoid an order effect. This was also evaluated statistically.

A similar procedure was followed to test the fish's tolerance to cold temperatures or CTmin. In this case, we placed the fish in a similar 2-liter glass container and after a 10-minute acclimation period, we continuously added cold water of 3^0C–4^0C to the system for a rate of temperature change of 1^0C/min. CTmin was measured at the point in which fish showed total absence of movement. No mortality was associated with the trials and after the experiments all fishes were returned to their respective stock tanks.

Climate index overlap: Monthly maximum and minimum temperatures for the 2010-2019 period were extracted from WorldClim database for the following localities included in table 1: La Zurza, Cabral, Arroyo del Agua, Roaring River, Rio Yuna, Rio Basima and Puerto Escondido. We calculated the average monthly temperature for each site during the last 10 years in order to measure pairwise thermal overlap between each focal site and the other localities using Janzen's (1967) equation:

$$\text{Overlap value} = \sum_{i=1}^{12} \frac{d_i}{\sqrt{} \; R_{1i}R_{2i}}$$

where d_i is the thermal overlap between the focal site and each other site for the ith month or the amount (in Celsius degrees) of one thermal regime that is included within the other, R_{1i} is the difference between the monthly mean maximum and minimum for the focal site and R_{2i} is the corresponding value for each other site of the study. As temperature overlap increases the overlap value increases up to a value of 12 which is the point where thermal regimes between two sites share identical monthly maximum and minimum temperatures throughout the year.

Phylogenetic signal: A common caveat of studies like this is that any pattern found might not necessarily reflect adaptations but be due to species relatedness. To test for this we used a recent phylogeny (Weaver *et al*., 2016 b) and conducted a test of phylogenetic signal to assess whether correlations in temperature tolerance among species may be due to their shared evolutionary history or to other factors (Gingras *et al*., 2013; Kamilar & Cooper, 2013; Gilbert *et al*., 2018;

Arnaudo *et al*., 2019). For this analysis we used Pagel's lambda (λ) (Pagel, 1994) as a quantitative measure of this relationship. The Pagel's λ has been shown to be a very robust indicator of a correlation between ecological and evolutionary processes even for incompletely resolved phylogenies (Molina-Venegas & Rodriguez, 2017; Leiva *et al*., 2019). We based this analysis on the phylogeny published by Weaver *et al.* (2016 b), which includes most of the species used in this study.

Data analysis: For data analysis of thermal tolerance, we used one-way ANOVA's, after confirmation of homogeneity of variances by Levene's tests and normality by Shapiro and Wilk's tests. We used three different ANOVA's to compare the mean CTmax, CTmin and temperature ranges (breadth) among eight different populations of *Limia*. Scheffe's post hoc tests were used to make comparisons between groups to distinguish populations that differed from others in extreme thermal tolerance or temperature ranges. The lack of order effect was statistically confirmed ($p>0.05$) using t-test analyses to compare CTmax and CTmin means of fish that were tested CTmax then CTmin versus fish that were tested CTmin then CTmax.

All statistical analyses were performed in SPSS 23. We performed independent ANOVA analyses because we considered CTmax and CTmin as ecologically and evolutionary independent variables with potentially different adaptive benefits. Similar approaches that consider the effects of these variables (and also acclimation temperature) as independent have been used in other studies examining thermal tolerances in ectothermic animals (Spotila, 1972; Layne & Claussen, 1982). To further explore a potential role for local adaptation in thermal tolerance within a widely distributed species, we also compared the same variables through separate ANOVA analyses in four populations of *L. perugiae*.

In order to calculate climate index overlap we used the R package raster (Hijmans, 2020) to extract minimum and maximum monthly temperatures values to our sampled locations. Finally, analysis of phylogenetic signal was computed using the R package (R Core Team, 2013) phytools

(Revell, 2012).

RESULTS

Analysis of phylogenetic signal: We performed analyses of phylogenetic signal using a previously-inferred phylogeny based on three mitochondrial (12S, ND2, Cytb) and two nuclear (MYH6, Rh) genes (Weaver *et al*., 2016 b) to determine whether more similar values in CTmin, CTmax and thermal range were associated with more closely related species more often than expected by chance. None of the analyses found a significant effect of phylogeny in explaining thermal breadth among the *Limia* species studied: CTmin (λ=6.257973e-05, p=1.000), CTmax (λ =0.4056982, p=0.78255460) and thermal range (λ=1.16242, p=0.362986) (Supplementary Information Fig. 1).

Analysis of climate index overlap: Overall, thermal overlap along altitudinal gradients among the study locations was relatively high but there was still some variation across pair of sites (Table 2). Climate index overlap decreased as the differences in elevations were more conspicuous. Particularly, Puerto Escondido (the highest collecting site included in the analysis) showed the lowest climate overlap with all other sites, which indicates that there are some differences in habitat temperatures.

Inter-specific analysis of thermal tolerance: The overall range of temperature tolerance for the eight species included in the analysis was 12^{0}C (CTmin) to 41.2^{0}C (CTmax), which may be considered as a broad range for tropical fishes when considering the overall climatic stability present in the tropics in terms of temperature fluctuations. A one-way ANOVA was conducted to compare the effect of varying distribution according to elevation on the temperature tolerance under CTmin and CTmax conditions. There were significant differences in thermal limits for both CTmin (One-way ANOVA, F (7, 72) = 41.977, p<0.001) and CTmax (One-way ANOVA, F (7, 72) = 14.878, p<0.001) among species after testing 80 individuals. The highest temperature tolerance was recorded for *L. sulphurophila* with an average CTmax of 40.9^{0}C. This species also showed the lowest tolerance to low temperatures with an average CTmin of 16.7^{0}C, which might suggest this species could be adapted to live in warmer habitats. A post hoc analysis also showed that *L. sulphurophila* differed significantly from all other species in both CTmin and CTmax (Scheffe, $p<0.05$) except for *L. zonata* in CTmin (Scheffe, $p=0.137$) (Fig. 2).

Another ANOVA was used to compare temperature ranges among populations. The analysis showed significant differences (One-way ANOVA, F (7, 72) = 15.993, $p<0.001$). *L. melanogaster* displayed the narrowest range of thermal tolerance, which differed from all other populations (Scheffe, $p<0.05$) but not from *L. sulphurophila* (Scheffe, $p=1.000$), *L. zonata* (Scheffe, $p=0.994$) and *L. versicolor* (Scheffe, $p=0.117$). Our data suggested that the most tolerant species to extreme temperatures were *L. perugiae*, *L. yaguajali*, and *L. vittata* (species distributed in low elevations) followed by *L. versicolor* and *L. dominicensis* (species which distribution extends into much higher elevations) since these two groups of species displayed broader ranges of temperature tolerance (Fig. 3) and their ranges did not differ significantly from each other (Scheffe, $p>0.05$).

Intra-specific analysis: Population analyses for *L. perugiae* showed significant differences in CTmax (One-way ANOVA, F (3, 36) = 6.118, $p=0.02$) and CTmin (One-way ANOVA, F (3, 36)= 20.982, $p<0.001$). The population from Lake Enriquillo differed from the other three in both CTmax (Scheffe, $p<0.05$) and CTmin (Scheffe, $p<0.001$) (Fig. 4). There were also significant differences in ranges of thermal tolerance among populations of *L.perugiae* (One-way ANOVA, F (3, 36) = 3.409, $p=0.028$). In this case the population from Lake Enriquillo had significant differences with the population from Azufrada (Scheffe, $p=0.037$). This is surprising as the Azufrada population is also from Lake Enriquillo, just the north shore, not the south shore.

DISCUSSION

Our evidence suggests that thermal tolerance and altitudinal distribution of the populations of *Limia* species analyzed in this study are not be related to temperature gradients expected in nature. The species studied here showed thermal tolerances not predicted by Janzen's hypothesis. Generally, even species from high altitudes (for the tropics) have broad thermal tolerances similar to species distributed in low elevations. Hence, the observed pattern does not separate the species as predicted.

Additionally, phylogeny did not explain species relationships according to thermal tolerance. However, failure to detect statistical significance may be due to the rather low number of species included in the analyses. Small sample sizes have been shown to influence the uncertainty and the expected values of most indices of phylogentic signal, including the Pagel's lambda (λ) (Munkemueller *et al*., 2012). Powerful phylogenetic comparative analyses typically demand trait and phylogenetic data for over 50 species and *Limia* only has 22 described species, which simply cannot satisfy the sampling requirements for robust macroevolutionary inferences.

Temperature tolerance ranges have been shown to shape species distributions and community compositions for some ectotherms in both tropical and temperate climates. Snyder and Weathers (1975) offered experimental evidence on the close relationship between the range of temperature tolerance and the environmental temperature variation in the distribution of several species of amphibians, showing that an increase in the environmental temperature variation also increases the range of temperature tolerance and consequently the distribution range of species. Estimating temperature tolerances and temperature ranges through the analysis of lower (CTmin) and upper (CTmax) thermal limits has been shown to be an efficient and useful method to assess species' capacity to acclimate to temperature changes in several ectotherms including terrestrial species (i.e. arthropods, reptiles and amphibians) and aquatic organisms (i.e. arthropods, mollusks and fish) (Van Berkum, 1988; Sunday *et al*., 2011; Buckley & Huey, 2016). However, when using this methodology, the results can be influenced by experimental protocols and conditions in which either CTmin or CTmax are measured. Factors such as acclimation temperature of individuals being tested, the cooling or heating rate used, and also the non-lethal endpoint chosen by the experimenter to determine CTmin or CTmax can influence the results (Lutterschmidt &

Hutchison, 1997). Fortunately, though, the method has been in use for a long time, which has allowed testing and standardizing protocols for different animal groups. Hence, the technique offers repeatable and rapid quantitative measure of the thermal limits as well as predicts optimal temperate ranges of multiple species (Fischer & Schlupp, 2009; Kingsolver & Umbanhowar, 2018). Although specimens used in this experiment were kept in common garden conditions for different lengths of time, which might influence their tolerance to critical thermal limits; we standardized the acclimation time to a specific temperature range (25^0C -27^0C) for 45 days under laboratory conditions. This acclimation time is considerably longer than others previously reported in studies of thermal limits in ectothermic organisms (Chanthy *et al*., 2012; Moyano *et al*., 2017; Tongnunui & Beamish, 2017), which ensures our data truly reflects the actual thermal tolerances of the species.

Janzen (1967) predicted that species occurring in high altitude in the tropics are specialized for lower temperatures than are low altitude species, which should be better adapted to cope with warmer temperatures. However, our results do not offer support for this prediction suggesting that factors other than temperature shape the distribution in populations of livebearing fishes of the genus *Limia*. In general, species and populations widespread in lowland habitats (*L. perugiae* populations from Hispaniola and *L. vittata* from Cuba) seem to be very tolerant to extreme temperatures (CTmax and CTmin) suggesting that they could also live in mountainous habitats. Given this, what could explain that none of these two species are found in high elevation streams either in Cuba or Hispaniola? We suggest that this is probably due to biotic factors such as competition. In the case of *L. vittata* in Cuba other dominant livebearing fishes (genera *Girardinus* and *Gambusia*) exploit available niches in mountain streams and on Hispaniola other *Limia* species and also species of *Poecilia* (*P. dominicensis*, *P. hispaniolana* and *P. elegans*) seem to be restricting *L. perugiae* to lowland environments. Evolutionary trade-offs between broad tolerance and competitive habitats have been shown to be common in different taxa. For instance, Robinson and Terborgh (1995) showed that interspecific aggression more that habitat suitability might explain spatial segregation patterns observed in Amazonian birds, and Griffis and Jaeger (1998) defined interspecific competition as cause of extinction of a species of salamander (*Plethodon shenandoah*) in the mountains of Shenandoah National Park, Virginia, USA.

Conversely, among species with distribution ranges that mostly include mid-elevations, there is a less consistent pattern in tolerance to extreme temperatures but with a general trend towards lower ranges of tolerance. Such are the cases of *L. melanogaster* from Jamaica and *L. zonata* from Hispaniola, which showed low tolerance ranges and were particularly sensitive to low temperatures. Our recent exploratory work in the Caribbean has recorded these two species associated with permanent freshwater springs and spring runs that buffer temperature fluctuations. The two species abovementioned seem to be physiologically adapted to relatively narrow fluctuations in temperature, which may explain their limited tolerance range.

Another result of our study that runs counter to Janzen's hypothesis is that two strictly mountainous and locally distributed populations of the species *L. versicolor* and *L. dominicensis*, exhibited relatively broad tolerance ranges similar to species that typically are widespread distributed in low elevation environments. Increasing altitude is always accompanied by a decrease in annual average temperature (Sarmiento, 1986), which may indicate high elevation organisms are better adapted to cope with low temperatures. This general pattern was also present in our analysis where the highest study site, in this case Puerto Escondido with one population of *Limia dominicensis*, exhibited the lowest climate overlap index with the rest of the other sites. However, in tropical ecosystems species living at high elevations might benefit from evolving broad thermal tolerance to deal with diurnal changes in temperature (Ghalambor *et al*., 2006), which in turn might explain why the two species also show a moderate tolerance for high temperatures. Temperature tolerances of high and mid elevation populations of *Limia* species relate to results of other studies in tropical amphibians (Navas, 1996; Navas *et al*., 2013), which showed that species occurring in intermediate elevations were likely stenothermic given the relative thermal stability of those habitats. Conversely, species from higher elevations seemed to have evolved to lead with more changeable temperature (differences between day and night temperatures) and consequently develop broader tolerance ranges.

Our results also provide insights into local physiological adaptations of thermal tolerance within species, which suggests that conspecific populations in diverse habitats have somewhat independent evolutionary pathways (Snyder & Weathers, 1975). First, *L. perugiae* from the south shore of Lake Enriquillo, near where *L. sulphurophila* is found differed from other three populations by showing a narrower thermal breadth. This result – together with the lack of phylogenetic signal in thermal tolerance for species – emphasizes the importance of biogeographical processes more than just phylogenetic patterns in analyses of climatic niche (Coelho *et al*., 2019). Second, *L. sulphurophila*, a locally distributed species mainly known from sulfur springs on the southeastern shore of Lake Enriquillo in the Dominican Republic (Rivas, 1980), seems to be locally adapted to live in a high temperature sulfidic environment and is also physiologically adapted to resist high temperatures. This may prevent competitive exclusion as shown in fish species from temperate climates (Ohlberger *et al*., 2008).

Our study has implications for conservation and is also pertinent in the context of climate change and species resilience to short-term temperature spikes. Our data provide evidence of species and populations that would be more vulnerable to temperature variation. In this case, the ones occurring in cool permanent freshwater springs in mid elevations and also *L. sulphurophila* (a local endemic species) seemed to be more susceptible to temperature fluctuations because of their narrower thermal breadth. Another issue that may affect conservation of native *Limia* species is the introduction of invasive livebearing fishes, such as *Poecilia reticulata* (Guppy). This species has recently been reported as one of the most tolerant ornamental fish to extreme temperatures (Yanar *et al*., 2019), which may be additional evidence of the invasive success of guppies and in some extend explain why this species becoming dominant in tropical ecosystems.

The implications of temperature for fish physiology and fitness (Niehaus *et al*., 2012; Payne *et* *al*., 2016) make the analysis of thermal limits particularly important in determining distribution of fishes (Culumber *et al*., 2012). Even though our study does not provide a comprehensive test for Janzen's hypothesis, it presents evidence at local scales to analyze how elevational gradients may affect the distribution of freshwater fishes, which is a barely studied zoological group in the Caribbean. While it does not offer evidence supporting Janzen's predictions about climatic variation across elevations, physiological adaptation and species distribution for this group of fish; the study emphasizes the importance of testing the validity of Janzen's mountain passes hypothesis across multiple taxa. In addition, like previous studies this work stresses the significance of other factors such as species interactions, diet specializations, and even thermoregulatory behavior (as shown by Muñoz and Bodensteiner (2019) in Caribbean anoles) when interpreting current altitudinal distribution patterns of species.

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TABLES

Table 1: List of species and populations of *Limia* included in this study with GPS coordinates and elevation in meters above mean sea level (m.a.m.s.l.) of original collecting sites. Number of individuals analyzed of each sex is also included in all cases.

Species	**Locality/GPS coordinates**	**Collection year**	**Sample size and sex**	**Elevation (m.a.m.s.l.)**
Limia perugiae	Lake Enriquillo 18° 24' 4.61" N 71° 34' 16.61" W	2014	7 females 3 males	0
Limia perugiae	Las Salinas (Bani) 18° 12' 43.488" N 70° 32' 27.095" W	2011	6 females 4 males	0
Limia perugiae	Azufrada 18° 33' 40.212" N 71° 41' 50.928" W	2003	5 females 5 males	0
Limia perugiae	La Zurza 18° 23' 56.08" N 71° 34' 13.22" W	2014	5 females 5 males	0
Limia vittata	Cuba (Unknown coordinates)	Unknown	5 females 5 males	Unknown
Limia sulphurophila	Cabral (Barahona Province) 18° 14' 45.902" N 71° 13' 23.944" W	2014	6 females 4 males	26
Limia yaguajali	Arroyo del Agua (Jamao al	2018	5 females	46

	Norte) 19° 37' 49.872" N 70° 26' 58.056" W		5 males	
Limia melanogaster	Roaring River 18° 17' 00.0" N 78° 03' 22.0" W	2017	9 females 3 males	71
Limia zonata	Rio Yuna (Bonao) 18° 57' 33.1" N 70° 24' 32.06" W	2014	6 females 4 males	158
Limia versicolor	Rio Basima 18° 42' 5.605" N 70° 11' 50.387" W	2018	5 females 5 males	181
Limia dominicensis	Puerto Escondido 18° 19' 6.93" N 71° 34' 14.24" W	2014	5 females 5 males	399

Table 2: Pairwise values of climate index overlap across the seven collecting sites showing different altitudinal gradients. Climate index overlap can take values up to a maximum of 12 where the higher values indicate more temperature overlap.

	La Zurza	Cabral (Barahona)	Jamao al Norte	Roaring River	Rio Yuna (Bonao)	Rio Basima	Puerto Escondido
La Zurza	-						
Cabral (Barahona)	11.47	-					
Jamao al Norte	10.63	11.15	-				
Roaring River	10.81	11.22	10.28	-			
Rio Yuna (Bonao)	10.29	11.39	11.59	9.96	-		
Rio Basima	10.05	10.55	11.74	9.65	11.84	-	
Puerto Escondido	8.93	9.38	10.17	8.45	10.57	10.79	-

FIGURES AND CAPTIONS

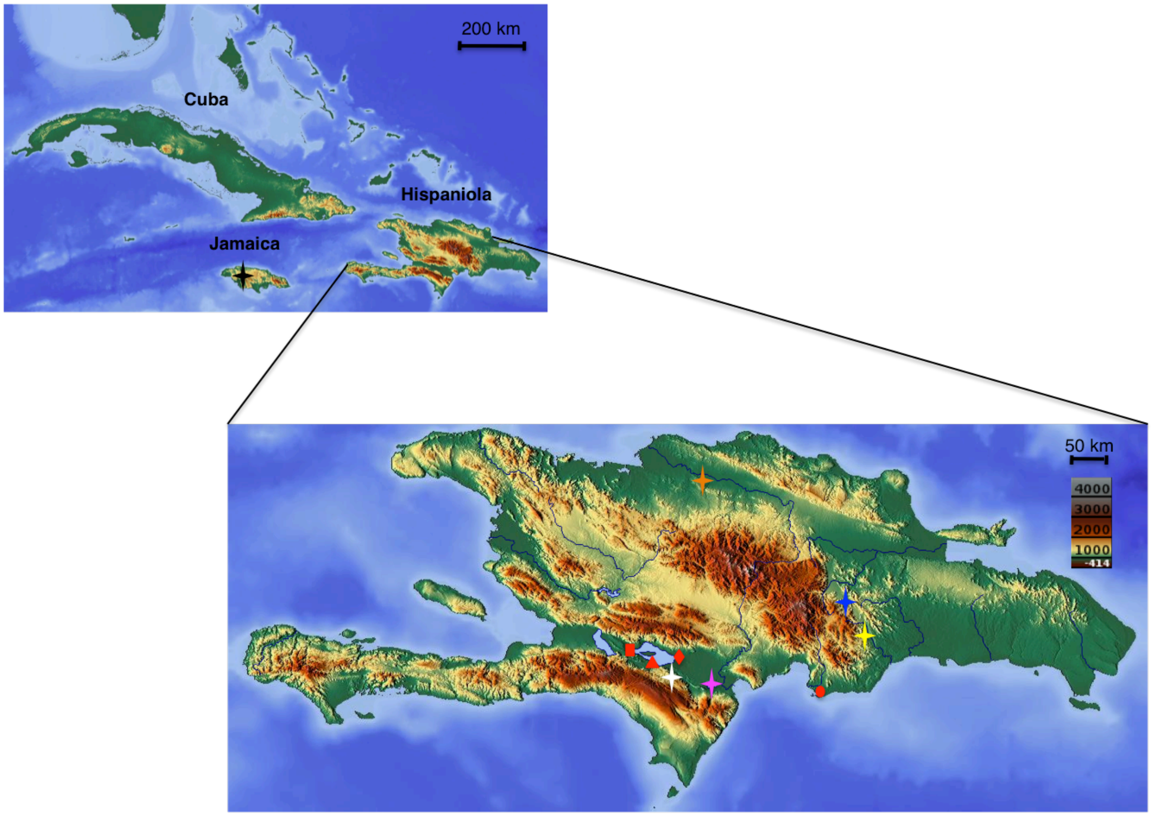

Fig.1. Geographic distribution of the populations of *Limia* (with known origins) analyzed in this study. Red rectangle: *L. perugiae* (Azufrada), Red triangule: *L. perugiae* (Lake Enriquillo), Red diamond: *L. perugiae* (La Zurza), Red circle: *L. perugiae* (Las Salinas), Orange star: *L. yaguajali*, Blue star: *L. zonata*, Black star: *L. melanogaster*, White star: *L. dominicensis*, Yellow star: *L. versicolor*, Pink star: *L. sulphurophila*.

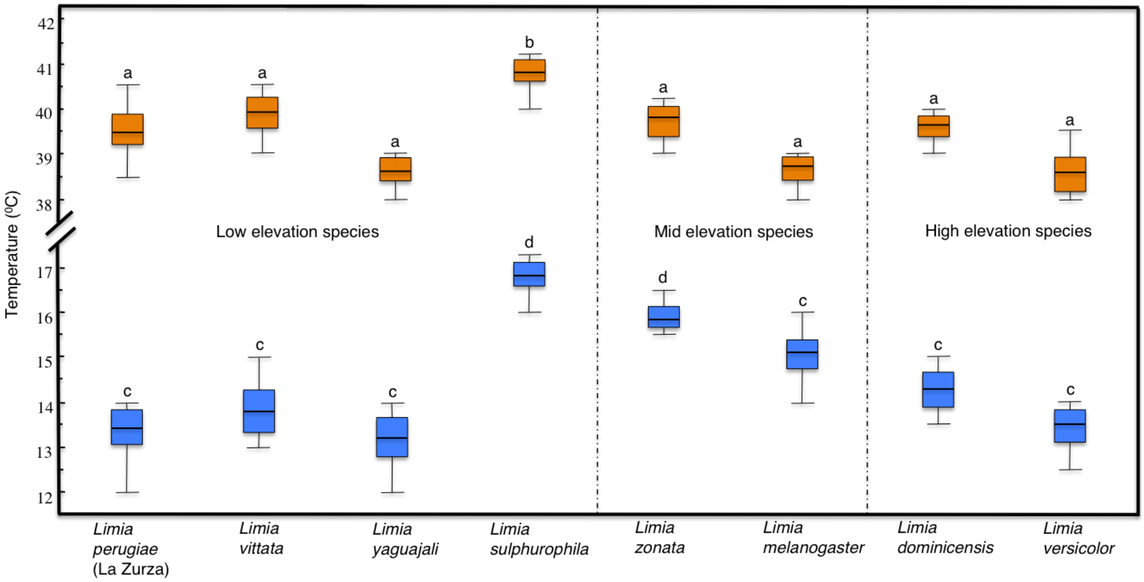

Fig. 2. Thermal tolerances of the eight species of *Limia* included in this study. Species are grouped according to their elevation distribution in three groups: low elevation species (left), mid elevation species (center) and high elevation species (right). Each box plot represents the median, interquartile ranges, maximum and minimum values of either CTmin or CTmax for each species. CTmin is shown by blue box plots and CTmax by orange box plots.

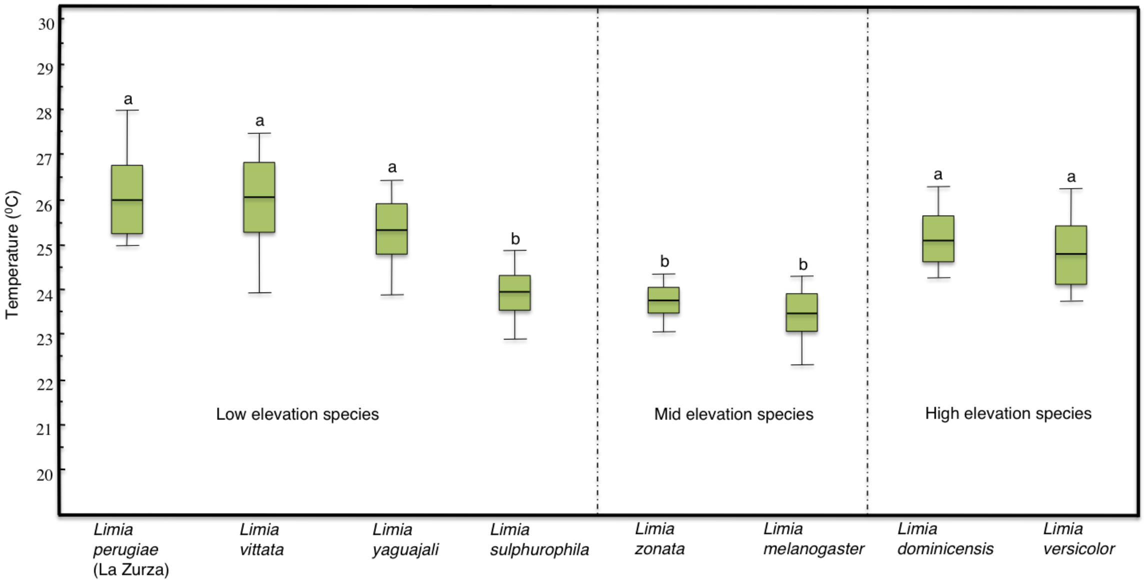

Fig. 3. Box plots of the thermal ranges of the eight species of *Limia* included in this study. Species are grouped according to their elevation distribution in three groups: low elevation species (left), mid elevation species (center) and high elevation species (right). Each box plot represents the median, interquartile ranges, maximum and minimum values of either CTmin or CTmax for each species.

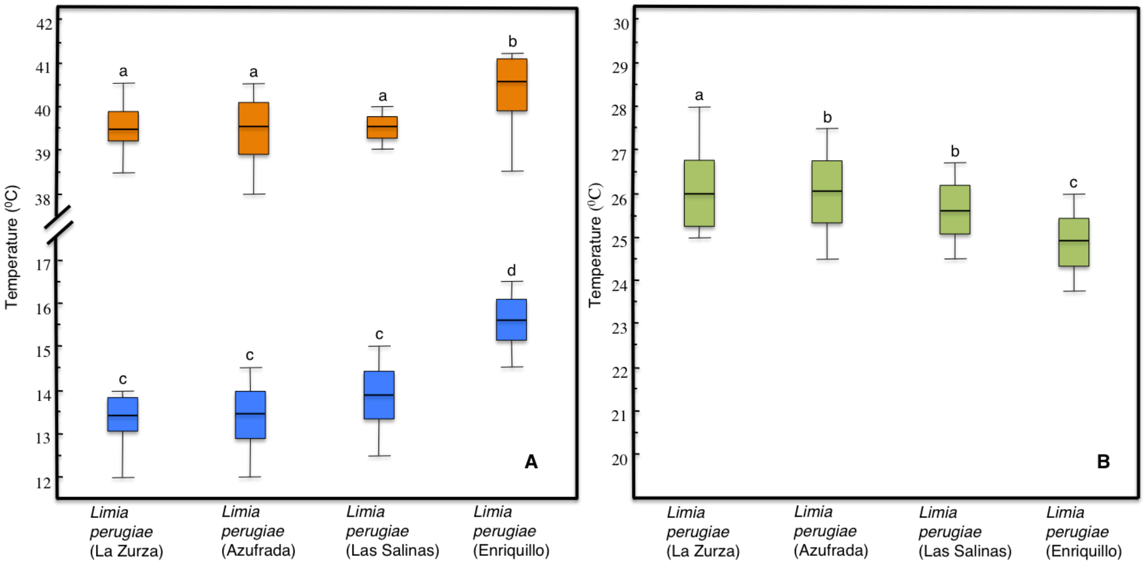

Fig. 4. Thermal tolerances of four populations of *L. perugiae* showing CTmin in blue box plots and CTmax in orange box plot (A), and box plots of thermal ranges of the four populations analyzed (B). Each box plot represents the median, interquartile ranges, maximum and minimum values.

SUPPLEMENTARY INFORMATION

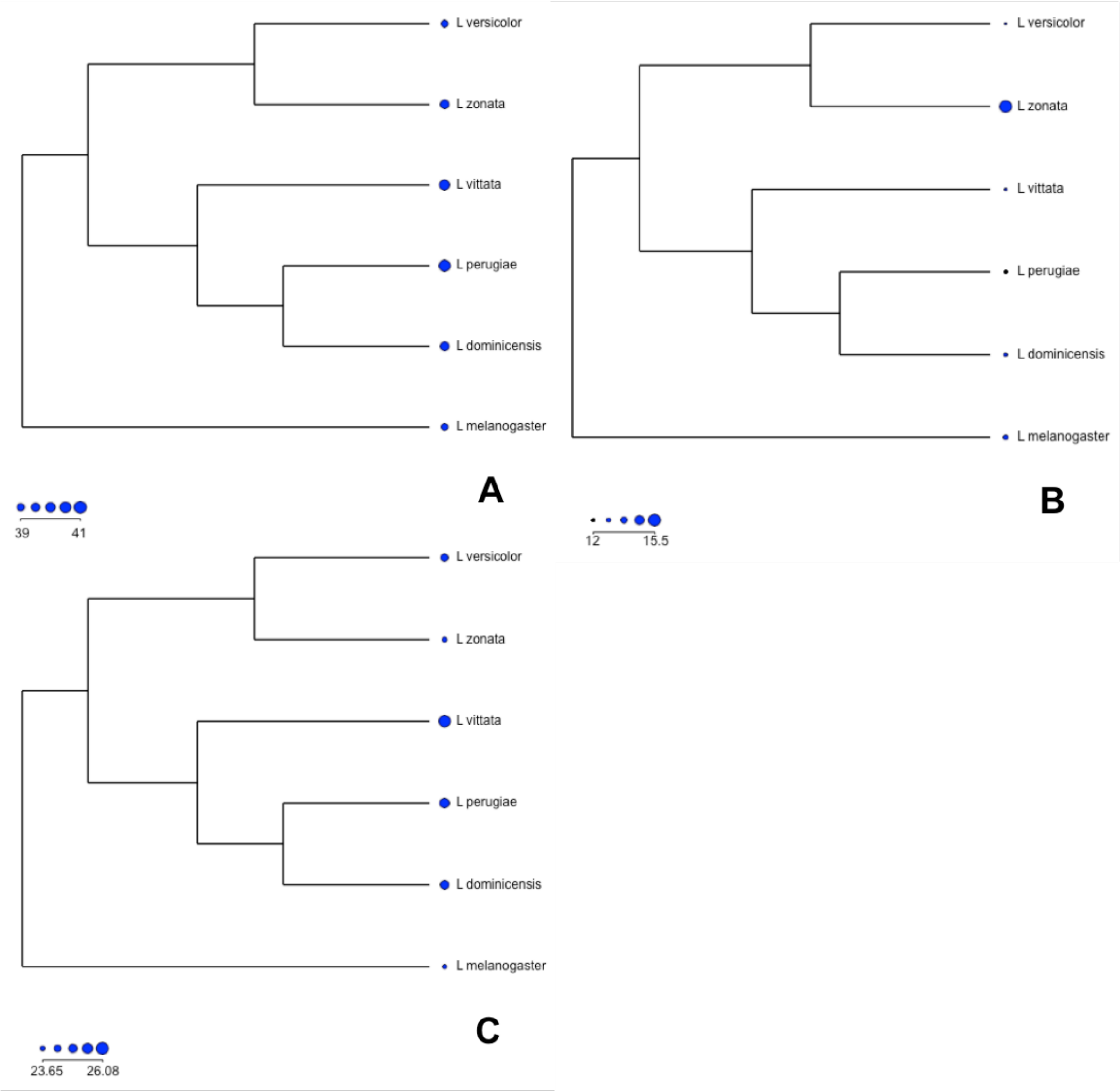

Figure 1. Analysis of phylogenetic signal using Pagel's lambda (λ). The phylogeny of species is based on mitochondrial (12S, ND2, Cytb) and nuclear (MYH6, Rh) gene sequences (Weaver *et al*., 2016 b). Circles at the tips of the phylogeny are sized in proportion to the average value of similarities in CTmax (A), CTmin (B) and thermal range (C) for each species. The legends in the figure also indicate the minimum and maximum values for each variable.

CHAPTER 3: FOOD RESOURCE PARTITIONING AND FEEDING STRATEGIES IN LIVEBEARING FISHES OF THE GENUS *LIMIA* (CYPRINODONTIFORMES: POECILIIDAE) IN THE GREATER ANTILLES

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INTRODUCTION

Feeding is relevant to every aspect of animal life and is tightly linked to the ecological niche a species occupies. In concordance with the enormous diversity found in teleost fishes, feeding specializations are also abundant in this group. One example for this is the order Cyprinodontiformes. This is the most diverse and speciose order of freshwater fishes within the Atherinomorphae. More than 1350 species are known in this order, which is distributed in temperate and tropical regions of the world usually inhabiting shallow freshwater environments or coastal brackish waters (*Malabarba & Malabarba, 2020)*. The Cyprinodontiformes contain approximately twice the number of species of the sister groups Beloniformes and Atheriniformes combined (Hernandez et al., 2009), and they are especially diverse in Central America representing 13% of the Neotropical ichthyofauna (*Malabarba & Malabarba, 2020)*. Despite this extraordinary diversity, the whole group has traditionally been considered as having generalized feeding habits mainly preying on benthic and planktonic organisms (Hunt, 1953; Weisberg, 1986; Motta et al., 1995; Mansfield & Macardle, 1998). This seems to contradict the notion that often feeding adaptations contribute to speciation, as for example found in cichlids. Furthermore, this general classification does not correspond with the diverse feeding behaviors and specializations that can be seen in some groups of cyprinodontiforms (Hernandez et al., 2009).

In this study we provide comparative data on gut content in *Limia*, a genus of livebearing fishes (family Poeciliidae) which allows us to take a more fine-grained look at the potential role of feeding ecology in the radiation this genus experienced in the Caribbean. We are especially interested in discovering if the species of this genus may show cryptic differences in their feeding adaptations.

A group of freshwater fishes that is exceptionally well understood in this context is cichlids. But, although not as notable as the examples of cichlid fishes (Cichlidae) from the East African Great Lakes where trophic specializations in both diet and mouth morphology have contributed to an extraordinary adaptive radiation (Kornfield & Smith, 2000; Takahashi & Koblmüller, 2011;

Meier et al., 2019), there are nonetheless a few examples of species showing feeding specializations in Cyprinodontiformes that deserve more attention. For instance, it has been shown that three species of *Cyprinodon* (Cyprinodontidae) that coexist sympatrically in hypersaline lagoons within the San Salvador Island, Bahamas have trophic partitioning (Martin & Wainwright, 2013) with one species (*C. variegatus*) having a broad geographic distribution and detritivorous feeding habits and the other two species being diet specialists: *C. brontotheroides* specializes in consuming hard prey (i.e. ostracods, gasteropods) and *C. desquamator* a specialized scale-eater (Martin & Wainwright, 2013). Another example of extreme feeding specialization in Cyprinodontiformes is the case of the Pike killifish (*Belonesox belizanus*) a poeciliid mostly distributed in Central America (Rosen & Bailey, 1963). This species exclusively feeds on fish that are captured by moveable elongate jaws and large and pointed unicuspid teeth (Greven & Brenner, 2008).

Furthermore, although not as specialized as the previously mentioned, a well-studied case within the Cyprinodontiformes are the predatory species of the genus *Gambusia* (mainly the invasive *G. affinis* and *G. holbrooki*), which have been shown to be primarily zooplanktivorous (Mansfield & Macardle, 1998; Blanco et al., 2004; Specziár, 2004; Singh & Gutpa, 2010). Against this backdrop, there is a surprising lack of studies that characterize the diet, annual shifts in dietary composition, and the morphology of the feeding apparatus for the majority of cyprinodontiforms. Particularly in livebearing fishes (family Poeciliidae), which are a dominant group in southern North America and Central America including the Caribbean islands, there are only few analyses of the diet in individual species. For instance, Zandonà et al. (2015) studied some differences in the diet of the Trinidadian Guppy (*Poecilia reticulata*) in relation to habitats with varying predation levels showing differences in dietary composition between high and low predation pressure environments. Furthermore, Sanchez and Trexler (2018) analyzed the adaptive evolution of herbivory in the Sailfin Molly (*Poecilia latipinna*) using enclosure cages in the Everglades in Florida revealing that this species fed selectively on epiphyton to compensate deficiencies in the available food. Scharnweber et al. (2010) explored the differences in feeding behavior and feeding efficiency between sexual (*Poecilia mexicana*) and asexual (*Poecilia formosa*) mollies concluding that food competition in these two species plays a minor role in mediating coexistence in natural habitats. While these studies provide useful information to understanding the ecology of the analyzed species, they do not offer a comparative perspective that allows inferring patterns of use of food resources and interpretations of species relationships or interactions are limited. Livebearing fishes from the West Indies are definitely a group of freshwater vertebrates that is worth to analyze in terms of dietary adaptations since it has experienced the largest radiation in aquatic environments of the West Indies (Rodriguez-Silva & Schlupp, 2021a). Particularly in the genus *Limia* the speciation process has resulted in the largest radiation of poeciliids in the Caribbean islands. There are 22 species of this genus that have been described so far from Cuba, Hispaniola, Jamaica and Grand Cayman. These species show a remarkably diverse ecology and are widespread in different types of habitats in the Antilles from freshwater springs in the mountains to hypersaline coastal lagoons (Haney & Walsh, 2003; Weaver et al., 2016a; Rodriguez-Silva & Schlupp, 2021b). The radiation center of *Limia* is found on Hispaniola with 19 species, while only one endemic species from Cuba, Jamaica and Grand Cayman each are currently known (Burgess & Franz, 1989; Hamilton, 2001; Weaver et al., 2016b; Rodriguez-Silva &Weaver, 2020; Rodriguez-Silva et al., 2020). The divergent species composition, mainly in the cases of Cuba and Hispaniola, is not predicted by the theory of island biogeography as the number of available ecological niches and island size is likely the same in both islands (Rodriguez-Silva & Schlupp, 2021a).

Niche specializations, and particularly foraging behavior coupled with resource partitioning and competition, are thought to be essential factors in disruptive selection based on resource specialization (Ackermann & Doebeli, 2004; Bürger et al., 2006). This is because specialization in feeding habits and adaptations in trophic morphologies can be important diversifying forces leading to speciation (Skúlason & Smith, 1995; Bolnick et al., 2003; Knudsen et al., 2010). The speciation process based on food resource-based divergence, and ecological speciation in general, is thought to imply initial stages of resource polymorphism in which the ancestral lineage exploits multiple niches (Smith & Skúlason, 1996; Nyman, 2010). Consequently, the ability by which individuals can utilize different resources lead to disruptive selection and, if successful use of the new resources is established, to lineage splitting (Nosil et al., 2009). In fact, selection acting on traits related to resource acquisition is considered to be critical in niche differentiation and even drive several cases of adaptive radiation (Schluter, 2000).

In this study we characterize the annual diet of eight species of *Limia* collected in different habitats from Cuba, Jamaica and Hispaniola to determine if temporal and spatial dietary changes occur in this genus. In addition, we analyze niche specializations and discuss the potential contribution of these specializations to the radiation process of this genus. We hypothesize that even if the majority of *Limia* species could be categorized as feeding generalists, there might be a few specialists especially on Hispaniola where the genus shows the highest species radiation.

MATERIALS AND METHODS

Study area, species and fieldwork methodology

The use of experimental animals complied with the University of Oklahoma animal welfare laws; guidelines and policies as approved by Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare under the assurance number A3240-01. Fishes were collected and euthanized in the field following the approved IACUC protocol R18-005.

We collected eight species of *Limia* between May 2018 and January 2019 in different localities on Cuba, Jamaica and Hispaniola (Figure 1). Fishes were sampled using a 7.0 m long, 1.5 m height and 3.0 mm mesh size seine, and euthanized in the field by immersion in a concentrated solution of MS 222 (tricaine methanesulfonate) and then preserved in 95% ethanol for further confirmation of species identity in the laboratory based on original descriptions and subsequent analysis of the gut content. All fishes were collected with the authorization of the corresponding authorities in each country. We sampled a variable number of specimens of the following species: *Limia vittata*, *L. melanogaster*, *L. perugiae*, *L. zonata*, *L. versicolor*, *L. yaguajali*, *L. nigrofasciata* and *L. islai*. The habitats where we collected these species showed notable variability. The species *L. melanogaster*, *L. perugiae*, *L. zonata*, *L. versicolor*, *L. yaguajali* were collected in freshwater springs or small creeks located in medium to low elevations while *L. nigrofasciata* and *L. islai* were sampled in a lentic freshwater system (Lake Miragoâne) from southwestern Hispaniola. Moreover, *L. vittata* specimens were collected in a hypersaline coastal habitat in southwestern Cuba (Figure 1). For some species we were able to do collections in both wet and dry seasons from the same site in order to obtain a more complete picture of variation in the annual diet. However, in other species we were only able to collect specimens during the wet period, specifically in the months of May or June (Table 1).

Diet analysis

Stomach content of a total of 181 individuals of eight *Limia* species were analyzed in order to characterize the diet of each species (Table 1). We only included adult specimens in this study to avoid effects of ontogenetic changes in diet. We measured the standard length of each individual and also the length of the corresponding digestive tract. For this, individual guts were placed in a petri dish and cut into 3 equal parts since the intestines can be very convoluted. Then we measured every part to obtain the total length of each gut.

To obtain dietary data, we analyzed the stomach contents found in the first third of the gut since the contents in this part of the digestive tract is not fully digested which allows identification and quantification of food items. Stomach contents were examined individually in the laboratory under an Olympus SZX7 stereomicroscope with direct and transmitted light and 50X magnification, and items were identified to the lowest possible taxonomic level using specific literature sources for algae (Bellinger & Sigee, 2010) and invertebrates (Merritt & Cummins, 1996). The proportions of each component of the diet were quantified according to the volumetric method established by Hynes (1950). Data on the volume of each particular food item was obtained through eye estimation in which the stomach contents were identified, separated and then squashed on a plate to a uniform depth (1 mm) and the area of the squash is measured. This method has been shown to be useful and reliable when measuring small stomach volumes (Hyslop, 1980; Gelwick & Matthews, 2006) which is the case in livebearing fishes.

Graphical representation of feeding strategies

We used the modification of the graphical method (Costello, 1990) made by Amundsen et al. (1996) that incorporates the prey-specific abundance into the graphical representation of dietary composition (Figure 2). In this graphical representation the prey-specific abundance is plotted against frequency of occurrence expressed in fraction. The final plot is used to assess three important elements of the fish diet: (1) prey importance (dominant versus rare), (2) feeding strategy (specialized versus generalized), and (3) niche width. Hence, this method augments the ecological insight that may be derived from stomach contents data (Amundsen et al., 1996).

Data analysis

We calculated the diet breadth for each species analyzed in this study according to the Levin's (1968) formula $B=1/\sum p_i^2$, where p_i is the proportion of the diet contributed by the food item i. This index tends to weight in favor of more abundant food types and is also an indicator of diet diversity for each species (Krebs, 1989; Terraube et al., 2011). Based on the assortment of dietary composition, we created six functional categories that summarized the variation in main type of consumed items (Delariva & Neves, 2020). These categories were: algae (mainly filamentous algae); aquatic invertebrates (larvae and pupae of Odonata, Hemiptera, Coleoptera, Diptera; and also Crustacea, Arachnida and Nematoda); terrestrial insects (Coleoptera and Hymenoptera); vegetal material (plant remains); fishes (fish remains) and detritus (sand grains and other sediment). Then, we calculated the alimentary index (IA_i) for each functional category of the diet

(Kawakami and Vazzoler, 1980). This index is also known in the literature as the index of preponderance (Natarajan & Jhingran, 1961), and it provides a summary representation of the frequency of occurrence as well as the volume of different food items. The IA_i offers a distinct and measurable foundation for grading the level of consumption of specific food elements and it is calculated through the formula $IA_i = FO_iV_i / \sum FO_iV_i * 100$, where FOi and Vi are the frequency of occurrence percentage and volume percentage respectively the of food item i with $i = 1, 2, 3…,$ n food items.

Diet breadth indexes were compared by a Student's t test to verify differences between wet and dry seasons (for the species analyzed in both periods) after confirming that the assumptions for normality and homogeneity of variance were met using the Shapiro-Wilk and Levene's tests respectively. Another Student's t test was used to determine significant differences in the alimentary index values for detritus and algae across all analyzed species since these were the only two food items found in all them. We tried to run an ANOVA test to compare the mean of the gut length among all the species analyzed but the data did not meet the assumption of normality and equal variances even after trying data transformations. Therefore, we ran a Quade's rank analysis of covariance (Quade, 1967) using gut length as dependent variable, species as independent variable and standard length of each individual as covariate. Then, we ran a Scheffe *post hoc* test to compare any contrast between means because we had different number of observations (gut samples) per treatment (species). These statistical tests were performed in SPSS version 26. Finally, we investigated the relationship between the proportion of invertebrates in the diet and the relative gut length in all species analyzed with the corresponding correction for phylogenetic independence. For this analysis we used a phylogenetic generalized least squares (PGLS) model based on a recent phylogeny of the genus *Limia* (Spikes et al., 2020). We ran this analysis in R (R Core Team 2014) using the R package Caper (Orme et al., 2018).

RESULTS

Feeding strategies

The analysis of feeding strategies provided evidence of mixed, generalized feeding habits for the majority of all species included in our study. Most of the *Limia* specimens exhibited preferences for detritus and algae in the two groups of species analyzed: the ones studied in wet and dry seasons (Figure 3, both seasons represented together) and the species analyzed only the wet period (Figure 4).

However, it can be noticed that in the first group the species *L. yaguajali* showed a population specialization towards detritus with most individuals feeding on this dominant food item. In this case there is also a small portion of other prey items but they were included only occasionally in the diet of a few individuals and there were neither high within- nor between-phenotype contributions to the niche width (see figures 2 and 3 for interpretation of the results). A similar trend towards specialization in consumption of detritus and also algae was observed in *L. nigrofasciata* with these two being the only food items found in the stomach of all analyzed individuals (Figure 4).

Surprisingly, the diet analysis in the *L. vittata* population demonstrated a situation in which there was a high between-phenotype contribution to the niche width, with most of the food items situated towards the upper left corner of the graph. In this case, individuals were specialized on fishes, crustaceans and detritus (in decreasing order) but each food category was consumed by only a limited fraction of them (see figures 2 and 4 for interpretation of the results).

Niche breadth (B_i) and alimentary index (IA_i)

There were significant differences in the niche breadth indexes between wet and dry seasons for the species analyzed in both periods (t (3) = 3.00899, p = 0.012). Niche breadth values were higher in the wet season (mean ± SD: 1.82 ± 0.18) than in dry season (mean ± SD: 1.31 ± 0.29) for all species but *L. yaguajali*, which had niche breadth values slightly higher in the dry season compared to the wet period (Figure 5).

The analysis of the importance of the functional food categories that summarized the variation in main type of consumed items was calculated in terms of frequency of occurrence, volume and alimentary index of each food category (Table 2). Overall, detritus and algae represent the two most important components of the diet for the analyzed species. We detected no significant differences in the alimentary index values for detritus and algae, which were the only two food items common to all species ($t (7) = -1.42167$, $p = 0.089$) revealing the importance of both items in the diet of *Limia*. The results of the analysis of covariance revealed significant differences between species in the gut length when controlling for size of each specimen ($F (7) = 13.36$, $p < 0.001$). Results of the post hoc test showed *L. versicolor* as the species that differ the most in gut length with the rest of analyzed species (Table 3).

We found a non-significant relationship between the proportion of invertebrates in the diet and the relative gut length in all species analyzed using a PLGS analysis ($r^2 = 0.01$, $p = 0.808$). However, most of the species showed to some extent a negative association between the proportion of invertebrates in the diet and relative length of the gut (Figure 6).

DISCUSSION

Our analysis of the gut contents of the eight *Limia* species we investigated, shows that they indeed – as predicted for these fishes in general - tend to be feeding generalists. However, we found that some degree of specialization occurs in certain species from Hispaniola were the diversity of the genus *Limia* is remarkably higher compared to the rest of the Greater Antilles. While the use of gut content examination has been widely used in analyses of dietary composition of fishes, how these findings can be interpreted has been and still is under debate (Hynes, 1950; Pinkas et al., 1971; Hyslop, 1980; Cortes, 1997). One of the main limitations of this method is that it only offers a detailed gut composition observed at one point in time. Thus, extrapolating from these snapshots should be done cautiously. Another problem is related to the quantification of food items as sometimes it is not possible to physically separate different prey types with a reasonable level of accuracy due to partial digestion (Baker et al., 2014). Still, the use of this methodology to determine the diet composition in fishes has been widely used in ecological studies, and is considered an established procedure that is easy to replicate and very useful in comparative analyses across multiple or single species in different habitats (Zandonà et al., 2015), seasons (Stefani & Rocha 2005; Liu et al., 2019), and ontogenetic stages (Andersen et al., 2005; Kreitzer et al., 2010; Hintz et al., 2017; Saraswati & Perdhana, 2020).

In our study we attempted to mitigate the issues inherent to the analysis of gut content by examining the diet of the studied species in both the wet and dry season in as many species as we could (half of the species in our case) in order to have a better picture of the dietary dynamic. In addition, we only examined the first third of the gut where it is moderately easier to separate different food items that are not completely digested.

Despite these issues we believe we made an important contribution to the field for several reasons. First, studies of the ecology, and especially the diet of the genus *Limia* are scarce. There are only a few published works that analyze the diet of species in this genus, mostly in the Cuban Limia (*L. vittata*) (Poey 1854; Barus et al. 1980; Ponce de Leon & Rodriguez, 2008). None of them, however, provide a thorough examination of the diet, analyze multiple species from a comparative perspective or study potential feeding specializations in the genus.

Second, our analysis of the feeding strategies showed a preference for detritus and algae independent of season for seven of the eight *Limia* species included in the study. This result was confirmed by the analysis of the importance of the functional food categories where detritus and algae represented the two most important components of the diet for the analyzed species. This finding sheds light on the importance and potential impact of the fishes of this genus, which is the dominant group of freshwater vertebrates on Hispaniola, on sediment reduction, organic matter decomposition, and their influence on the carbon flow in tropical streams (Taylor et al. 2006). By reducing sediment resources these fish species may indirectly affect populations of other grazers consuming the same food item. This has been experimentally tested in a tropical river in Venezuela where the temporary exclusion of the dominant detritivorous fish, *Prochilodus mariae,* from the order Characiformes, caused a significant increase in the standing stock of benthic organic sediment and biomass of aquatic insects (Hall et al., 2011).

On the other hand, *L. vittata* from Cuba was the only species that showed a different prey preference unveiling a certain degree of specialization for the consumption of fishes and crustaceans, although these food items were consumed by only a limited fraction of individuals (Figure 4). This is an unexpected result as this Cuban endemic has been reported as a primarily detritivorous species since its original description by Poey (1854) (Barus et al. 1980; Ponce de Leon & Rodriguez 2008; Falcón-Hidalgo et al., 2011). It is important to note that the few dietary analyses that have been published for this species have only included populations from freshwater environments and the individuals analyzed here were collected in a hypersaline lagoon.

There are studies showing diet shifts associated with habitat type in fishes. For instance, Andersen et al. (2005) confirmed a considerable variation in the diet of juvenile flounder (*Platichthys flesus*) in habitats that differed in algae abundance. Specifically, they showed that in heavily vegetated sites this species fed on a varied diet of aquatic invertebrates including copepods, polychaetes and oligochaetes, while in the less vegetated sites the species fed primarily on amphipods. Yang et al. (2018) also documented significant changes in diet for the largemouth bronze gudgeon (*Coreius guichenoti*) under three different environmental conditions in the Yangtze River basin. Zandonà et al. (2015) also showed differences in the diet of the Trinidadian Guppy (*Poecilia reticulata*) related to different predation levels. Our results of the diet analysis in *L. vittata* draw attention to the importance of developing comparative analyses in populations of this species that live under different saline gradients to determine its dietary plasticity in relation to the habitat type. Recently, it has been discovered that salinity levels *L. vittata* may be linked to maintaining different color morphs in natural populations (Rodriguez-Silva et al. in preparation).

Niche breadth comparisons between wet and dry seasons for the species analyzed in both periods revealed that *L. versicolor*, *L. zonata* and *L. perugiae* expanded their dietary niche breadth in the wet period while *L. yaguajali* had similar values for this variable in both seasons, even though niche breadth was slightly higher in the dry period for *L. yaguajali*. Annual variations in diet composition have been commonly reported in several fish species (Stefani & Rocha 2005; Novakowski et al. 2008; Liu et al. 2019). Concurrently, the three species that we observed that expanded their niche breadth during the wet season were the ones that showed a more diverse feeding strategy with trends towards an omnivore diet (Figure 2, Table 2). The increasing number of aquatic and terrestrial invertebrates in the diet of these three species may be due to the fact that these preys are more abundant in the wet season. Conversely, *L. yaguajali* showed a narrower niche breadth with evident specialization in ingesting detritus. For this species in particular, this food category displayed the highest value of alimentary index (over 73%) among the eight species included in our study (Table 2).

Gut length is known to correspond with the feeding patterns in a way that absorption of nutrients could be more efficient (Karasov et al., 2011). In this sense, our analysis of the relative gut length in relation to the dietary composition revealed similar trends found in previous studies in fish where species with shorter guts presented a more carnivorous diet (more invertebrates) and species with longer guts corresponded to a more herbivorous dietary mode (more detritus and algae) (Kramer & Bryant, 1995; German & Horn, 2006). Although the two variables did not show a significant association we found that regardless phylogenetic relatedness some species seemed to be more specialized as primary consumers of detritus and algae such is the case of *L. nigrofasciata* from Lake Miragoane, and other species showed a more omnivore feeding habit that included more invertebrates in their diet (e.g. *L. versicolor*, *L. islai*, *L. zonata*) (Figure 6). In this analysis we also found one species, *L. perugiae*, which showed some deviation from the pattern seen in the rest of other species. Particularly, *L. perugiae* showed a large relative gut length and also a high proportion of invertebrates in the diet. This result might indicate that this species, which is the most widespread *Limia* on Hispaniola, may have some dietary plasticity mirroring different types of environments with divergent food availability.

While our findings revealed common trends toward a generalized feeding strategy in *Limia*, we found that certain diet specializations may also occur in this genus. Particularly some species from Hispaniola, where the genus *Limia* shows a notable radiation, seemed to have narrow feeding strategies moving in the direction to detritivory. The novel and comparative nature of our study provides a basis for deeper analyses of the trophic relationships in the genus *Limia* incorporating morphological examination of the trophic apparatus that has been hypothesized to play a role in speciation in *Limia* species from Lake Miragoane (Rodriguez-Silva et al., 2020). Although not conclusive on whether diet specializations may drive speciation in the genus *Limia*, our findings support the idea that a certain degree of diet specialization in the genus might be a factor to be considered when exploring the causes of diversification of this group of fishes in the Caribbean.

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TABLES

Table 1: Number of individuals analyzed per species in each season of the year with information on the collecting sites, size ranges and gut lengths for each species. N: sample size, SL: standard length, GL: gut total length.

Species	**GPS coordinates**	**N (Wet season)**	**N (Dry season)**	**SL range (mm)**	**GL (mean ± SD) (mm)**
Limia versicolor	18°42'5.61" N 70°11'50.39" W	15	15	23 - 45	41.47 ± 15.60
Limia zonata	19°18'44.64" N 70°31'27.23" W	17	15	27 - 42	64.03 ± 23.16
Limia yaguajali	19°37'49.87" N 70°26'58.06" W	15	14	31 - 41	57.72 ± 13.72
Limia perugiae	18°14' 20.76" N 70°26'41.82" W	10	15	30 - 53	80.16 ± 27.20
Limia nigrofasciata	18°25'33.2" N 73°02'56.9" W	15	-	37 -43	119.00 ± 19.48
Limia melanogaster	18°02'31.30" N 76°54'36.87" W	15	-	24 - 47	44.67 ± 16.33
Limia islai	18°25'59.4" N 73°03'33.5" W	13	-	28 - 31	43.46 ± 3.76
Limia vittata	22°10'40.76" N 81°15'13.18" W	22	-	22 - 36	44.32 ± 12.32

Table 2: Percentage values of frequency of occurrence (FO), volume (V) and alimentary index (IA) for the six functional categories that summarized the variation in main type of food items in the eight *Limia* species analyzed. Total values that represent wet and dry seasons together are presented for *L. versicolor*, *L. zonata*, *L. yaguajali* and *L. perugiae* while values that represent only the wet season are presented for *L. nigrofasciata*, *L. melanogaster*, *L. islai* and *L. vittata*.

Food type	**%FO**	**%V**	**%IA**
Limia versicolor			
Algae	100	40.64	49.37
Aquatic invertebrates	65.50	10.79	8.59
Plants	3.45	0.35	0.01
Detritus	72.4	47.78	42.03
Limia zonata			
Algae	93.75	68.65	74.83
Aquatic invertebrates	59.38	6.25	4.32
Plants	28.13	1.44	0.47
Detritus	68.75	25.50	20.38
Limia yaguajali			
Algae	96.55	26.73	26.53
Aquatic invertebrates	17.24	0.96	0.17
Plants	10.34	1.14	0.12
Detritus	100	71.17	73.18
Limia perugiae			
Algae	72.00	11.52	10.71
Aquatic invertebrates	56.00	20.43	14.78
Terrestrial invertebrates	56.00	3.37	2.44
Plants	20.00	3.48	0.90
Detritus	92.00	59.89	71.17
Limia nigrofasciata			
Algae	73.33	38.26	31.24
Detritus	100	61.74	68.76
Limia melanogaster			
Algae	100	55.18	60.95
Aquatic invertebrates	26.67	0.94	0.28
Plants	80.00	8.85	7.82
Detritus	80.00	35.03	30.95
Limia islai			
Algae	83.33	22.59	23.60
Aquatic invertebrates	16.67	3.65	0.76
Fishes	16.67	2.33	0.49
Plants	50.00	13.29	8.33
Detritus	91.67	58.14	66.82
Limia vittata			
Algae	23.08	3.66	2.90
Aquatic invertebrates	23.08	4.19	3.32
Fishes	30.77	53.40	56.43
Detritus	38.46	28.27	37.34

Table 3: Results of the Scheffe's post hoc analysis comparing mean of gut lengths among species. Probability values are presented in the table for all possible comparisons. Values highlighted in bold represent significant differences with an alpha level of 0.05 (*L. ver.*: *Limia versicolor*, *L. zon.*: *Limia zonata*, *L. yag.*: *Limia yaguajali*, *L. per.*: *Limia perugiae*, *L. nig.*: *Limia nigrofasciata*, *L. mel.*: *Limia melanogaster*, *L. isl.*: *Limia islai*, *L. vit.*: *Limia vittata*).

	L. ver.	*L. zon.*	*L. yag.*	*L. per.*	*L. nig.*	*L. mel.*	*L. isl.*	*L. vit.*
L. ver.		**0.000**	**0.028**	**0.001**	**0.000**	1.000	**0.039**	**0.000**
L. zon.	**0.000**		0.983	1.000	0.206	**0.025**	1.000	0.793
L. yag.	**0.028**	0.983		0.984	**0.025**	0.249	0.999	0.250
L. per.	**0.001**	1.000	0.984		0.281	**0.034**	1.000	0.858
L. nig.	**0.000**	0.206	**0.025**	0.281		**0.000**	0.371	0.974
L. mel.	1.000	**0.025**	0.249	**0.034**	**0.000**		0.189	**0.000**
L. isl.	**0.039**	1.000	0.999	1.000	0.371	0.189		0.879
L. vit.	**0.000**	0.793	0.250	0.858	0.974	**0.000**	0.879	

FIGURES AND CAPTIONS

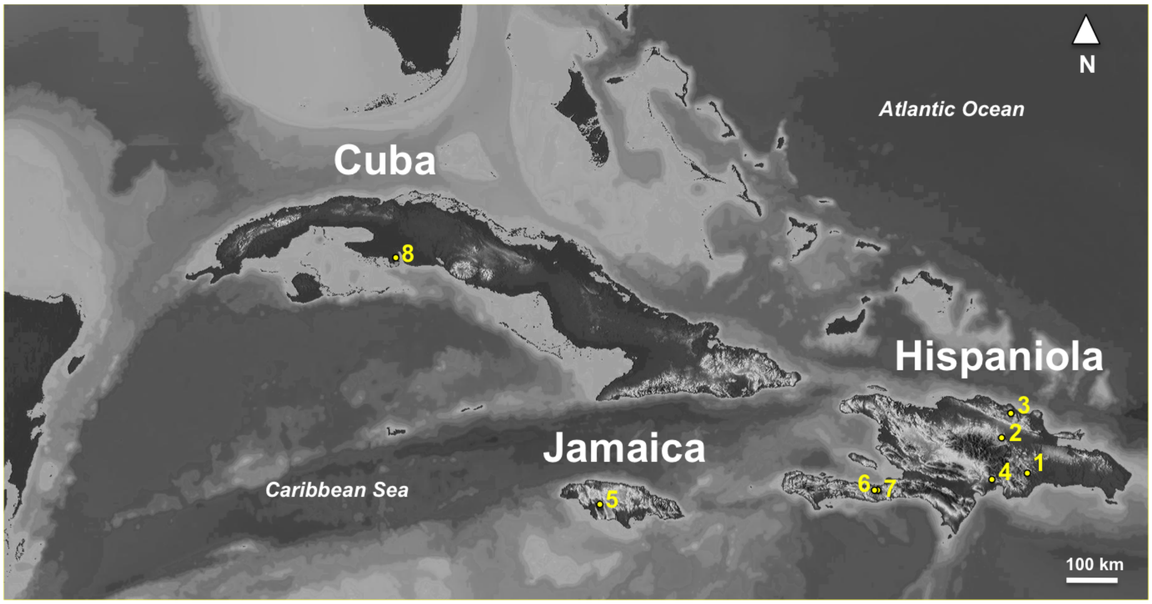

Figure 1: Collecting sites of the *Limia* species analyzed in the study. 1. *L. versicolor*, 2. *L. zonata*, 3. *L. yaguajali*, 4. *L. perugiae*, 5. *L. melanogaster*, 6. *L. islai*, 7. *L. nigrofasciata*, 8. *L. vittata* (Map credit: OpenStreetMap contributors).

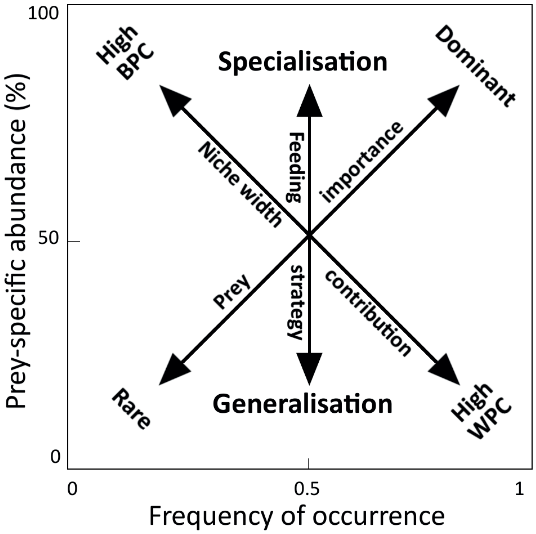

Figure 2: Explanatory diagram for interpretation of feeding strategy, niche width contribution and prey importance according to Amundsen et al. (1996). (BPC - between-phenotype component; WPC - within-phenotype component (figure modified from Amundsen et al., 1996).

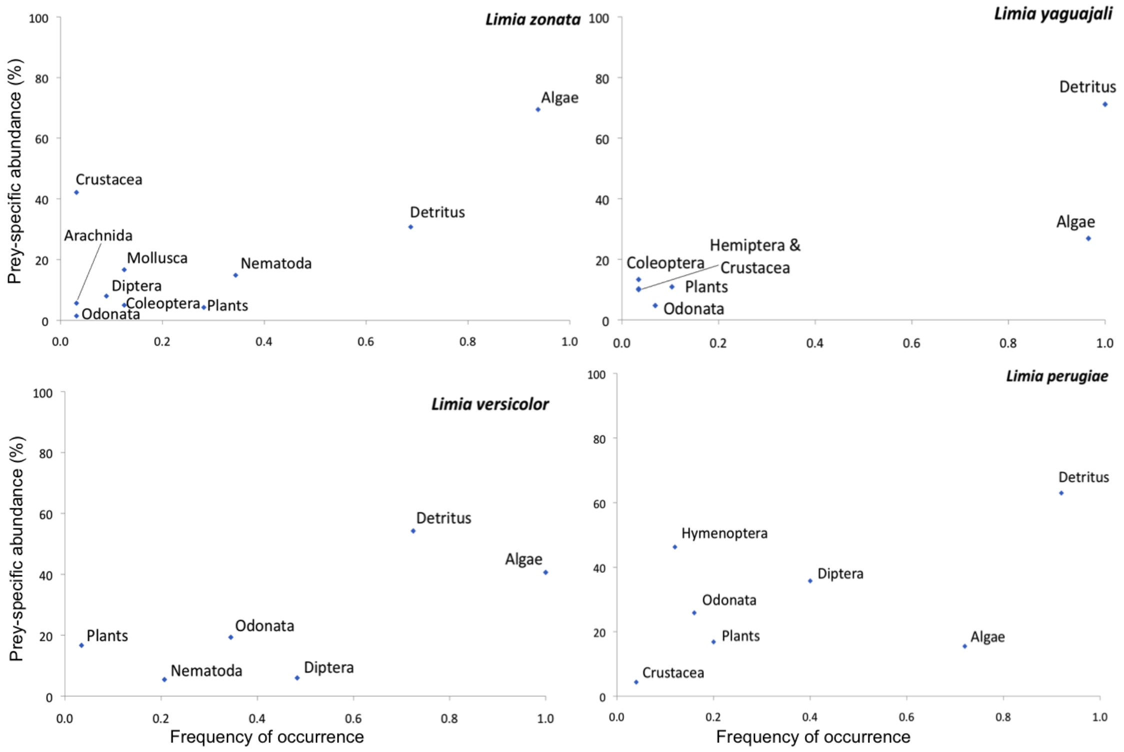

Figure 3: Graphic representation according to Amundsen et al. (1996) of the feeding strategies of four *Limia* species from Hispaniola (*L. zonata*, *L. yaguajali*, *L. versicolor* and *L. perugiae*) analyzed during wet and dry seasons.

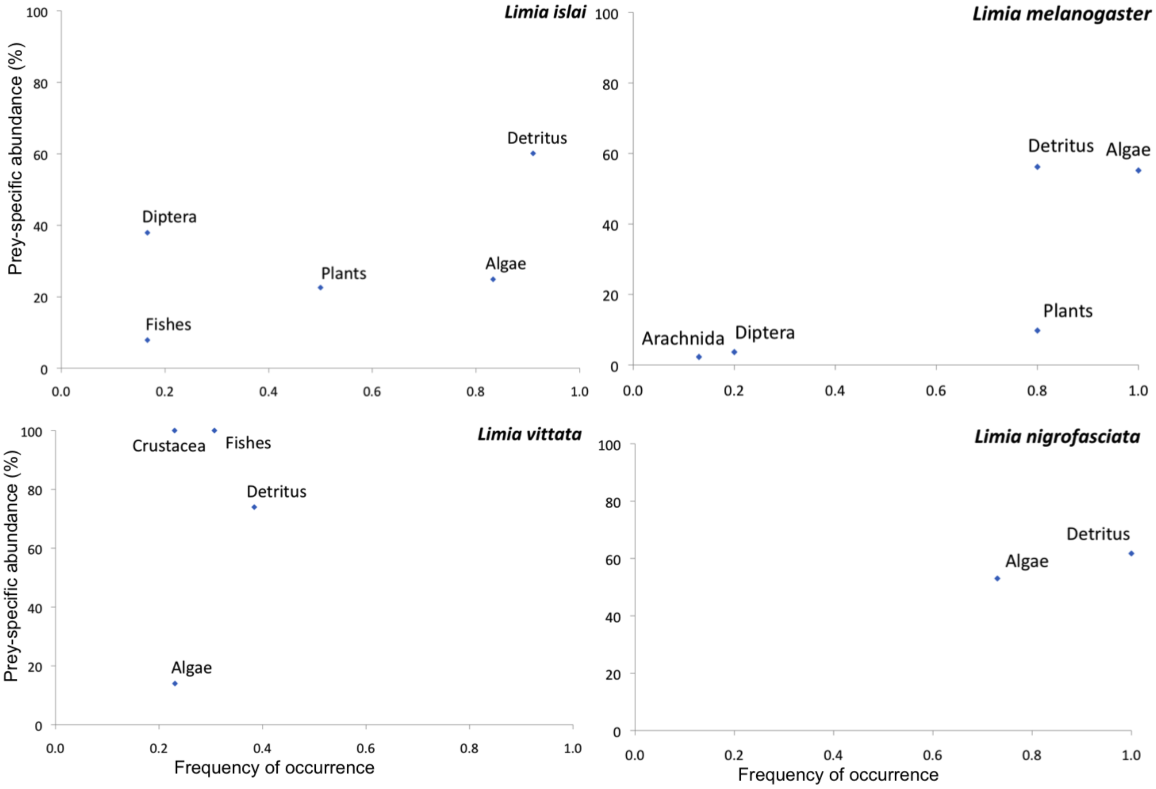

Figure 4: Graphic representation according to Amundsen et al. (1996) of the feeding strategies of two *Limia* species from Hispaniola (*L. islai*, *L. nigrofasciata*), the single endemic species from Jamaica (*L. melanogaster*) and Cuba (*L. vittata*). Specimens analyzed were collected only in the wet season.

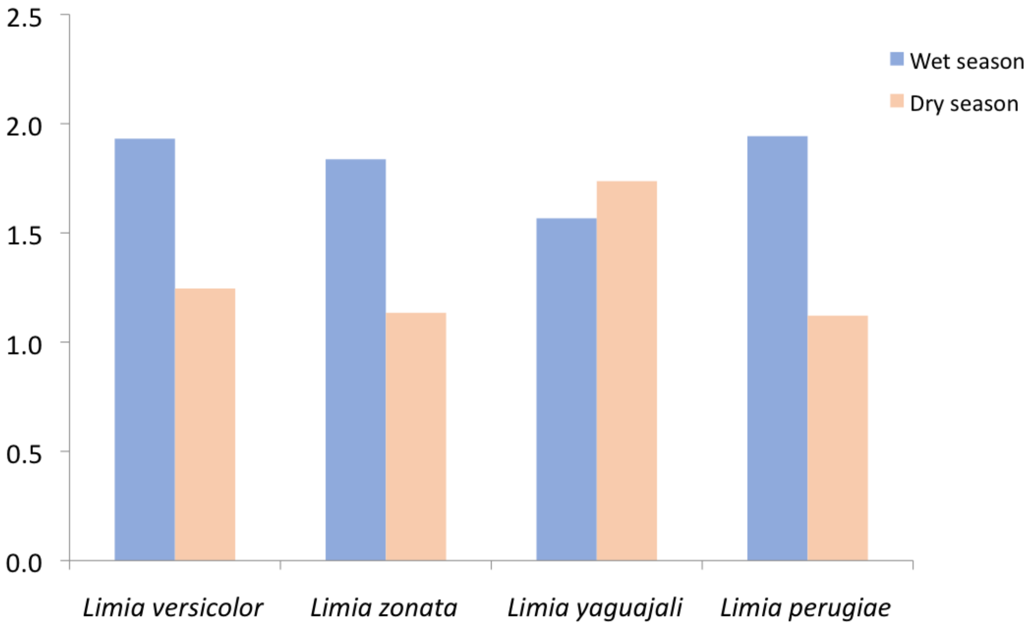

Figure 5: Seasonal variations in dietary niche breadth of the four *Limia* species analyzed in wet and dry seasons. Values in the vertical axis are the niche breadth (Levins' index) for each period.

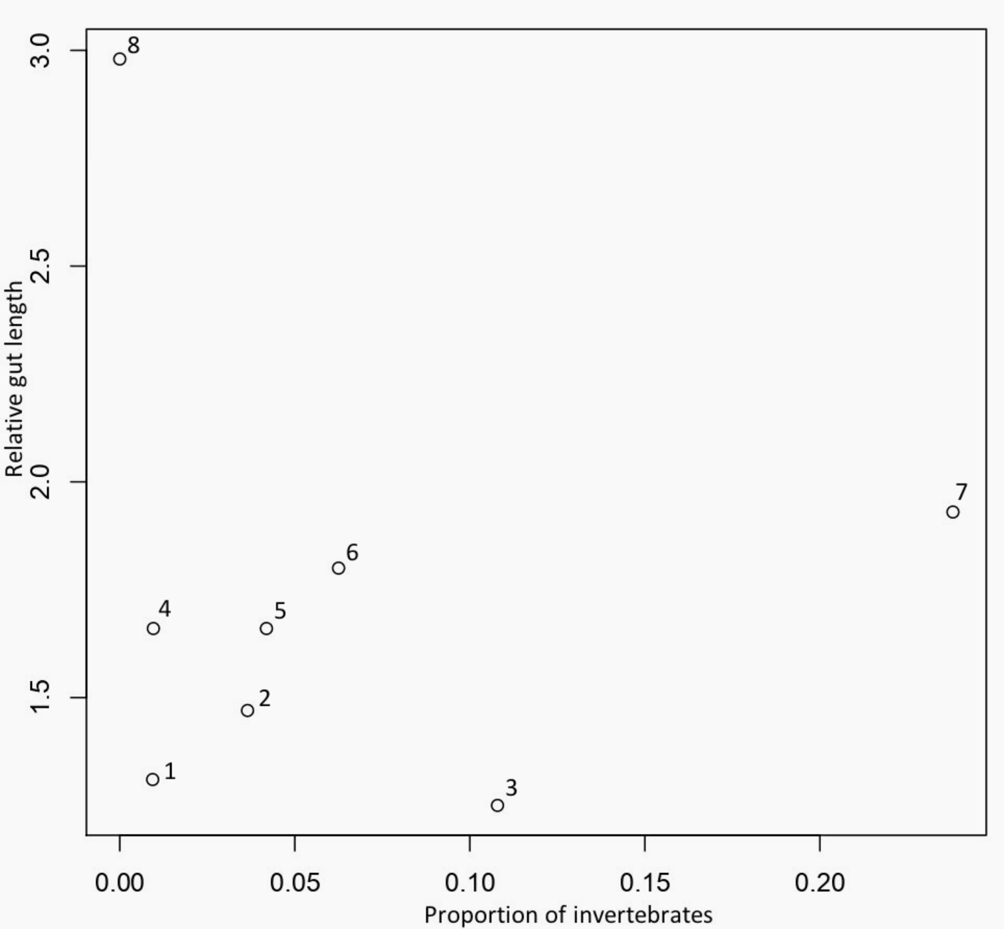

Figure 6: Relationship between the proportion of invertebrates in the diet and the relative gut length of *Limia* species. 1. *L. melanogaster,* 2. *L. islai*, 3. *L. versicolor*, 4. *L. yaguajali*, 5. *L. vittata*, 6. *L. zonata*, 7. *L. perugiae*, 8. *L. nigrofasciata*).

CHAPTER 4: COLOR POLYMORPHISM IN THE CUBAN ENDEMIC LIVEBEARING FISH *LIMIA VITTATA* (TELEOSTEI, POECILIIDAE): DOES SEXUAL OR NATURAL SELECTION DETERMINE THE PREVALENCE OF BLACK SPOTTED INDIVIDUALS?

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color polymorphism, *Limia*, poeciliid, sexual selection, natural selection, salinity gradient

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Authors declare that they have no conflicts of interest.

Availability of data and material

Photos of specimens and data from the sexual selection experiment will be available in Dryad after publication.

Code availability (Not applicable)

Authors' contributions

RRS and IS designed the study; RRS and MS planned and performed sexual selection experiments; RRS, IS and MIM collected specimens in the field; RRS and MIM collected digital images of preserved fish, RRS, MS and IS analyzed the data, RRS wrote the manuscript; RRS, IS, MS and MIM read and edited different versions of the manuscript.

Ethics approval (Not applicable)

Consent to participate (Not applicable)

Consent for publication (Not applicable)

INTRODUCTION

Polymorphism can be defined as the occurrence of multiple discrete forms or morphs within populations of a species. This is a common phenomenon that can be caused and modulated by selection, drift, mutations and gene flow (Charlesworth and Charlesworth 1975; Oxford 2005; Guerrero and Hahn 2017; Kang et al. 2017; Rojas et al. 2020). As a special case of polymorphism, phenotypes can show discrete variation in color. Such color polymorphisms are common and widespread (White and Kemp 2016; Gray and McKinnon 2007), and have been well studied across several taxa, such as aposematic poison frogs of the genus *Dendrobates* (Hoogmoed and Avila-Pires 2012; Stuckert et al. 2019); different species of lizards (Bleay and Sinervo 2007; Vercken et al. 2007); Gouldian finches (*Erythrura gouldiae*) (Gilby et al. 2009), among others.

Color polymorphism (and polymorphism in general) can be explained by sexual selection or by natural selection acting separately when any of them act disparately in a temporal or spatial context (Gosden and Svensson 2009; Hughes et al. 2013; Iserbyt et al. 2013). In the latter case, if natural selection were invoked, one would assume a benefit to a particular phenotype that, for example, is better suited to avoid predators (Punzalan et al. 2005). Often this is governed by frequency dependent selection with morph variants that are usually rare having a selective advantage. This phenomenon results in a higher fitness that causes negative frequency-dependent selection (NFDS), which has been shown in both classical and recent studies (Ford 1945; Kettlewell 1961; Clarke and Donald 1962; Ayala and Campbell 1974; Heino et al. 1998; Svardal et al. 2015; Chouteau et al. 2017; Kurbalija et al. 2020). Conversely, one would assume sexual selection to maintain a polymorphism if, for example, females show preferences for one of several color phenotypes in males. In this case, a particular color morph influences mating interactions by changes in mating preferences, which alters morph-specific fitness and also maintains genetic diversity (Iserbyt et al. 2013; Hughes et al. 2013; Roulin and Ducrest 2013). Yet in some cases neither natural selection nor sexual selection alone explains how polymorphisms are maintained and scenarios where both agents act simultaneously are required to explain the presence of color polymorphism (Wellenreuther et al. 2014). This has been shown in populations of guppies (*Poecilia reticulata*) where color morphs are exposed to strong sexual and natural selection as agents that uphold the diversity in color patterns in males. Studies have shown that males of this species that exhibit rare color morphs seem to have a reproductive advantage over males with common phenotypes (Olendorf et al. 2006; Hampton et al. 2009; Hughes et al. 2013).

The most common pattern of color polymorphism observed in natural conditions is when both sexes exhibit the polymorphism within a population. This is the case in the poeciliid species *Limia vittata* that is endemic to the island of Cuba in the West Indies. The genus *Limia* is endemic to the Caribbean where it shows a notable radiation on the island of Hispaniola with 19 described species so far (Chambers 1987; Burgess and Franz 1989; Weaver et al. 2016; Rodriguez-Silva et al. 2020). However, *L. vittata* is the sole species in the genus found in Cuba, where it has a ubiquitous presence in lowland water bodies that range from freshwater to brackish and hyper-saline habitats (Barus et al. 1980; Ponce de Leon and Rodriguez 2010). This species shows a complex pattern of melanistic color polymorphism with intra- and inter-population variation in the frequencies of black spotted individuals in both sexes. Other species in the same genus do not show this polymorphism, but a few other species in the family do even though the occurrence of male-limited color polymorphism is more frequent in poeciliids in general (Lindholm et al. 2004, 2015; Hurtado-Gonzalez et al. 2010, 2014; Zerulla and Stoddard 2021). The fact that both sexes are polymorphic and that the variation in melanism is across populations in *L. vittata* allows using this species as a model to ask what might maintain spotted and non-spotted phenotypes on a species, population and individual level.

To tackle this, we investigated the role of sexual selection with two mate choice studies using traditional binary choice tests, looking at both female and male mate choice. For this we used black spotted aquarium stocks because they have likely been selected by breeders to have more spots. In other words, if there are preferences to be detected, likely they can be found here. Furthermore, we investigated the role of habitat variation and natural selection in color polymorphism by comparing populations that live in environments with different salinity levels. To do this, we collected information about the frequency and geographic distribution of melanistic and non-melanistic morphs of *L. vittata* of natural populations in Cuba. Concretely, we tested the hypothesis that melanistic phenotypes are associated with habitats of different salinity. Together, we test which mechanism (sexual selection, natural selection or a combination of both) may more likely contribute to maintain the polymorphism found in the Cuban Limia.

MATERIALS AND METHODS

Classification of spotted area for sexual selection experiment

In this study we included two populations of *L. vittata* obtained from stocks that are kept in common garden conditions at the OU International Stock Center for Livebearing Fishes in a greenhouse at the Aquatic Research Facility at the University of Oklahoma. The collecting site and date information for these populations is not known. Prior to the experiment all individuals were transported to an indoor fish-room with the temperature held at 26ºC (±3ºC) on a 12-hour day-night cycle. Fish were fed *ad libitum* with a varied diet that included bloodworms, *Daphni*a, brine shrimp, and TetraMin flakes twice daily. Once in the fish-room, the fish were separated by sex and kept in 75 L tanks for at least 30 days as an acclimation period.

To measure body area covered by black spots on each individual, 60 males and 80 females were anesthetized one at a time with tricaine-methanesulfonate (MS-222) and photographed with a Nikon D5200 camera. The photos were saved digitally and are available upon request. We captured both lateral views. The photos were used to measure the standard length and total body area covered by black spots for each fish using ImageJ 1x software (Schneider et al. 2012). Then, we analyzed the distribution of less and more spotted individuals in our sample for males and females and split them in different groups according to the spotting pattern. Individuals that fell in the lower quartile or 25th percentile (smaller total spotted area) were considered low spotted and the ones that fell in the upper quartile or 75th percentile (greater total spotted area) were classified as high spotted individuals (Figure 1). We only used stimulus fish in both treatments (males and female choice) that were similar to each other in size (± 1.5 mm) and also included size of stimuli fish as a covariate in our statistical analysis.

Sexual selection experiment

Dichotomous choice tests were conducted in a 75 L tank that was divided in three equal sections in length by drawing two vertical lines on the front glass of the tank. Within each section was an 8.5 x 8.5 x 46 cm clear, unperforated Plexiglas prism with rectangular shape. These containers were used to restrict movement, mechanosensory signals, and chemical cues, while still allowing visual communication of fish. The experimenter then sat 3 m away directly in front of the test tank and recorded the amount of time a focal fish spent within the outer sections (stimuli fish) of the tank. The three sections of the tank, demarcated as left choice zone, middle neutral zone and right choice zone were of the same size (32 x 32 x 43 cm). In other experiments, we successfully used this method to detect preferences (Makowicz et al. 2016).

The tests began by us placing focal a fish (male or female) in the Plexiglas container within the neutral zone of the testing tank. Then, a high spotted and a low spotted stimulus fish of the opposite sex was placed on either side of the focal fish, in a randomized outer section of the test tank. Fish were then undisturbed for 5 minutes to acclimate to the test tank (habituation phase). After acclimation, the focal fish was released from the neutral zone to swim freely throughout the test tank for 5 minutes. The experimenter then recorded the amount of time the focal fish spent within the section with either the high spotted or low spotted stimulus fish (association time). After the 5 minute trial the focal fish was returned to the container in the neutral zone and the stimulus fish were switched to the opposite outer section of the tank. We performed a second trial with the stimulus fish swapped, to detect any side bias. We gave the fish (focal and stimuli) another 5 minutes of acclimation and then released the focal fish to record the association time for another 5 minutes. Once each trial was completed, we measured the standard length of the focal fish by placing it on a millimeter grid. Although we attempted to use different stimulus fish in every trial during the two dichotomous choice assessments (male preference and female preference), it was necessary to reuse some individuals to create sufficient numbers of stimulus pairs with contrasting spotted areas. However, no stimulus fish were reused more than once and all of the reused individuals were tested in different dyadic combinations (Sommer-Trembo et al. 2016). No focal fish were reused in the experiment.

We calculated the association time of the focal fish with each stimulus fish by adding the time the focal fish spent associated with each choice zone in the two parts of each trial in males and females, and then calculated the strength of preference (SOP) as: SOP= (time spent with more spotted stimulus – time spent with less spotted stimulus)/time spent with both stimuli. Thus, SOP-values could range from +1 (maximum preference for the high spotted stimulus to -1 (maximum preference for the low spotted stimulus). We assessed female preference in 24 individuals with different male choices according to the spotted area (SA): low spotted males (mean spotted area ± SE: 9.02 ± 2.08 mm^2) and high spotted males (mean spotted area ± SE: 21.66 ± 2.94 mm^2) (t (7) = -9.91, p<0.00001). In the case of males, we tested 25 individuals to determine any preference for less of more spotted females: low spotted females (mean spotted area ± SE: 19.94 ± 3.43 mm^2) and high spotted females (mean ± SE (SA): 64.50 ± 13.12 mm^2) (t (6) = -8.70, p<0.00001).

Frequency and geographic distribution of melanistic and non-melanistic morphs in natural populations

We analyzed individuals of *L. vittata* collected by us from multiple localities in Cuba in 2018 and 2019 as well as voucher specimens from additional localities from the scientific collections at the Instituto de Ecología y Sistemática in Cuba (CZACC) (Figure 2, Table 1). For the coastal localities (represented by blue dots in Figure 2), salinity levels were confirmed either by direct measurements on site using a conductivity meter or refractometer (collections made in 2018 and 2019) or through personal communication with the collector Tec. J. Rodolfo Sánchez Correa. We recorded the number of melanistic and non-melanistic morphs by sex in each location (Figure 3). In the case of melanistic individuals, we measured the body area covered by black spots or blotches on each fish by using the same procedure described for aquarium-raised specimens used in the sexual selection experiment.

Statistical analysis

Sexual selection experiment

Equal variances and normality of residuals were checked using Levene's test and Shapiro-Wilk's test, respectively prior to inferential analyses, and both assumptions were met for our data. In this experiment we predicted that sexual selection would explain the presence of color polymorphism in some populations of *L. vittata* and that at least one sex would show a preference for high spotted mates. Hence, we explored whether SOP-values explained sexual preference in both mating situations (male and females). We conducted a univariate general linear model (GLM) using SOP-values as dependent variable, sex as factor, and size of focal and stimulus fish as covariates.

In addition to the GLM using SOP-values as dependent variable, we also corroborated focal fish preferences for less or more spotted stimuli using paired t - tests considering total association time with both types of stimuli in the two mating situations (male and females) as the dependent variable. As a post-hoc procedure, we then performed Bayes Factor analysis in R (R Core Team 2014) using the BayesFactor R package (Morey et al. 2018) to estimate if negative results were likely due to a true absence of a preference or if the results are inconclusive. Bayes factor analyses compute Bayes factors, using Bayesian inference, to compare multiple mixed models. The Bayes factor quantifies the support for or against the null hypothesis.

We used a linear regression to model the relationship between fish size and spotted area. In addition, we calculated the Pearson correlation coefficient to determine any significant correlation between the two variables.

Color polymorphism in natural populations

We evaluated the prevalence of spotted morphs in natural populations from habitats with different salinity levels. We used Chi-square tests to determine whether the frequency of spotted and non-spotted individuals was dependent on the type of habitat or related to sex. Moreover, we compared the total body area covered by black spots of individuals collected in brackish/saltwater versus freshwater habitats using a Mann-Whitney U test after checking that the assumptions of homogeneity of variances and normality were violated through Levene's and Shapiro-Wilk's tests, respectively. All analyses were performed on SPSS version 26.

RESULTS

Sexual selection: Mating preferences relative to spotted area

Neither females nor males showed a preference for mating partners that differed in spotted area. SOP-values for both sexes ranged from -1.00 to 1.00, which suggests no clear mating preference relative to total body spotted area. For males, the mean SOP-value was 0.02 ± 0.53 (mean ± SE), and for females the mean SOP-value was -0.03 ± 0.48 (mean ± SE) (Figure 4). Overall, our GLM detected no significant difference between males and females for SOP-values, F (1, 45) = 0.019, p = 0.890. In addition, we detected no significant effects of size of the focal fish (F (1, 45) = 2.242, p = 0.125), size of the low spotted stimulus (F (2, 44) = 0.276, p = 0.602) and size of the high spotted stimulus (F (2, 44) = 0.190, p = 0.665). As is typical for livebearing fishes, focal females were on average larger than males (t = 12.923, p < 0.001), (female size (mean ± SE: 35.17 ± 4.19; male size (mean ± SE: 21.68 ± 3.05)).

Because we did not find a significant preference, we used a Bayes factor analysis as a *posthoc* test to ask if the absence of a preference is likely due to a true absence of a preference or not. To prepare for that, we used simple paired t-tests on association time to confirm that neither males nor females preferred more or less spotted individuals as mates. Focal females spent roughly equal time with low spotted (mean ± SE: 220.75 ± 130.50 s) and high spotted stimulus males (mean ± SE: 201.25 ± 111.45 s) (t (23) = 0.50, p=0.62). Similarly, we did not find differences in the association time of males with low spotted (mean ± SE: 251.84 ± 145.76 s) or high spotted females (mean ± SE: 267.28 ± 153.04 s) (t (24) = -0.26, p=0.79).

After the t-tests, we ran a Bayes factor analysis (BFA) as a *posthoc* analysis to assess the two statistical models used in our traditional, Fisherian statistical test. In this analysis, the Null Hypothesis of an absence of preference is evaluated against the Alternative Hypothesis of a presence of a preference and the Bayes factor is used to quantify the support for or against the null hypothesis. The BFA for the female preference data showed moderate support for a lack of preference, suggesting that females actually do not possess a pre-copulatory preference (BFA= ± 1.94%). In the case of males, the full model had strong support, which suggests that the factors influencing male association time are interacting (BFA= ± 2.99%). This means that our results are inconclusive, and we cannot say definitively that there is no preference for female body spotted area in male *L. vittata*.

Finally, we tested for a correlation between fish size and spotted area. The two variables were found to be significantly correlated in females (r (43) = 0.594, p < 0.001), but not in males (r (35) = 0.094, p = 0.587) (Figure 5).

In summary, there is little support for a role of sexual selection in maintaining the polymorphism for spots in *L. vitatta*.

Natural selection: Prevalence of black spotted morphs in natural populations of L. vittata

We found that the frequency of black spotted morphs in natural populations of *L. vittata* changed with habitat. The frequency of spotted individuals was significantly higher in brackish and saltwater environments compared to freshwater habitats, where they were rare or completely absent in some localities (X^2 (1, N=529)=9.9984, p=0.001) (Figure 6).

However, the type of habitat did not have an effect on the frequency of spotted and non-spotted individuals by sex: X^2 (1, N=111)=1.2087, p=0.271 (freshwater habitats) and X^2 (1, N=419)=0.7794, p=0.377 (brackish/saltwater habitats). In addition, we compared the degree of pigmentation of individuals (measured as total body area covered by black spots) collected in these different habitats. We detected no significant differences in the spotted area between individuals of brackish/saltwater (N=68) and freshwater (N=5) populations (U= 106, p= 0.177).

DISCUSSION

The results of our study support natural selection generated by variation in habitat salinity, rather than sexual selection, as the most probable mechanism that explains the presence of color polymorphism in *L. vittata*. It has been shown that characteristics of the physical environment where fish species live can play an important role in changing the frequencies of melanistic morphs temporally and spatially (Horth and Travis 2002; Zerulla and Stoddard 2021). For instance, in the Green Swordtail (*Xiphophorus hellerii*) and the Atlantic Molly (*Poecilia mexicana*) spotted fish are thought to have a social advantage for mating in low visibility conditions where spotted individuals are likely to be more visible than unspotted fishes (Franck et al. 2001, Culumber et al. 2014). However, it is not clear how salinity favors melanin spotting in *L. vitatta* as the effect of this and other physical factors (e. g. temperature, oxygen concentration, light exposure) on pattern expression and morph fitness remain unexplored for livebearing fishes (Meyer et al. 2006; Petrescu-Mag et al. 2008). Our comparison between spotted area of individuals collected in brackish/saltwater and freshwater habitats showed no differences in spotting patterns, which reinforces the idea of natural selection as determinant of the higher frequency of spotted individuals in saline environments over a physiological factor linked to salinity in melanin spotting expression.

The combination of two different lines of evidence such as the assessment of mate choice preferences and collection of ecological data from the field offer a unique, interdisciplinary approach to explain the causes of color polymorphism. However, we acknowledge some limitations of both approaches. In the sexual selection experiment we only analyzed individuals from one population in the case of females, and two populations in the case of males. In particular for males, even though we did not find preferences for female spotting, the Bayes factor analysis showed that our results were inconclusive and we could not say definitively that there was no preference for female body spotted area. There is the possibility that we obtained inconclusive results in this case because we analyzed spotted males from two different populations or maybe due to the fact that females spotted area was associated with female size while males exhibited no significant association between the two variables.

It has been shown that most of the short-term experiments that assess how sexual selection acts in maintaining polymorphism are performed under simplified environments that constrain the interpretation of results. This is because mating preferences can be context dependent where social and ecological conditions, time and inter-population variation can influence the outcome of each study (Rolán-Álzarez et al. 1999; Alonzo and Sinervo 2001). Conversely, long-term studies that incorporate analyses of social effects in mating behavior and/or the quantification of different phenotype frequencies in natural habitats represent have been used as an alternative to avoid the limitations mentioned above (Schartl et al. 1993; Hurtado-Gonzalez and Uy 2010; Culumber et al. 2013). Regarding the field component of our study, one caveat is that the locations were sampled only once, and the collection times are far apart, which may not offer a complete view of the dynamic and temporal variation of color polymorphism as well as changes in salinity levels in natural environments.

Our results offer compelling evidence that connects the prevalence of spotted morphs in wild populations of *L. vittata* with the salinity of the habitat, suggesting that color polymorphism could be maintained in this species either directly or indirectly by natural selection. According to our results, the positive association between melanin spotting and moderate to high salinity levels may be a result of negative frequency dependent selection where the rare color variants (black spotted individuals in this case) are favored or have a selective advantage over the most common color morph (non-spotted individuals) (Ayala and Campbell 1974; Chesson 2000; Wellenreuther et al. 2014). Coastal habitats show spatially, highly variable physical and chemical features since their position at the boundaries of terrestrial and marine ecosystems creates a patchwork of environmental gradients (Prado et al. 2014; Namba et al. 2020). This environmental heterogeneity inherent to coastal ecosystems makes them distinctive settings in terms of the high levels of biodiversity that they contain (Palmer et al. 2011). Populations of *L. vittata,* a primarily detrivorous species, that live in coastal habitats can benefit from high productivity and food availability (Barus et al. 1980; Ponce de Leon and Rodriguez 2010; Rodriguez-Silva et al. in preparation). However, higher productivity and the connectedness of coastal ecosystems also brings an increased predation threat by some diadromous and marine fishes that are known as major predators of livebearing fishes; for example, the bigmouth sleeper, *Gobiomorus dormitor* (Bacheler et al. 2004; Langerhans et al. 2007). Hence, *L. vittata* populations that live in estuarine habitats are likely to face higher predation pressure compared to populations that occur in purely freshwater environments suggesting that a tradeoff between productivity/food availability and predation may be responsible for maintaining the polymorphism

Predation pressure can have strong effects on the composition and structure of aquatic communities (Matthews 1998; Jackson et al. 2001). It is also known to be a key selective agent facilitating speciation and favoring particular phenotypes and adaptive behaviors in natural populations. This has been thoroughly tested using both laboratory and field studies in the Trinidadian guppy (*Poecilia reticulata*) from low and high predation environments, where changes in life history traits, morphology and behavior have been reported (Reznick and Endler 1982; Fraser and Gilliam 1987; Reznick et al. 1997; Kelley and Magurran 2003; Magurran 2005; Reznick et al. 2008; Broder and Angeloni 2014; Kolluru et al. 2015). Contrasting predation pressure has been shown to be related to divergence in body shape coupled with assortative mating in *Gambusia* fishes from the Bahamas (Langerhans et al. 2007). Particularly in melanic side-spotting poeciliid species, studies have found that predators prefer unspotted over spotted morphs, which may contribute to a selective survival advantage for melanic side-spotted individuals (Zerulla and Stoddard 2021). This pattern has been shown in populations of spotted Green Swordtail (*Xiphophorus hellerii*) where the predator species *Belonesox belizanus* ate more frequently unspotted than spotted specimens (Franck et al., 2001). Likewise, it has been described that unspotted individuals of the poeciliid species *Gambusia holbrooki* are more likely to be eaten by sunfishes (genus *Lepomis*) than unspotted morphs (Martin 1977; 1986).

Other empirical experiments have also demonstrated, not only in fishes but also in other vertebrates, the significance of color patterns; especially background matching coloration (e.g. crypsis), in evading detection by predators (Gray and McKinnon 2007). For instance, color polymorphism is maintained by selective predation in the case of the mottled rock rattlesnake (*Crotalus lepidus lepidus*) where individuals with color patterns contrasting with the substrate were more vulnerable to avian attacks (Farallo and Forstner 2012). The analysis of three lizard species from a white sand ecotone (*Holbrookia maculata*, *Sceloporus undulatus* and *Aspidoscelis inornata*) also demonstrates that phenotypic variation in color is quite common in heterogeneous habitats and substrates, which contributes to maintain color polymorphism through selection by differential predation (Rosenblum 2006). There are also examples on how background matching and spatiotemporal variation could be implicated in maintaining polymorphism in poeciliids. In *Poecilia parae*, for instance, changes in the visual background resulted in variations in which different male morphs were most conspicuous to females across multiple localities. Also, the most colorful morphs were the most conspicuous morphs to predators (Hurtado-Gonzalez et al. 2014). However, in the specific case of melanic side-spotting pattern background matching has not been thoroughly studied (Zerulla and Stoddard 2021).

Our findings are important to understanding the mechanisms that can potentially explain color polymorphism in species naturally occurring across highly heterogeneous environments such is the case of *L. vittata* that lives in freshwater and saline habitats. We provide novel evidence on how a measurable variable like salinity level influences polymorphism. In the case of our studied system it seems that this variable is indirectly responsible of maintaining different color morphs likely due to the regulatory effect on predation regimes across saline gradients.

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TABLES

Table 1 Number of *L. vittata* specimens analyzed from multiple populations in Cuba. Populations were selected based on the association with habitats of different salinity levels. Locality code is in parenthesis after the locality name. M: males, F: females

Locality	Collection date	GPS coordinates	Number of melanistic individuals by sex	Number of non-melanistic individuals by sex	Total
Río San Juan, Sierra del Rosario, Candelaria, Artemisa (SJA)	Apr/1982	22.848217, - 82.945286	3 F, 0 M	12 F, 4 M	19
Río San José, Jovellanos, Matanzas (JJM)	Sep/1982	22.776520, - 81.249290	1 F, 0 M	6 F, 3 M	10
Canal del Indio, Surgidero de Batabano, Mayabeque (CIB)	Nov/1984	22.687590, - 82.2268841	4 F, 2 M	28 F, 11 M	45
Zanja del Indio, Surgidero de Batabano, Mayabeque (ZIB)	May/1985	22.691526, - 82.268179	8 F, 1 M	67 F, 3 M	79
Surgidero de Batabano, Mayabeque (SBM)	Feb/1986	22.685079, - 82.290201	4 F, 6 M	16 F, 41 M	67
Laguna de Facundo, Ciénaga de Zapata, Matanzas (LFM)	Mar/2014	22.280148, - 81.163460	3 F, 1 M	6 F, 10 M	20
Península de Hicacos, Matanzas (PHM)	Nov/2015	23.192912, - 81.162099	4 F, 0 M	76 F, 7 M	87
Guanímar, Artemisa (GUA)	Aug/2018	22.694176, - 82.651991	3 F, 2 M	19 F, 15 M	39
Boyeros, Havana (BOH)	Aug/2018	23.045658, - 82.370904	0 F, 0 M	13 F, 0 M	13
El Venero, Morón, Ciego de Ávila (MCA)	Jan/2019	22.075944, - 78.496111	0 F, 0 M	17 F, 14 M	31
Cauto Embarcadero, Granma (CEG)	Jan/2019	20.624414, - 76.928889	0 F, 0 M	6 F, 3 M	9
Río Jobabo, Las Tunas (RJT)	Jan/2019	20.881805, - 77.300997	1 F, 0 M	6 F, 16 M	23

Las Salinas de Brito, Ciénaga de Zapata, Matanzas (LSL)	Jan/2019	22.177989, - 81.253661	28 F, 2 M	49 F, 3 M	82
Río Matagua, Cumanayagua, Cienfuegos (RMC)	Jan/2019	22.052714, - 80.291944	0 F, 0 M	4 F, 1 M	5

FIGURES AND CAPTIONS

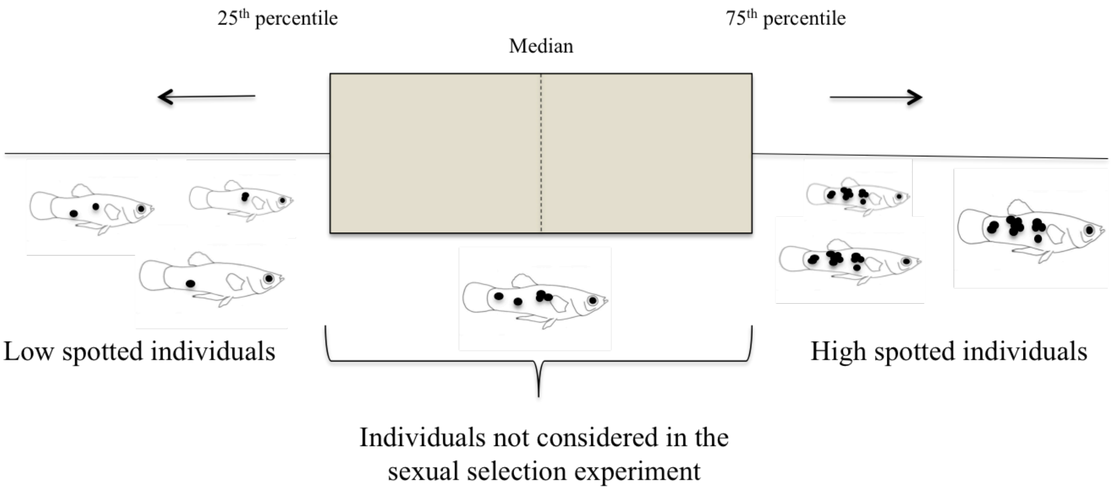

Fig. 1 Classification of stimuli fish in the sexual selection experiment based on the total body area covered by black spots

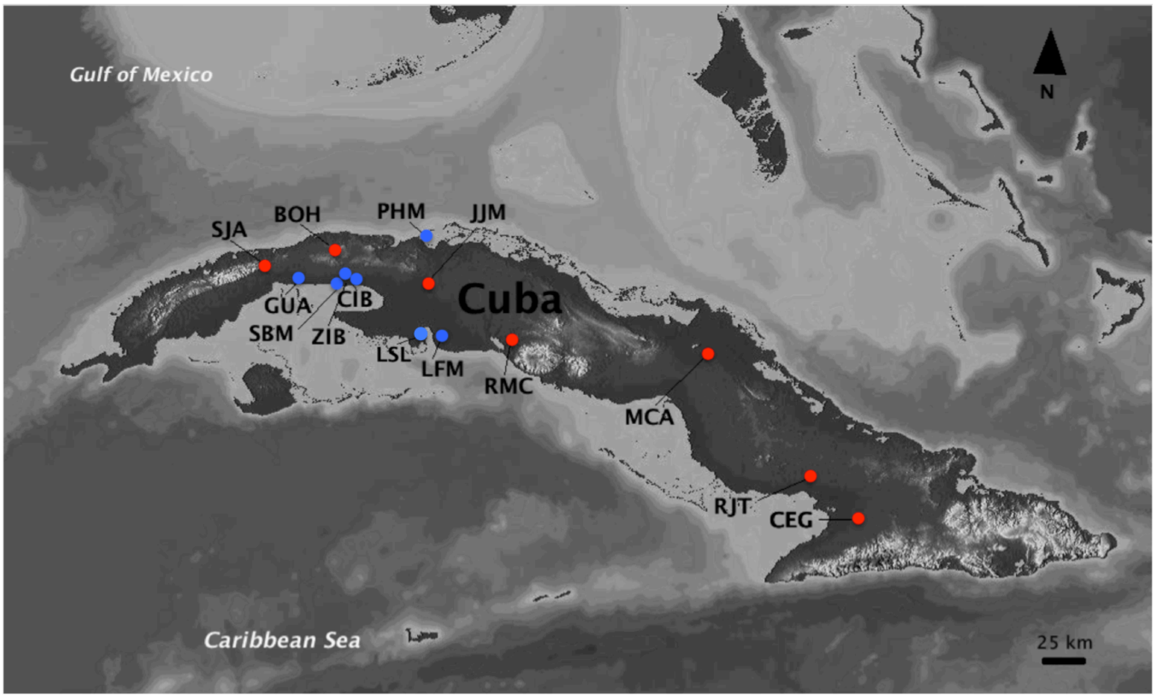

Fig. 2 Geographic distribution of the field sites where *Limia vittata* specimens were collected to evaluate the natural occurrence of melanistic morphs. Locality codes represented by three capital

letters are described in Table 1. Red dots represent freshwater habitats and blue dots brackish or saltwater environments. (Map source: StreetMap contributors)

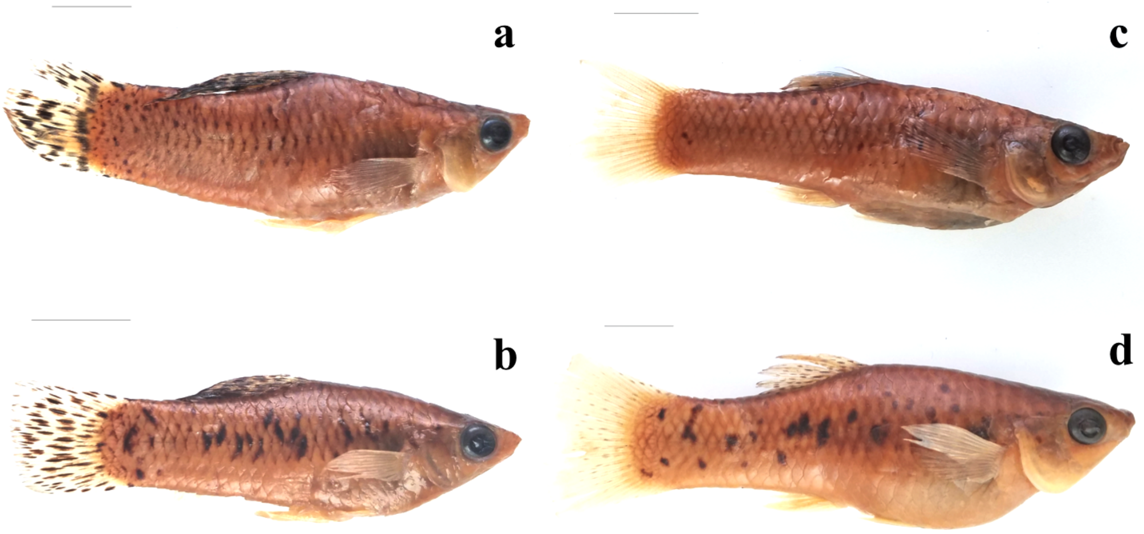

Fig. 3 Photographs of non-melanistic (top) and melanistic morphs (bottom) deposited in the collections of the Instituto de Ecología y Sistemática in Cuba. a.) non-melanistic male (CZACC 9.07), b.) melanistic male (CZACC 9.01), c.) non-melanistic female (CZACC 9.21), d.) melanistic female (CZACC 9.02)

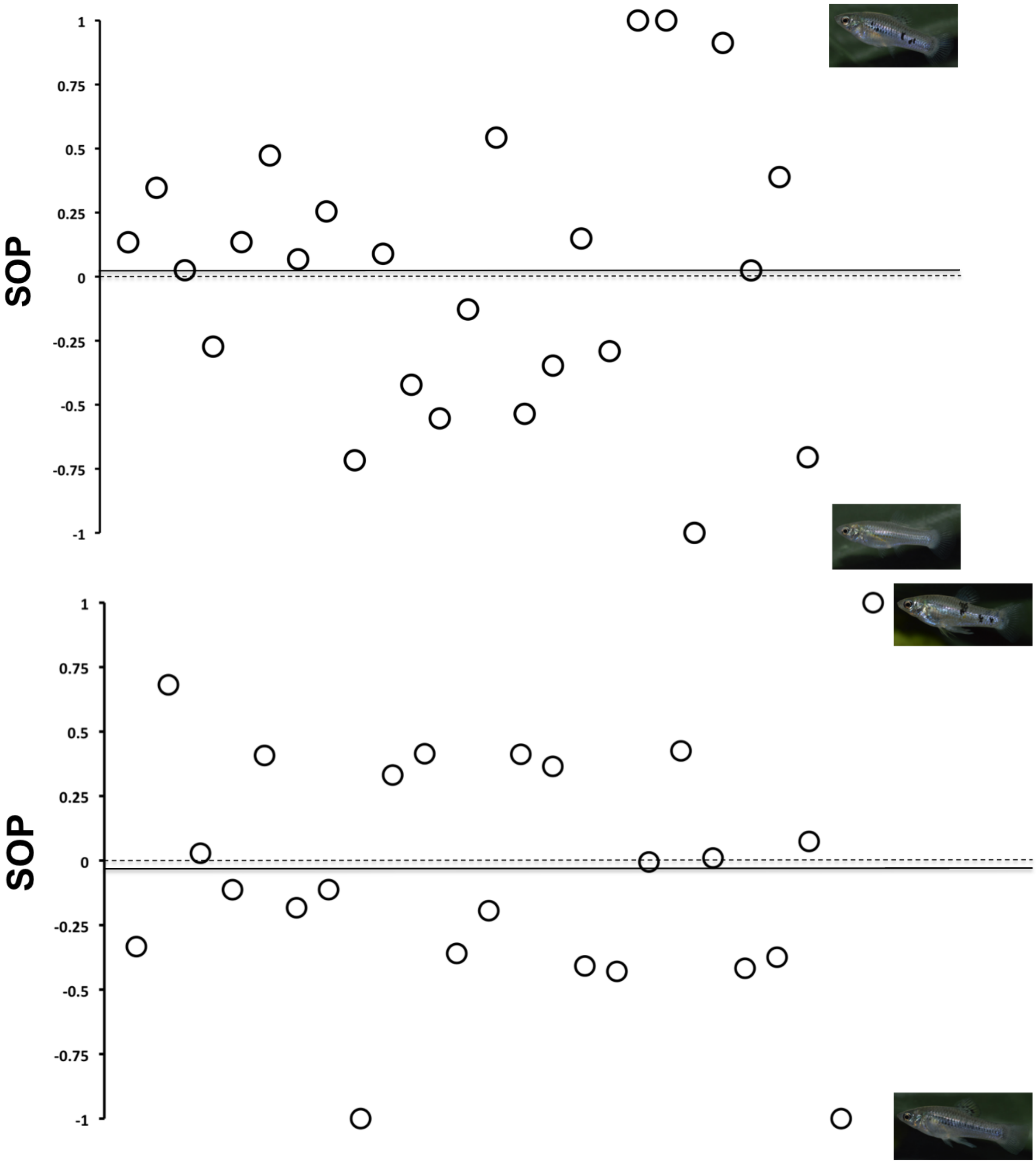

Fig. 4 Distribution of individual strength of preference values (SOP) that resulted from the dichotomous association preference tests. Solid lines in the panels (in both cases close to the dashed lines representing the SOP-value equal 0) correspond to the mean SOP-value for males (top) and females (bottom).

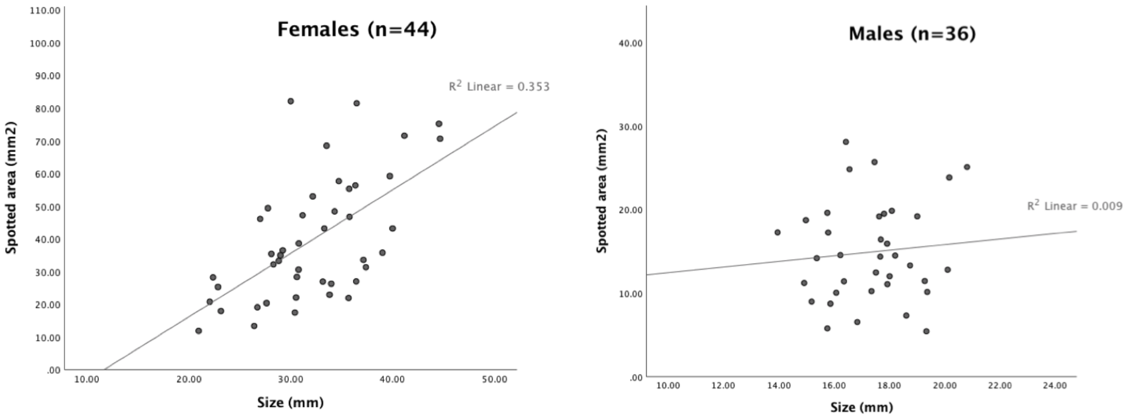

Fig. 5 Representation of the linear regression analysis between size of the fish and spotted area. Females (left panel) showed a positive, strong association between size of the fish and spotted area ($R^2 = 0.353$) while males (right panel) exhibited a positive, weak association between the two variables ($R^2 = 0.009$).

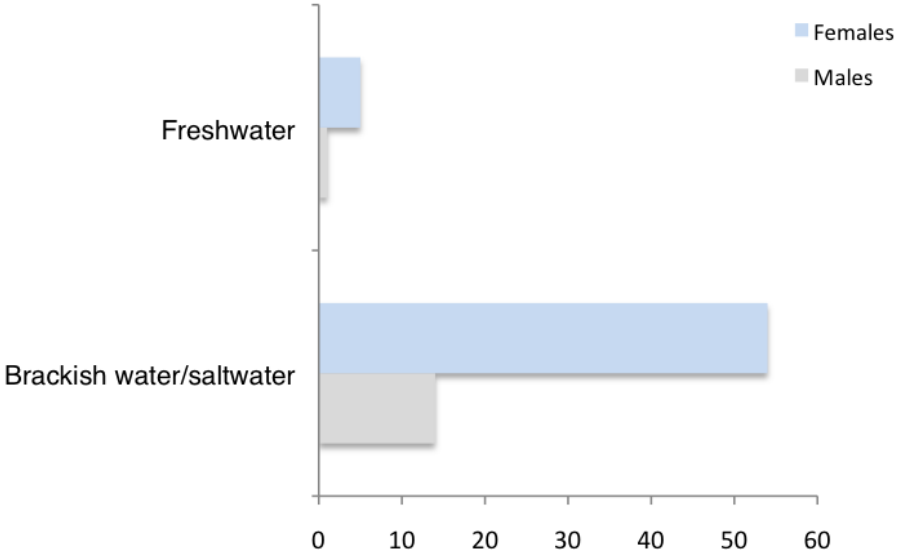

Fig. 6 Frequency of spotted morphs in natural populations of *L. vittata* according to the type of habitat.