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Sympatric speciation is reinforced by predation, parasite load, reproductive character displacement, and sexual selection in two species flocks of pupfish (Cyprinodon spp)

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SYMPATRIC SPECIATION IS REINFORCED BY PREDATION, PARASITE LOAD, REPRODUCTIVE CHARACTER DISPLACEMENT, AND SEXUAL SELECTION IN TWO SPECIES FLOCKS OF PUPFISH (*CYPRINODON SPP*)

by

RHIANNON J.D. WEST

B.S., Psychology, University of New Mexico, 2005

DISSERTATION
Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy

Biology

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DEDICATION

To Mom, Taber, and Joshua,
for always believing in me.

And, in memory of my grandparents,
for supporting me in my favorite endeavors.

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Sympatric speciation is reinforced by predation, parasite load, reproductive character displacement, and sexual selection in two species flocks of pupfish (*Cyprinodon spp*)

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ABSTRACT

Sympatric speciation, the divergence of one lineage into two or more lineages within one geographic range, is often driven by disruptive selection on niche utilization. While niche specialization is found in sympatrically speciated groups, it is often reinforced by other strong selective pressure. This dissertation examines the role of predation, morphology, mate choice, and parasite load in maintaining reproductive isolation in two sympatrically speciated species flocks of pupfish (*Cyprinodon*).

The role that predation, morphology and male and female mate choice play in reproductive isolation is examined in the Bahamian species flock. To examine the role of predation, we focus on two species, a generalist detritivore and a predator/scale-eater. We show that premating isolation is based primarily on visual cues and is asymmetrical. It is well developed in the less abundant scale-eater but is much less well developed in the more abundant detritivore. Responses of detritivore females from three lakes to conspecific and scale-eater males suggest that abundance of the predator also affects development of premating isolating mechanisms in the prey species. These results highlight the importance of frequency-dependent selection and predators in the evolution of premating isolating mechanisms.

The three species, a detritivore, an ostracod-eater, and a scale-eater which preys upon the other two, inhabit distinct trophic niches. We use geometric morphometrics to examine variation in body shape among these three species. Results show that there are significant differences in male, but not female body shape. These results suggest that male body shape, specifically nuchal hump height, is under sexual selection and may contribute to reproductive isolation between species.

Female mate choice reinforces reproductive isolation in sympatrically speciated species. Male mate choice, while not as strong a selective pressure as female mate choice, could also act as a reproductive isolation mechanism in sympatric species. Using a binary choice design, we examine the importance of visual and olfactory cues in female mate choice and focus on the ostracod-eater, the least abundant of the three sympatric species of pupfish. We also examine male mate choice in the scale-eater and the detritivore. Females use visual and not olfactory cues and prefer conspecific males. Males also preferentially associate and court conspecific females. Thus, mutual mate choice acts a strong premating isolation mechanism in these sympatrically speciated pupfish.

Recognition and behavioral avoidance of parasitized conspecifics or individuals with slightly different immunities, either due to differences in genotype and/or regional parasite load, would be favored by natural selection to maintain a healthy phenotype. This avoidance of parasitized conspecifics may play a role in species differentiation in two ways: 1) rapidly changing genes that encode for immune function, such as the Major

Histocompatibility Complex may lead to small, localized differences in immune function. If small differences in immune function and parasite load drive behavioral avoidance of conspecifics, then reproductive barriers within a population are effectively formed through non-random mating. If such nonrandom mating barriers persist, they may lead to isolation of gene pools and subsequently, sympatric speciation; 2) Changes in parasite resistance may be tightly coupled with trophic differentiation as parasitic infection often occurs when organisms are foraging. To address this question, we performed two experiments to examine how parasites affect female choice in sympatrically speciated pupfish (*Cyprinodon spp.*) from the Yucatan, Mexico. We infected and sham infected male *C. labiosus* and *C. maya* with *Ichthyophonus hoferi.* In a series of binary choice trials, we presented female *C. labiosus* and *C. maya* with olfactory cues from infected and non-infected conspecific males. Then we presented the females with odors from infected conspecific males and non-infected heterospecific males. Results indicate that infection was successful in both species but varied in infectivity. Our findings show that females preferred non-infected conspecific males and suggest that reproductive barriers may be formed by female preference for healthy male conspecifics. Additionally, reproductive barriers between species are strong and prevent mating with heterospecific males.

Overall this dissertation shows that multiple selective pressures contribute to and reinforce reproductive isolation. Future studies should take care to examine the roles of sexual selection, frequency dependence, ecological pressures beyond trophic niche usage, mate choice, morphology and other common methodologies such as genetic inference.

Realistic models of sympatric speciation likely include a non-random collection of these and similar variables.

Table of Contents

Introduction

Sympatric speciation is the divergence of one lineage into two or more lineages within a geographic region (Coyne and Orr 2004). There are two commonly studied hypotheses of what selective pressures create and maintain sympatric species. The first, ecological speciation, occurs when gene flow is limited by ecologically-based disruptive selection. Selection favors specialists and disfavors intermediate phenotypes. The fitness cost of intermediate offspring leads to reproductive isolation between morphotypes. Distinct ecomorphs are then under both natural and sexual selection leading to limited gene flow and, ultimately, speciation (e.g. Dieckmann et al. 2004). This form of sympatric speciation has many studies with supportive results indicating that it does occur (as reviewed in Weissing, Edelaar, and van Doorn 2011). The second process of sympatric speciation occurs when there is divergence of (generally) male mating types linked to female preference for diverse mating types. The disruptive selection in this case acts upon male mating types and linked female preferences. There is little empirical evidence in the literature to support a primarily sexually selected sympatric speciation event. The literate does support examples of strong sexual selection facilitating ecological speciation (as reviewed in Ritchie 2007 and Weissing, Edelaar, and van Doorn 2011).

In this dissertation, we examine the role of two previously under examined ecological pressures in sympatric speciation and the role of both female and males mate choice in maintaining species boundaries. First, we examine the role of predation in reproductive isolation in a sympatrically speciated flock of pupfish (*Cyprinodon spp.*) from The Bahamas. Then we use geometric morphometrics to see what shape characters differ between species, sexes. These differences are likely used in female choice for both species discrimination and intraspecific mate choice. Finally we discuss the role of both female and male mating preferences in maintaining species boundaries in all three species in the Bahamian species flock. The last chapter delves into the role of parasite in maintaining species boundaries in a second sympatrically speciated flock of Mexican pupfish.

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Chapter 1: Asymmetries in premating isolating mechanisms of a sympatric species flock in pupfish (*Cyprinodon*)

Astrid Kodric-Brown and Rhiannon J.D. West

Abstract

We examine cues used in species recognition and assortative mating in a sympatric species flock of pupfishes (*Cyprinodon*) from San Salvador Island, The Bahamas. The species are morphologically, genetically, and ecologically distinct. We focus on two species, a generalist detritivore and a predator/scale-eater. Experiments show that premating isolation is based primarily on visual cues and is asymmetrical. It is well developed in the less abundant scale-eater but is much less well developed in the more abundant detritivore. Responses of detritivore females from three lakes to conspecific and scale-eater males suggest that abundance of the predator also affects development of premating isolating mechanisms in the prey species. These results highlight the importance of frequency-dependent selection and predators in the evolution of premating isolating mechanisms.

Key words: visual cues, olfactory cues, sympatric speciation, frequency-dependent selection, scale-eater, detritivore

Introduction

Premating isolating mechanisms in sympatric species have been the focus of many theoretical and empirical studies. Models of sympatric speciation focus on the role of female choice and assortative mating (Machteld 2005; Servedio 2011; Bolnick and Kirkpatrick 2012) distribution of resources (Thibert-Plante and Hendry 2011) and reinforcement (Kraak and Hart 2011). Empirical studies of model systems such as sticklebacks (Schluter 2000), cichlids (Schliewen et al. 2001; Seehausen 2006) arctic char (Jonsson and Jonsson 2001), walking-stick insects (Nosil 2007), *Rhagoletis* fruit flies (Hood et al. 2012), *Heliconius* butterflies (Jiggins et al. 2001), and poison frogs (Maan and Cummings 2008) have shown how the proposed mechanisms operate singly or in combination in these systems. However, behavioral studies focusing on breeding behavior and mate choice are still needed to better understand how non-random mating develops and leads to the formation and maintenance of sympatric species.

Sympatric speciation is the divergence of one species into two or more lineages without geographic isolation (Coyne 2007). The resulting species are monophyletic, descended from a common ancestor that invaded and diverged without geographic isolation. Disruptive selection to occupy distinct ecological niches leads to assortative mating and evolutionary divergence, culminating in genetically distinct species. Recent studies have focused on behavioral and ecological mechanisms underlying this process of divergence. There is general agreement about the importance of environmental processes, such as trophic differentiation to exploit different diets, with corresponding ecological, morphological, physiological, behavioral and genetic divergence (Schliewen et al. 2001; Barluenga et al. 2006; Elmer et al. 2010a,b). The role of sexual selection in this process is still debated, however, because assortative mating by itself does not necessarily result in speciation (e.g. Arnegard and Kondrashov 2004; Leithen et al. 2012). However, sexual selection may facilitate differentiation if it leads to assortative mating based on traits such as diet, body size, or habitat (Herder et al. 2008; Maan and Seehausen 2011; Weissing et al. 2011).

Empirical studies of sympatric species ‘flocks’ have inferred the existence of premating isolation based on genetic structure and differentiation, or on observations of assortative mating based on color patterns (Jiggins et al. 2001; Maan and Cummings 2008; Seehausen et al. 2008; Elmer et al. 2009; Rudh et al. 2011), or body size (McKaye 1986). Although there are many studies on female mating preferences for male traits (e.g. Kozak et al. 2008), relatively few of them have addressed mate choice in sympatric species (but see: pupfish: Strecker and Kodric-Brown 1999, 2000; Kodric-Brown and Strecker 2001; sticklebacks: Ward et al. 2004; Rafferty and Boughman 2006; cichlids: Seehausen et al. 2008). Traits which function in both ecological differentiation and mate choice (e.g.‘magic traits’: Gavrilets 2003; review by Servedio et al. 2011) can lead to rapid speciation. If females identify conspecific males based on traits associated with ecological characteristics (e.g. diet, predation) then a genetic association between ecological and mating traits can potentially lead to rapid speciation (e.g. Ward et al. 2004; Reynolds and Fitzpatrick 2007; Rudh et al. 2011; Merrill et al. 2012).An interesting aspect of this process is the role of predators, which can potentially inhibit or promote divergence between sympatric species (Vamosi 2005). Both theory and some experimental studies suggest that predators can inhibit the evolution of premating isolating mechanisms if females pay increased survival costs while searching for mates (e.g. Forsgren 1992; Milinski and Bakker 1992; Godin and Briggs 1996). Other studies suggest that predators may enhance the formation of premating isolating mechanisms (Jiggins et al. 2001; Nosil and Crespi 2006; Maan and Cummings 2008). Nosil and Crespi (2006) showed that adaptive divergence in the color patterns linked to host-plant use of two species of walking stick insects was greater in the presence of predation. Similarly, divergence of benthic and limnetic sticklebacks was greater in the presence of predatory fish (Rundle et al. 2003).

Frequency-dependent selection can also facilitate assortative mating in sympatric species. With high frequencies of conspecifics, probabilities of encountering individuals of another species are low, so selection for cues promoting assortative mating should be relaxed. However, if frequencies of conspecifics are low and encounter rates with heterospecifics are high, then selection for cues promoting assortative mating should be strong (Strecker and Kodric-Brown 1999, 2000). This suggests that premating isolating mechanisms will be stronger in the rarer species in sympatric species flocks.

In this study we quantified premating isolation and examined the sensory cues used to identify conspecifics in two sympatric species of pupfish (*Cyprinodon*) in three lakes in The Bahamas. We focused on the behavior of females, which choose mates and spawn with males on lek-like territories. We compared premating isolation in the less-abundant predator species (scale-eater), a trophic specialist, with that of the more abundant generalist prey species (detritivore) to determine if the strength of premating isolation is frequency-dependent and better developed in the trophic specialist than in the generalist.

The system –

The pupfish species complex on San Salvador Island (24^0 0' N 74^0 40' W) provides a unique opportunity to study the behavioral mechanisms of speciation, especially processes that drive the evolution of reproductive isolation. Since one of the species is a predator (scale-eater), it also provides an opportunity to examine the role of predation on the development of reproductive isolation in the prey (detritivore). This is the only case of scale-eating in *Cyprinodon*. Scale-eating is rare, and most species flocks of freshwater fish have at most a few scale-eating species (e.g. Fryer et al. 1955; Fryer 1972; Sazima 1983).

The species flock consists of three morphologically and genetically distinct and as yet undescribed species of pupfish (Cyprinodontidae:*Cyprinodon*) that occur sympatrically in some of the inland lakes on San Salvador Island, The Bahamas. The three species represent a monophyletic clade (Martin and Wainwright 2011). The likely ancestor is *Cyprinodon variegatus*, which is widely distributed and ranges from the Atlantic coast of North America throughout the Gulf of Mexico, the Caribbean, and The Bahamas. The three species are of recent origin, since the inland lakes were dry as recently as 6,000 years ago (Pacheco and Foradas 1986; Milliken et al. 2008). The lakes are hypersaline and have a depauperate ichthyofauna consisting of pupfish (*Cyprinodon spp*.), mosquito fish (*Gambusia hubbsi*) and silverside (*Atherinomorus stipes*)(Barton and Barton 2008). The three species of pupfish differ in diet and exhibit corresponding morphological specializations (Holtmeier 2001; Martin and Wainwright 2011). The most abundant species in all lakes is the detritivore, which feeds on detritus and algae. The scale-eater is a predator and consumes scales and fin parts of other pupfish, mostly the abundant detritivore. The molluscivore feeds on hard-shelled invertebrates. The scale-eater and the molluscivore are less abundant than the detritivore and occur only in a few lakes (Holtmeier 2001; Barton and Barton 2008).

Based on microsatellite variation, the three species are genetically distinct, and hybrids are rare in the field, suggesting that there is a high level of assortative mating (Turner et al. 2008). Postmating isolation is not complete, however, since viable hybrids are produced in the laboratory (Holtmeier 2001; Martin and Wainwright 2013). The detritivore and scale-eater differ in morphology, behavior, and in male breeding coloration (Fig. 1A,B). Breeding scale-eater males acquire a nearly black coloration on the body and fins. Breeding detritivore males display the typical color patterns seen in most pupfishes –blue iridescence on the nape and reddish orange fins and belly. However, as in most pupfishes, male breeding coloration is variable and can be turned on or off almost instantaneously (Kodric-Brown 1998; Barton and Barton 2008). Males of both species establish territories, but they differ in location and types of substrates. Scale-eaters establish ‘leks’, clusters of territories with well-defined boundaries on heterogeneous substrates, primarily rocky outcrops covered with algae and mangrove oysters, while detritivore males defend more dispersed territories with poorly defined boundaries over sandy or muddy substrate and vegetation. The differences in breeding habitat are only qualitative and partial, however, and females of both species have ready access to breeding territories of heterospecific and conspecific males. Male courtship of scale-eaters consists of a series of rapid darting movements toward the female, while detritivores show the typical courtship behavior of most pupfish species, circling below a female and guiding her toward the substrate of their territory. The third species, the molluscivore, is rarely collected and occurs in deeper water (Barton and Barton 2008). Little is known about their biology and interactions with the other two species.

Methods

We determined the presence and strength of premating isolating mechanisms in wild-caught scale-eaters and detritivores from three lakes (Crescent, Osprey and Little Lake; Fig. 1C) by quantifying female preferences for conspecific males. The lakes are shallow, with a maximum depth of 4.5 m, hypersaline (over 35 ppt), have similar habitats (including both rocky outcrops and sandy/muddy areas) but differ in size and relative abundance of scale-eaters (Holtmeier 2001; Barton and Barton 2008; Turner et al. 2008). In each lake both species were collected with minnow traps baited with crushed mangrove oysters. Trapping efforts at each lake were standardized by sampling 10 different sites for 80 minnow trap days at each site. In order to adequately sample all habitats, we placed traps in open areas favored by the detritivores, and on, or adjacent to, rocky outcrops where scale-eaters males established their territories. Fish were placed in buckets, transported to the Gerace Research Center, and the water in the buckets was gradually diluted with sea water. After 24 hr of acclimation, fish were transferred to concrete holding tanks filled with sea water. Species were kept in separate holding tanks but sexes of each species were kept together. After completion of the trials, fish were returned to the locality where they were collected.

We examined the role of visual and olfactory cues using a binary choice design. We used 41 scale-eaters and 41 detritivores (25 females and 16 males of each species) from Little Lake (May 10-27, 2011), 32 scale-eaters (22 females and 10 males) and 36 detritivores (22 females and 14 males) from Osprey Lake (May 27- June 6, 2011) and 41 scale-eaters and 41 detritivores (25 females and 16 males of each species) from Crescent Lake (May 7-20, 2012). All fish were adults, and males were in breeding coloration.

The size distribution of scale-eaters and detritivores was similar (scale-eater males: 22.6 mm – 34.6 mm, females: 23.8 mm – 33.9 mm; detritivore males: 23.7 mm – 33.7 mm, females: 23.5 mm – 36.1 mm). Each female was used once and tested for both visual and olfactory mate choice. However, some male scale-eaters from Osprey Lake were used more than once in novel combinations with different females.

Visual Cues

A pool (91.5 cm in diameter) was filled to a depth of 10 cm with ~65.8 L of sea water. Two clear 7.6 L Ziploc® bags (33.02 cm x 39.68 cm) were suspended from the ceiling, filled with 4 L of sea water to the level of the water line in the pool, and positioned 10 cm from the walls of the pool. Males of each species were size-matched within 1.62 mm for trials using scale-eating females and 2.34 mm for trials using detritivore females. Two male detritivores were placed into one bag and two male scale-eaters were placed into the other bag. The males remained in their respective bags for 24 hours and were only removed once for feeding. Air stones were used to oxygenate the males' water except during the visual trials. A female was placed in the pool equidistantly from the two sets of males. The trial began when the female swam freely around the pool and visited both sets of males. Her behavior was recorded for 30 min. with a pair of stopwatches. Female visual preference for males of each species was quantified as the time she spent actively touching or swimming within 1 cm of the bags. We videotaped each set of males for 15 min to quantify time spent actively swimming courtship displays, and agonistic behavior (chases, fights). Courtship displays consisted of a male orienting and facing the female, often touching the partition next to the female, swimming up and down or side to side with exaggerated movements of the pectoral fins and closely following the female’s movements. Fighting involved two males circling each other and occasionally biting the opponent.

Olfactory Cues

Olfactory trials tested whether females of each species could recognize (1) the odors of conspecific males and (2) discriminate between the odors of conspecific and heterospecific males. Each part of the trial lasted 15 min. A 22 L aquarium (PETCO Bookshelf aquarium, 60 cm long x 20.65 cm wide) was filled with 20 L of sea water. Two lines were drawn on the front of the aquarium, delineating 3 “compartments.” The two outer compartments were 17 cm wide and the middle one was 26 cm wide. A one L capacity (Tyco Healthcare “Kendall Kangaroo” IV) intravenous bag was suspended from the ceiling over each end of the aquarium. In the first part of the trial one of the IV bags was filled with 200 mL of sea water (control) and the other with water in which two conspecific males were kept for 24 hr during the visual trials (see above). We used these bags because we could accurately calibrate the drip rate of the ‘male stimulus’ water and dispose of them after use. The IV bags were calibrated to deliver 1 drop per s (45 mL during each trial). A female was introduced into the central ‘compartment’ of the tank. A trial began when the female started to swim and explored all parts of the aquarium. Females of each species were first presented with water from conspecific males and seawater. At the end of the trial the female was moved into another tank, allowed to acclimate, and given a choice between water from conspecific males and water from heterospecific males. The position of the IV drip that delivered water from conspecific males was changed between the first and second part of the trial to control for any potential side bias.

Male Behavior:

We recorded the behavior of each set of males during visual trials with a Panasonic camcorder (HDC-TM80) placed on a tripod and a visual field that encompassed the container with the male and a 5 cm area around it. To determine if males of each species differed in courtship of conspecific and heterospecific females, we divided the time males spent courting a female by the total time the female was actively investigating males of each species. We also analyzed the proportion of time each set of males was active (swimming, courting and chasing).

Statistical analysis

Visual and Olfactory trials

To determine if visual and olfactory preferences were correlated, we used the same females and the same males in both the visual and olfactory trials. Since the data on visual and olfactory preferences were normally distributed in all trials, no transformations were required. Results of the visual and olfactory trials were analyzed with Lawley-Hotelling MANOVAS to assess the effects of species, lake, and lake-by-species interaction on time females spent associating with conspecifics and heterospecific males. Significant effects were then examined with post-hoc Tukey's HSD tests. To determine if female preferences were consistent between the visual and olfactory trials, MANOVAs with female species, lakes and a lake-by-species interaction were performed on the difference between time a female spent assessing conspecific males in visual and olfactory trials. Post-hoc Tukey's HSD tests were used to examine significant effects. Data on male activity during visual trials failed normality tests, even after transformation;

a Kruskal-Wallis test was used to determine if male activity differed between males, between species of females, or between lakes. Post-hoc Bonferroni corrected ($\alpha = 0.002$) Mann-Whitney tests were used to examine pairwise comparisons. All data were analyzed with Minitab 16 software.

Results

In all three lakes scale-eaters were less abundant than detritivores. The ratio of scale-eaters to detritivores was 1:185 in Little Lake and 1:189 in Osprey Lake. Relative abundance of scale-eaters was an order of magnitude higher in Crescent Lake where the ratio was 1:19 scale-eaters to detritivores. To obtain sufficient scale-eaters for behavioral trials, additional collecting was done on or near rocky outcrops where scale-eater breeding territories were concentrated.

Visual trials

Visual preferences of all females differed between species ($F_{2,136}= 4.857$, $p<0.009$), lakes ($F_{4,272,}=11.977$, $p<0.000$), and there was a significant interaction between species and lakes ($F_{4,272,}= 3.991$, $p<0.004$). Scale-eater females from all three lakes spent a significantly greater proportion of each trial with conspecific males (Fig. 2). Strength of visual preferences of scale-eater females for conspecific males differed between lakes. Scale-eater females from Little Lake and Crescent Lake spent significantly more time with conspecific males than those from Osprey Lake (Fig. 2).

Visual preferences of detritivore females for conspecific males differed between lakes. Only detritivore females from the smallest lake, Crescent, spent significantly more time with conspecific males (Fig. 2).

Olfactory trials

A MANOVA on all females from the three lakes showed a significant effect of lake ($F_{8,262}$=1.73, p=0.09) and species-by-lake interaction ($F_{8,262}$=3.02, p=0.003) but not of species ($F_{4,131}$=0.68, p=0.61). Scale-eater females did not discriminate between the odors of conspecific males, heterospecific males, or water (Fig. 3). In Osprey Lake detritivore females preferred the odor of conspecific males to heterospecific males (Fig 3).

Male behavior

Males differed in their activity (H_{11} = 37.18, P = 0.0001). Males of both species spent most of their time courting, followed by swimming and chasing. Overall levels of activity (courting, chasing and swimming) differed between lakes but not species (Table 1). Both scale-eater and detritivore males from Crescent Lake were more active than males from Little Lake (P<0.001) and Osprey Lake (P<0.000; Table 1). Scale-eater males spent more time courting scale-eater females than detritivore females (P<0.001). Detritivore males divided their time equally courting detritivore and scale-eater females (Table 1).

Discussion

Comparisons of breeding behaviors and mate choices in Mexican and San Salvador Island species flocks, the only two well-studied examples of sympatric speciation in pupfish, provide unique insights into the roles of ecological factors and sexual selection in the formation of isolating mechanisms. Both sympatric species flocks formed within the last 12,000 years (Covich and Stuyver 1974; Milliken et al. 2008) and occur in shallow saline lakes with few competitors (Turner et al. 2008; Humphries and Miller 1981). Both species flocks are monophyletic, descended from morphologically and ecologically similar sister species (San Salvador Island: *C. variegatus*, Mexico: *C.*

artifrons; Martin and Wainwright 2011), and both show genetic and trophic divergence among members of the species flock (Holtmeier 2001; Horstkotte and Strecker 2005; Strecker 2006). Comparisons of these two species flocks provide insights into three aspects of the formation of isolating mechanisms in sympatric populations.

Difference signals

The two species flocks differ in the signals that are used in mate recognition and result in assortative mating. Species isolation in San Salvadoran pupfishes is based on both visual and olfactory cues. Scale-eaters and detritivores differ in body shape, jaw morphology and breeding coloration (Fig. 1A,B; Martin and Wainwright 2011). In all three lakes, scale-eater females showed a strong preference for conspecific males based on visual, but not olfactory cues. In detritivores, both visual and olfactory cues were used to identify conspecifics: visual cues in Crescent Lake and olfactory cues in Osprey Lake (Figs. 2,3). Strength of preference for conspecific males also varied for detritivore females, from strong (Crescent and Osprey) to non-existent (Little Lake). Thus the two species differ not only in the development of premating isolating mechanisms but also in the sensory modalities used to identify conspecifics.

In two of the three species of Mexican pupfish (*C. labiosus* and *C. maya*) examined to date, premating isolation is based on olfactory cues which reflect differences in diet, rather than on visual cues (Strecker and Kodric-Brown 1999, 2000; Kodric-Brown and Strecker 2001). Thus the only two cases of sympatric speciation in pupfish do not show parallel evolution, a familiar paradigm of many other species flocks (Schluter 2000; Langerhans and Dewitt 2004; Landry et al. 2007; Elmer et al. 2010a,b). Diversification in both systems is based on trophic divergence, but resource partitioning differs for the top predators. On San Salvador Island the top predator is a scale-eater, similar in size to the other pupfish species, while in the Mexican species flock it is a specialist (*C. maya*), feeding on mollusks as well as other fish (Horstkotte and Strecker 2005) and is much larger than the other pupfishes. Superficially similar ecological conditions have resulted in sympatric formation of species flocks with different trophic niches.

Premating isolation is asymmetrical

Premating isolating mechanisms are well developed in the scale-eater, a trophic specialist, but reduced or absent in the detritivore. An experimental study of hybrids generated from crosses and back-crosses between detritivores and scale-eaters showed that hybridization resulted in reduced growth and lower survival of scale-eater phenotypes compared to the detritivore phenotypes (Martin and Wainwright 2013). The scale-eater species appears to have specialized coevolved traits adapted for its specialized trophic niche. There is strong selection for positive assortative mating in scale-eaters because their complex of traits is lost after hybridization. Our results suggest that frequency-dependent processes also contribute to the development of premating isolation. Because of their specialized trophic niche, scale-eaters are less abundant than detritivores in all three lakes. Stronger preferences for conspecifics in scale-eaters than in detritivores suggest that selection for positive assortative mating is stronger in the less abundant scale-eaters than in the more abundant detritivores.

Comparisons with the Mexican species flock provide further insights into the importance of relative abundance and frequency-dependent selection in the development of premating isolation and reinforcement of assortative mating. A molecular phylogeny of the *Cyprinodon* clade suggests that frequency-dependent selection accentuates the rate of morphological diversification in the San Salvador Island and Mexican sympatric species flocks (Martin and Wainwright 2011). The highest rates of morphological diversification occurred in the two trophic specialists (scale-eater and *C. maya*) with small population sizes. Given selection for positive assortative mating is required to preserve the specialized traits of predators, such selection should be much stronger on the rarer species. The much more common detritivore species would encounter and mate with conspecifics frequently by chance alone, even in the absence of assortative mating. The rare trophically specialized species, however, would require cues to selectively find and mate with conspecifics and avoid hybridization. The evolution and maintenance of rare, trophically specialized species in sympatric species flocks appears to depend on the coevolution of strong conspecific mating preferences along with ecological traits related to feeding niches. *Predators facilitate premating isolation*. Abundance of scale-eaters and the proximity of breeding areas of the two species in the two smaller lakes, Crescent and Osprey, may reinforce premating isolation based on visual and/or olfactory cues in detritivores, which appear to actively avoid scale-eaters. It is difficult to generalize from other studies on the effects of predators in reinforcing diversification in sympatric species (Rundle et al. 2003; Vamosi 2005; Nosil and Crespi 2006), since in the San Salvador pupfishes the predator (scale-eater) is a member of the species flock and is closely related to the prey (the detritivore). More than 40% of detritivores in our samples showed signs of scale removal by scale-eaters (personal observation). Thus the impact of scale-eaters on their prey may be substantial and may select for recognition of conspecifics and assortative mating in detritivores in Crescent Lake and Osprey Lake.

The results of this study on the development of premating isolation in sympatric pupfishes support several assumptions of models of sympatric speciation. Strong reproductive isolation requires substantial phenotypic divergence and assortative mating (Bolnick and Langerhans 2012); cues used in assortative mating are associated with ecological parameters that reflect trophic specialization, thus represent ‘magic traits’ (Gavriletts 2003; Servedio et al. 2011). Under these conditions premating isolation can be established quickly and leads to speciation. Our results also agree with findings of empirical studies of other sympatric closely related species, which identify cues associated with conspecific matings based on color patterns (e.g. cichlids: Seehausen et al. 2008; poison frogs: Maan and Cummings 2008) or chemical cues (e.g. sticklebacks: Ward et al. 2004; Rafferty and Boughman 2006). Other results of our study seem not to have been addressed in theoretical or empirical studies of sympatric speciation. For example, our results highlight the importance of frequency-dependence in the development of premating isolation, and of asymmetries in the strength of premating isolation which result from strong directional selection on trophic specialists and normalizing (relaxed) selection on generalists.

Our results call attention to the need for additional studies of the behavioral aspects of mate choice in species flocks. An understanding of the divergence of sympatric lineages requires knowledge of the cues used in assortative mating. For example, in the Mexican species flock, females of the abundant detritivore species (*C. beltrani*) did not distinguish conspecific males from males of a less abundant trophic specialist (*C. labiosus*), but female *C. labiosus* preferentially mated with males of their own species (Strecker and Kodric-Brown 1999). These results, and those presented herein, show that divergence between sympatric species can occur even when mating preferences are strong in one species and weak or non-existent in another.

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Tables

Table 1: Behavior of scale-eater and detritivore males toward conspecific and heterospecific females during visual trials in Little Lake (LL), Osprey Lake (OL) and Crescent Lake (CL). Data were converted to proportion of time during a 15 min. trial. Comparisons are for species between lakes and for species within lakes. Results are Bonferroni corrected Mann-Whitney U post-hoc pairwise comparisons significant at $P < 0.002$. Significant differences are in bold.

Behavior	Comparisons	N	Median	U	P-value
Court, Chase, Swim	Scale-eater males vs.	393	0.048	150723	0.2922
	Detritivore males	390	0.064		
Court, Chase, Swim	Both male species: CL vs.	273	0.093	85178	0.0001
	LL	297	0.039		
Court, Chase, Swim	Both male species: CL vs.	273	0.093	73852	0.0000
	OL	213	0.021		
Court, Chase, Swim	Both male species: LL vs.	297	0.039	77506	0.2416
	OL	213	0.021		
Court	Scale-eater male/ scale-eater female vs.	69	0.240	5268.5	0.0010
		62	0.082		

	Scale-eater male/ detritivore female				
Court	Detritivore male/ scale-eater female vs.	68	0.238	4580.5	0.5569
	Detritivore male/ detritivore female	62	0.168		

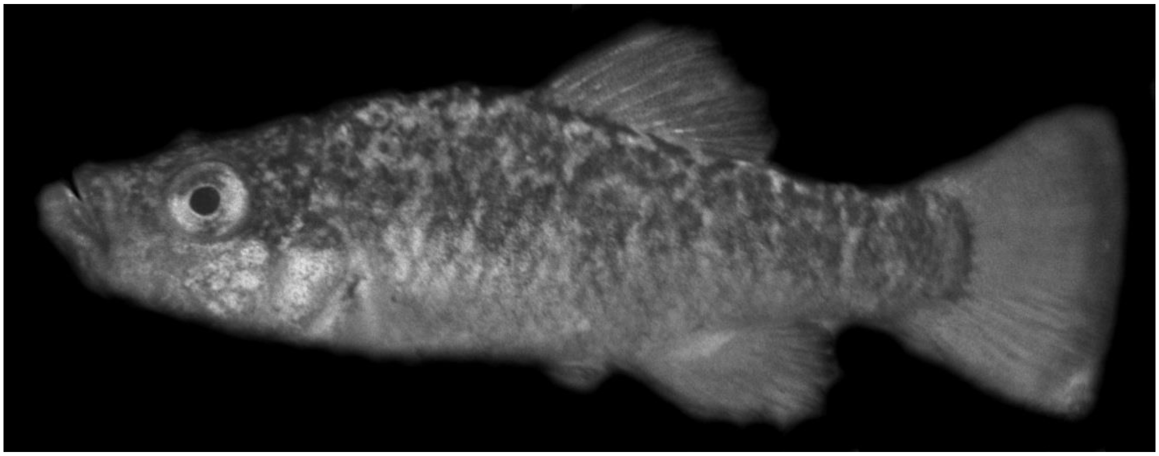
Figures

Figure 1.(A). Scale-eater male from Crescent Lake (B). Detritivore male from Crescent Lake. (C). Map of the north end of San Salvador Island, The Bahamas, showing the location of Crescent Lake, Osprey Lake and Little Lake.

Figure 2. Association time (s) of scale-eater females and detritivore females from Crescent Lake, Osprey Lake and Little Lake with conspecific and heterospecific males in visual trials. Pairwise comparisons that do not share a letter are significant at $p < 0.05$.

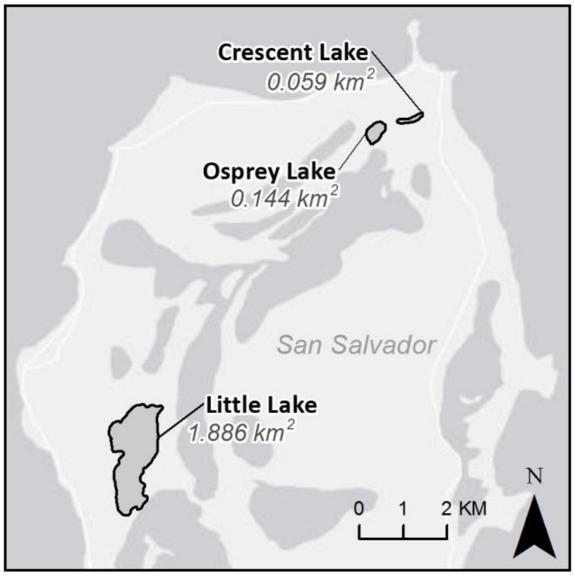
Figure 3. Association time (s) of scale-eater females and detritivore females from Crescent Lake, Osprey Lake and Little Lake with conspecific and heterospecific males in olfactory trials. Pairwise comparisons that do not share a letter are significant at $p < 0.05$.

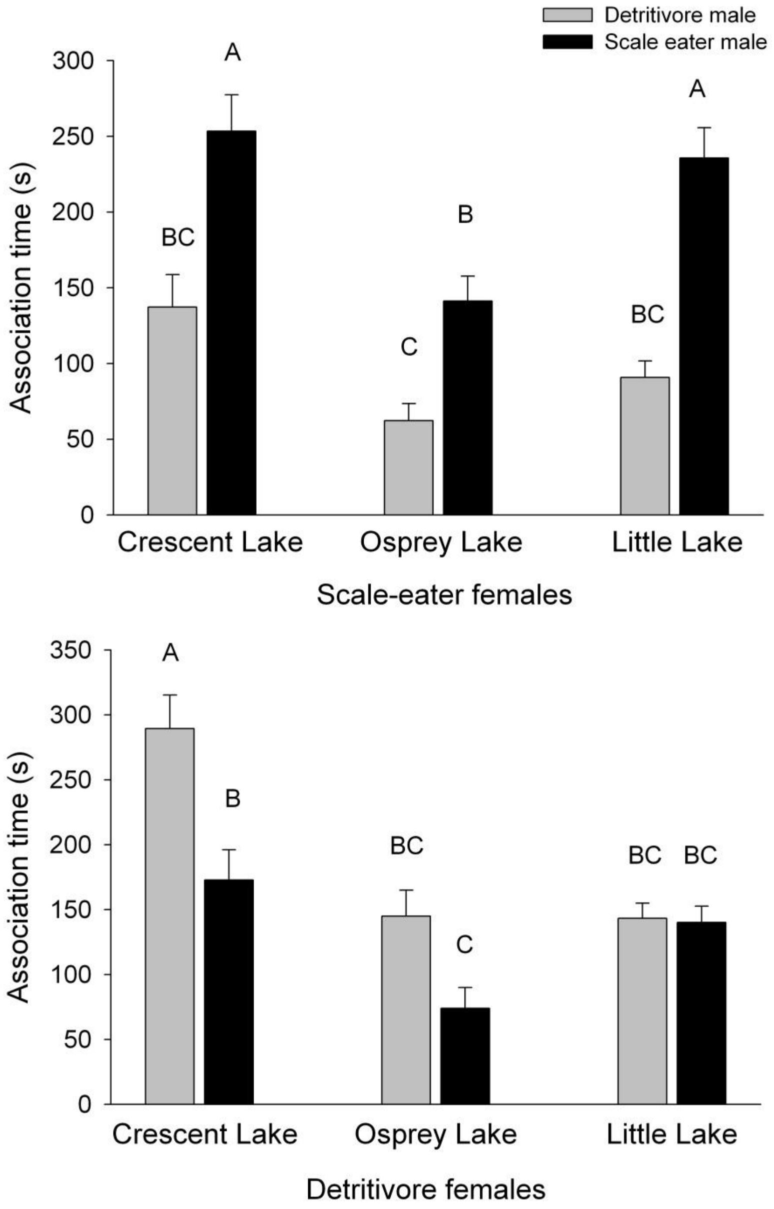
A.

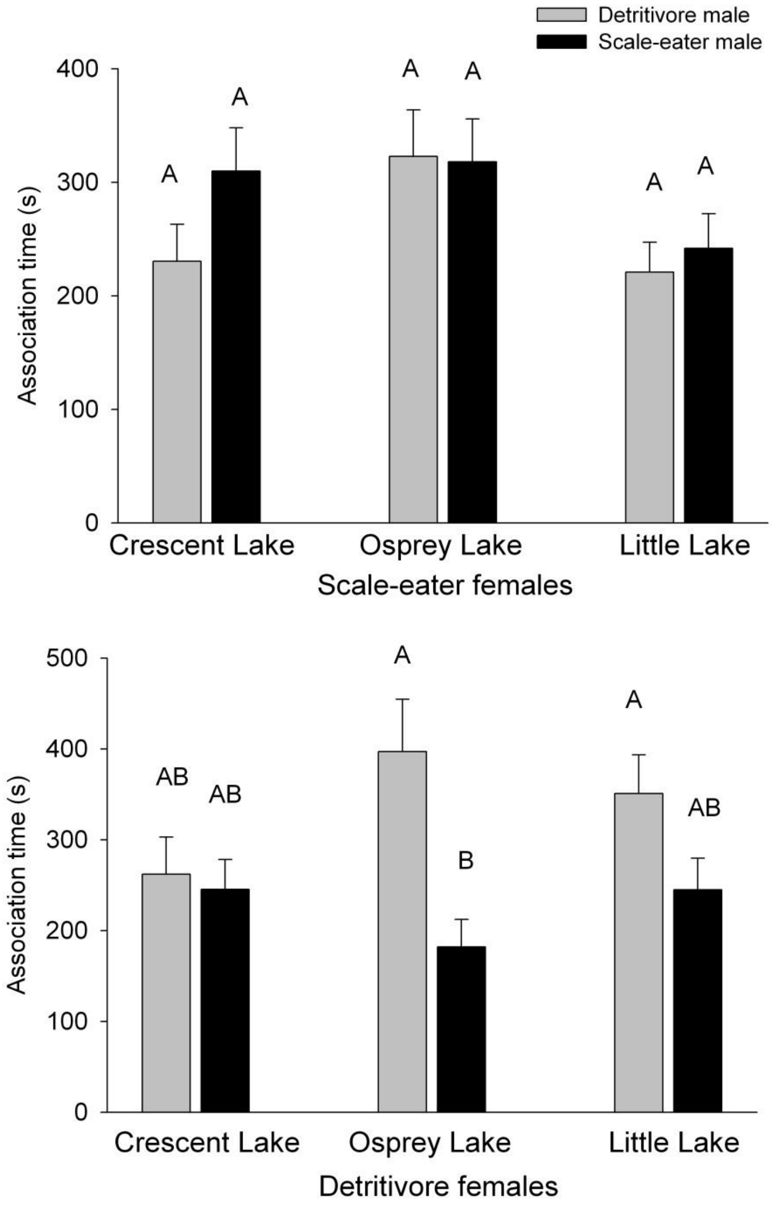


B.

C.


Crescent Lake
0.059 km²
Osprey Lake
0.144 km²
San Salvador
Little Lake
1.886 km²
0
1
2 KM
N


Detritivore male
Scale eater male
Association time (s)
300
250
200
150
100
50
0
A
BC
B
C
A
BC
Crescent Lake
Osprey Lake
Little Lake
Scale-eater females
Association time (s)
350
300
250
200
150
100
50
0
A
B
BC
C
BC
BC
Crescent Lake
Osprey Lake
Little Lake
Detritivore females


Detritivore male
Scale-eater male
400
300
200
100
0
Association time (s)
A
A
A
A
A
A
Crescent Lake
Osprey Lake
Little Lake
Scale-eater females
500
400
300
200
100
0
Association time (s)
AB
AB
A
B
A
AB
Crescent Lake
Osprey Lake
Little Lake
Detritivore females

Chapter 2: Reproductive character displacement and speciation in three pupfish species (*Cyprinodon*)

Rhiannon J.D. West and Abbie J. Reade

Abstract

Saline lakes on San Salvador Island, The Bahamas, are home to a sympatric species flock of pupfish (*Cyprinodon spp.*). The three species, a detritivore, an ostracod-eater, and a scale-eater which preys upon the other two, inhabit distinct trophic niches. We used geometric morphometrics to examine variation in body shape among these three species. Results show that there are significant differences in male, but not female body shape. These results suggest that male body shape, specifically nuchal hump height, is under sexual selection and may contribute to reproductive isolation between species.

Keywords: Reproductive character displacement; Sympatric speciation; Morphometrics; Prezygotic isolation; Pupfish; Secondary sexual trait

Background

Ecological character displacement and sexual selection are two evolutionary processes that contribute to morphological differences between species and sexes, respectively (Brown and Wilson 1956; Grant 1972). Ecological character displacement is a form of disruptive selection, which occurs when sympatric species use and compete for the same resources. It leads to increased differences in shape between species. Additionally, it is thought to be a driver of sympatric speciation (Pfenning and Pfenning 2010) and adaptive radiation (Schluter 2000). Geometric morphometrics (Bookstein 1991; Adams, Rohlf and Slice 2002) has spurred an interest in using shape analysis in ecological and evolutionary studies (e.g. Schluter 1993; Klingenberg and Ekau 1996; Caldecutt and Adams 1998; Douglas et al. 2001; Rüber and Adams 2001; Hulsey and Wainwright 2002; Langerhans et al. 2004). Interest in trophic differentiation and evolution led to analyses of the morphospace of sympatric species. These studies revealed that sympatric species diverge in morphological traits that are related to trophic differences, (e.g., ecological character displacement; Adams and Rohlf 2000). Geometric morphometrics have been used to both support the ecological character displacement hypothesis and to indicate that sympatric species differ in shape (e.g. Cichlids: Barluenga et al. 2006).

Sexual dimorphism, defined as differences in morphology between males and females of the same species, is commonly used to form *a priori* hypotheses about mating systems (see Clutton-Brock 2007). There are numerous studies quantifying sexual dichromatism (e.g. Stevens et al. 2007; fish: Kodric-Brown 1998; birds: Badyaev and Hill 2000, 2003; frogs: Hoffman and Blouin 2000, Bell and Zamudio, 2012; lizards:

LeBas and Marshall 2000). However, quantitative comparisons of sexual dimorphisms of shape are rarer (but see Quinn and Foote 1994), even though sexual selection studies often rely upon differences in shape between the sexes. There are even fewer studies using morphometrics to examine characters that underlie reproductive isolation in sympatric species (but see Elmer et al. 2010; Cooper, Gilman and Boughman 2011; Pfaender et al. 2011). Like ecological character displacement, sexual selection has been hypothesized to lead to speciation by creating disruptive selection on divergent mating types (Kirkpatrick and Ravinge 2002; Ritchie 2007). Here we use morphometric analysis to examine the underlying differences in body shape that may inform female mate choice in a sympatric species flock of Bahamian pupfish (*Cyprinodon spp.)*.

The sympatric species flocks are found in three lakes on San Salvador Island, The Bahamas. They consist of three as of yet undescribed species which differ primarily in feeding ecology: a generalist detritivore, a piscivorous scale-eater, and an ostracod-eater. The three species differ in abundance, their ecological niches overlap to varying degrees and there are no clear habitat boundaries. As predicted by the ecological character displacement hypothesis, jaw morphology differs significantly between the three species (Martin & Wainwright, 2011; 2013). There are apparent differences in shape between the sexes and between species which suggests the action of sexual and/or natural selection. In the Bahamian pupfish, sexual dimorphism is most likely a sexually selected trait as both sexes utilize the same habitats and are exposed to similar predation risks (pers. observ.). This is reinforced by the finding that females of all three species have strong visual preferences for males of their own species (Kodric-Brown & West, *in press*; West & Kodric-Brown, *in prep*.). Here we test the hypothesis that body shape is under sexual selection. If this is the case, then interspecific body shape differences should be greater in males than in females. Additionally, we test the ecological character displacement hypothesis that body shape should differ between species (Rundle and Nosil 2005). The two hypotheses are not mutually exclusive and, testing both hypotheses allows for inferences regarding the contribution of both sexual and natural selection in this system. Thus we examined shape and size differences across sexes, species, and lakes and focused on those traits that are likely under sexual selection. Sexually selected traits are important because they facilitate assortative mating and reproductive isolation in sympatrically speciated species flocks such as this one.

Community structure and the habitats

The three species are monophyletic, diverged from a common ancestor approximately six thousand years ago and speciated in sympatry (Turner et al, 2008; Martin and Wainwright, 2011). The three species inhabit three hypersaline lakes in the interior of the island. San Salvador Island is a karst outcrop and the lakes are connected to the ocean via conduits of varying size and flow (Mylroie & Carew 1995). The lakes vary in salinity, size, depth, community structure, and habitat type. The ichthyofauna in all San Salvadorian lakes is depauperate and consists primarily of pupfish (*Cyprinodon* spp.), mosquitofish (*Gambusia hubbsi*), and the occasional silverside (*Menidia menidia*). The flora tends to grow on rocky substrate in shallow (0.5-2m) water and primarily consists of algaes. Female scale-eaters, detritivores and juveniles of all three pupfish species forage in shallow water, ~0.25-1.5m (pers. observ.). Male scale-eaters defend contiguous, lek-like territories on shallow karst outcrops. Male detritivores defend territories on sandy substrate that is usually surrounded by vegetation. Ostracod-eaters are found in deeper (~2m) water. Little is known about their behavior (Barton and Barton 2008; Martin and Wainwright 2011).

The lakes

The three lakes differ in size, salinity, available breeding habitat and densities of predatory scale-eaters. Little Lake is significantly larger than the other two lakes ($1.886km^2$). The salinity ranges from 40-60ppt (Rothfus 2012). The substrate is comprised of finely ground shells and patchy algae. Osprey Lake, while an order of magnitude smaller ($0.144km^2$), is similar to Little Lake in species composition and habitat structure. Salinity is significantly higher in Osprey than Little Lake and ranges between 55-76ppt (Rothfus 2012). Crescent Lake is the smallest of the three lakes ($0.059km^2$). It has a large ocean conduit, is semi-tidal, and only slightly more saline than the ocean at 35.5ppt (Rothfus 2012). The substrate of most of Crescent Lake consists of very light flocculent organic materials up to 2m deep, and seems unsuitable for territory establishment and as an oviposition substrate. The edges of the lake consist of a narrow vegetative littoral zone that abuts rocky karst extrusions from the shore. All rocky substrate appears to be divided into contiguous male territories occupied by both scale-eaters and detritivores.

Methods

Overall, 249 detritivores (Crescent: n=74, Little Lake: n=70, Osprey: n=105), 93 scale-eaters (Crescent: n=41, Little Lake: n=25, Osprey: n=27), and 12 ostracod-eaters (Crescent: n=7, Osprey: n=5) were collected from the 3 lakes. Ostracod-eaters are not very abundant and thus both low capture rates and concerns regarding the unknown impact of sampling led to a small sample size. Fish were collected with both minnow traps and seine hauls, and transported in buckets to the Gerace Research Station. They were photographed next to a scale-bar with a Canon SX230HS© camera that was held a constant distance of 16cm from the fish. The fish were then returned to the site they were collected from.

Sixteen landmarks describing the body shape of the 3 species were digitized with tpsDig2 (Rohlf, 2010). Landmark coordinates were then imported into PAST (Hammer et al., 2001) and statistically analyzed. Procrustes coordinates were obtained by removing variation due to position, size and rotation from the landmark coordinates. These transformations on the original landmark data leave shape as the only variance in the data. Pairwise comparison on Euclidean Procrustes distances were performed with a non-parametric MANOVA and used Bonferroni corrected p-values. A shape principal component analysis (PCA) was performed on the transformed coordinates to identify the variables that accounted for the largest variance in the data. A MANOVA was used to determine if there are significant differences in shape between species, sexes and across lakes. Finally, a post-hoc canonical variate analysis (CVA) examined the maximal differences in among group variation relative to the pooled within-group variation between the species, sexes, and across lakes (Zelditch et al. 2004). CVAs produce maximal and second to maximal separation between all groups (multigroup discriminant analysis). As in PCA, the axes are linear combinations of the original variables and eigenvalues indicate the amount of variation explained by these axes.

Results

Between Species

As expected in sister-species, the three species had similar overall body shape but significantly differed in body shape variation (PCA on mean differences: figure 1; CVA:

Bonferroni corrected MANOVA Wilk's λ=0.046; $F_{64,664}$=36.65; P<0.000). Both the CVA and the non-parametric MANOVA indicated that there was no overlap in shape between the three species (NPMANOVA: F=30.5, P=0.0001: figure 2a,b). The mean shape deformations of canonical variate 1 (CV1) indicate that the main differences in body shape between scale-eaters and ostracod-eaters and detritivores were due to differences in mouth placement, nuchal hump height, and anal fin placement (figure 2c). The differences in body shape between scale-eaters and detritivores are mainly explained by CV1 and not CV2 (figure 2b). CV2 describes differences in eye placement, postorbital length and inclination, and body depth (figure 2d). The scale-eaters and detritivores were not significantly different in these respects. The ostracod-eaters have eyes that are closer to their snout and a more abrupt nuchal hump resulting in significant differences in CV2.

Among species, by lake

A non-parametric MANOVA (F=17.25, P=0.0001; table 1) and a PCA on pooled detritivore and scale-eaters between all three lakes indicated that the shapes of the fish between lakes overlap but differ in the amount of shape variance. This variance in the PCA required a post-hoc CVA which showed that body shape differed between lakes (Bonferroni corrected MANOVA Wilk's λ=0.366; $F_{64,660}$=6.74; P<0.000). Fish from Crescent Lake had the least variance in shape and Little Lake had the most (figure 3). A PCA on the Procrustes coordinates without size removed indicated that the species are smaller in the smallest lake (Crescent) and show the most variance in size in the largest lake (Little). Within detritivores, shape differs significantly (P<0.000) between all three lakes (Bonferroni corrected MANOVA Wilk's λ=0.301; $F_{64,\,430}$= 5.53; P<0.000). Within scale-eaters, shape differs between Crescent and Little (P<0.000) and Crescent and Osprey (P<0.000) but not between Little and Osprey Lakes (P=0.778; Bonferroni corrected MANOVA Wilk's λ=0.068; $F_{64,120}$=5.315; P<0.000). A PCA indicates that in both detritivores and scale-eaters, Crescent Lake's variation is explained by PC2 whereas both Little and Osprey vary on PC1.

Between and within species and between sexes

A pairwise comparison indicates that all species and sexes differ from each other with the exception of scale-eater and ostracod-eater females (table 1b, *F*=10.56, P=0.0001). Male ostracod-eaters were not included in this analysis due to a small sample size. In detritivores, a pairwise comparison shows significant differences in shape with size removed (F=2.766 P=0.0091). A PCA on Procrustes coordinates without size removed shows that detritivore females and males differ both in shape and size (figure 4a,b). Detritivore males are larger than females, and sexes differ primarily on the height of their dorsal fin, the placement of their anal fin, the width of their pectoral fins, and eye placement (figure 4b). In scale-eaters, male and female shape also differs significantly and males are larger (*F*=3.738 *P*=0.0007; Figures 5a-c) although there is less variance in shape in scale-eaters (figure 5a) than in detritivores (figure 4a). Overall, scale-eaters are fusiform but females range from fusiform to deeper-bodied. This variation is likely due to female gravidity. Sample sizes were too small to determine differences in body shape between male and female ostracod-eaters.

Discussion

Between Species

Martin and Wainwright (2011) posited that niche specialization, measured by mouth morphology, led to the rapid divergence of sympatric pupfish. Our results show that head shape differs between the Bahamian species but so do male secondary sexual characters (figures 2, 4, and 5). Since male scale-eaters form leks (breeding aggregations) with clear territorial boundaries on karst outcroppings in shallow water whereas detritivore males generally defend heterogeneous, dispersed territories across vegetation and sandy or muddy substrates, both body shape and breeding territory sites (e.g., Kodric-Brown, 1983) may be important in facilitating assortative mating. As the three species of Bahamian pupfish mate assortatively and show strong visual preferences for conspecifics (Kodric-Brown & West, *in press*), these characters likely function in visually-mediated mate recognition and assortative mating (figures 3&4). Species in sympatry with habitat overlap are expected to display strong prezygotic reproductive isolation to lessen fitness costs of intermediate offspring (Dobzhansky 1940; Coyne & Orr 2004). There is no post-zygotic isolation, since Lab-reared hybrids are viable to the F2 generation (Holtmeier 2001; Martin & Wainwright 2013). The use of visual cues suggests reproductive character displacement (Brown & Wilson 1956; Crozier 1974) to minimize hybridization between species (Blair 1955; Waage 1979; Pfenning & Pfenning 2009).

Between Species by Lake

Overall shape and body size differ between lakes. Shape and body size in detritivores and scale-eaters is less variable in the smallest lake (Crescent; figure 3); and most variable in the largest lake (Little) suggesting that selective pressures on shape are less intense in larger lakes with more variation in habitat (e.g. adaptive plasticity: Klopfer & MacArthur 1960; Bradshaw 1965; Cook & Johnson 1968; West-Eberhard 1989; Moran 1992). Within species, differences between Osprey and Little Lake are less extreme than differences between Crescent and either Little or Osprey. Crescent Lake is not only smaller than the other two lakes, it also has the most limited breeding area, thus competition for mating sites is quite strong compared to the other lakes. While drift could also account for the disparities in shape between lakes, a more parsimonious explanation is that differences between lakes, such as lake size and available breeding territory, are causing differences in shape between lakes.

Sexual dimorphism and sexually-selected traits

Male detritivores and ostracod-eaters differ from scale-eaters primarily in nuchal hump height and anal fin placement (figs 2, 4, and 5). Females of both species did not exhibit these traits. Evidence of sexual dimorphism is strongest in detritivores. Males are larger, have abrupt nuchal humps with more anterior dorsal fins. They also have larger pectoral fins which are, like most pupfish, sexually dichromatic and range from orange to black in males and translucent in females. Male scale-eaters are larger than females and females vary significantly in body depth but otherwise there is little shape sexual dimorphism in this species. Whether the nuchal hump is exaggerated in this system as compared to other pupfish species is unclear but if so it may be due to the shared nuchal hump coloration with the sympatric scale-eater. Ostracod-eaters are generally found in deeper (1.5-2m) water (pers. obsv and communication with C. and M Barton, C. Martin, and D. Lonzarich) and like the other two species use visual cues in mate choice (West and Kodric-Brown *in prep*). As ostracod-eaters and detritivores and the distantly related *C. tularosa* (Collyer et al, 2005) share the nuchal hump trait, it is likely an ancestral character. Thus nuchal hump height is probably a secondary sexual trait, and part of the visual cues that maintain premating isolation (as reviewed in Ptacek 2000). Barlow and Siri (1997) have shown that it functions as a signal for sex but not species recognition in Midas cichlids (*Cichlasoma citrinellum*). The function of nuchal humps in this system warrants additional study.

All species and sexes except detritivore males have similar placement of the anal fin (figures 2, 4, and 5). Anal fins may assist in steady swimming and rapid movement (Standen and Lauder 2005), and so may be used by female detritivores and ostracod-eaters to escape attacks by predatory scale-eaters. It is likely that the placement of the anal fin in male detritivores is not due to differences in sexual selection as male scale-eaters defend and maintain territories in a similar manner to males of the other two species. Kinematic tests of the function of anal fins (using 3D high-speed photography) in pupfish would untangle this relationship.

Detritivore males show two distinct clustering of phenotypes (figure 4a) which are not explained by lake. In the field, we observed small, black-finned detritivore males defending territories and spawning with females. We also observed larger, red finned males with territories on seemingly less desirable habitat, such as the vegetative littoral zone. Since territorial, satellite and sneaker males are common in pupfish (e.g. Kodric-Brown, 1977; Kodric-Brown, 1986; Leiser & Itzkowitz, 2003), it is likely that the two types of detritivore males pursue alternative reproductive tactics (ARTs; Oliveira et al. 2008). Such ARTs may provide the initial phenotypic variance underlying the speciation process (West-Eberhard 1986; Pfenning and Pfenning 2011)

Conclusion

There has been much debate about the role of sexual selection in sympatric speciation. Multiple models have demonstrated the feasibility but there have not been many examples of the process itself (see Ritchie 2007 for a review). Furthermore, many models have indicated that magic traits, a single trait related to both ecological divergence and sexual selection (Gavrilets 2003), may act as the sole character underlying reproductive isolation. Here we have shown that multiple characters differ between species and traits under sexual selection differ from ecologically divergent traits. In this system strong mating preferences based on visual traits suggest a role of sexual selection in the evolution of sexually-dimorphic traits that differ between species. Additionally, there is avoidance of heterospecifics due to predation of the scale-eating sister-species (Kodric-Brown and West, *in press*). Therefore, sexual selection, predator-prey dynamics, and niche use are thus likely reinforcing reproductive isolation between species. Future studies should examine the interaction between ecological characters, reproductive characters, mate choice, and habitat use to disentangle the evolutionary history of sympatrically speciated species.

Acknowledgements

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Figure legends

Table 1: Pairwise comparison p-values on Bonferroni corrected Euclidean Procrustes distances on across lakes differences of shape.

Table 2: Pairwise comparison p-values on Bonferroni corrected Euclidean Procrustes distances on sexes and species. * denotes significance at the $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Figure 1: Overall mean shape differences between species, sexes and lakes.

Figure 2: Body shape differences between species from PCA (a; left) and CVA (b; right) with 95% confidence intervals. c: Mean shape deformations of PC1 across species from principle component analysis (PCA) show that the main difference in shape between the three species is the placement of the mouth, the height of the nuchal hump and the placement of the anal fin.d: The mean shape deformations on CV2 are between the ostracod-eaters and the scale-eaters and detritivores. The main differences are eye placement, brow placement and the depth of the body. Ostracod-eaters have eyes that are closer to their snout, protruding “lips,” and shorter body lengths than scale-eaters and detritivores.

Figure 3: A principal component analysis on pooled species shows the overall differences in body shape between lakes with 95% confidence ellipses.

Figure 4a: A principal component analysis shows the differences in body size by sex within detritivores with 95% confidence ellipses. The clustering of males and females is not explained by lakes.

Figure 4b: Mean shape deformations of CV1 from the canonical variate analysis (CVA) indicate that male and female detritivores differ primarily on nuchal hump height and placement of anal fin and eye.

Figure 5a: Ellipses of 95% confidence interval of all scale-eaters show that the sexes differ significantly in shape with most of male shape explained by CV1 and most female shape explained by CV2.

Figure 5b: Male scale-eaters vary in body height and length.

Figure 5c: Female scale-eaters vary in dorsal height.

Figures

Table 1

Species		Detritivores			Scale-eaters			Ostracod-eaters
	Lake	Crescent	Little	Osprey	Crescent	Little	Osprey	Crescent
Detriti-vores	Little	0.0028						
	Osprey	0.0028	0.5208					
Scale-eaters	Crescent	0.0028	0.0028	0.0028				
	Little	0.0028	0.0028	0.0028	0.0028			
	Osprey	0.0028	0.0028	0.0028	0.0028	0.1932		
Ostracod-eaters	Crescent	0.0028	0.0056	0.0056	0.0028	0.0028	0.0028	
	Osprey	0.0224	0.0672	0.0308	0.0252	0.0084	0.0392	1

Table 2

Species			Scale-eaters		Ostracod-eaters
Detritivores	Females	Males	Females	Males	Females
Males	0.078				
Scale-eater					
Females	0.0015**	0.0015**			
Males	0.0015**	0.0015**	0.012*		
Ostracod-eater					
Females	0.0015**	0.0015**	0.0015***	0.003**	
Males	0.2085	0.225	0.0135**	0.8715	1

Figure 1

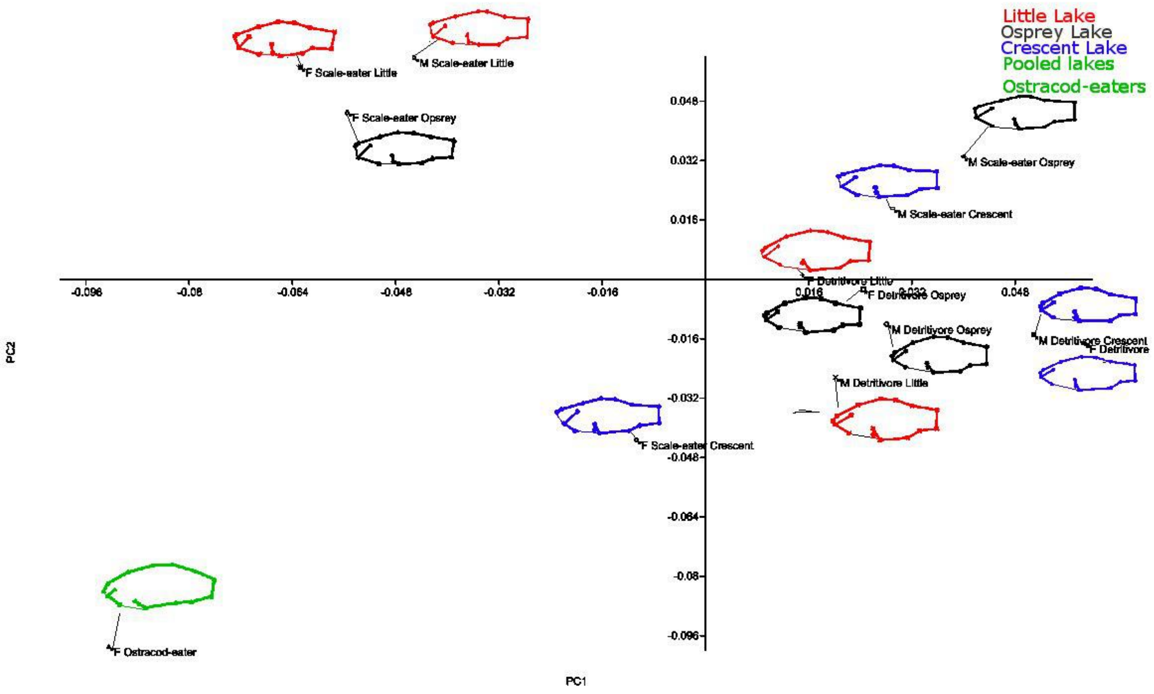

Little Lake
Osprey Lake
Crescent Lake
Pooled lakes
Ostracod-eaters
F Scale-eater Little
M Scale-eater Little
F Scale-eater Opsrey
M Scale-eater Osprey
M Scale-eater Crescent
F Detritivore Little
F Detritivore Osprey
M Detritivore Osprey
M Detritivore Crescent
F Detritivore
M Detritivore Little
F Scale-eater Crescent
F Ostracod-eater
0.048
0.032
0.016
-0.016
-0.032
-0.048
-0.064
-0.08
-0.096
-0.096
-0.08
-0.064
-0.048
-0.032
-0.016
0.016
0.032
0.048
PC1
PC2

Figure 2

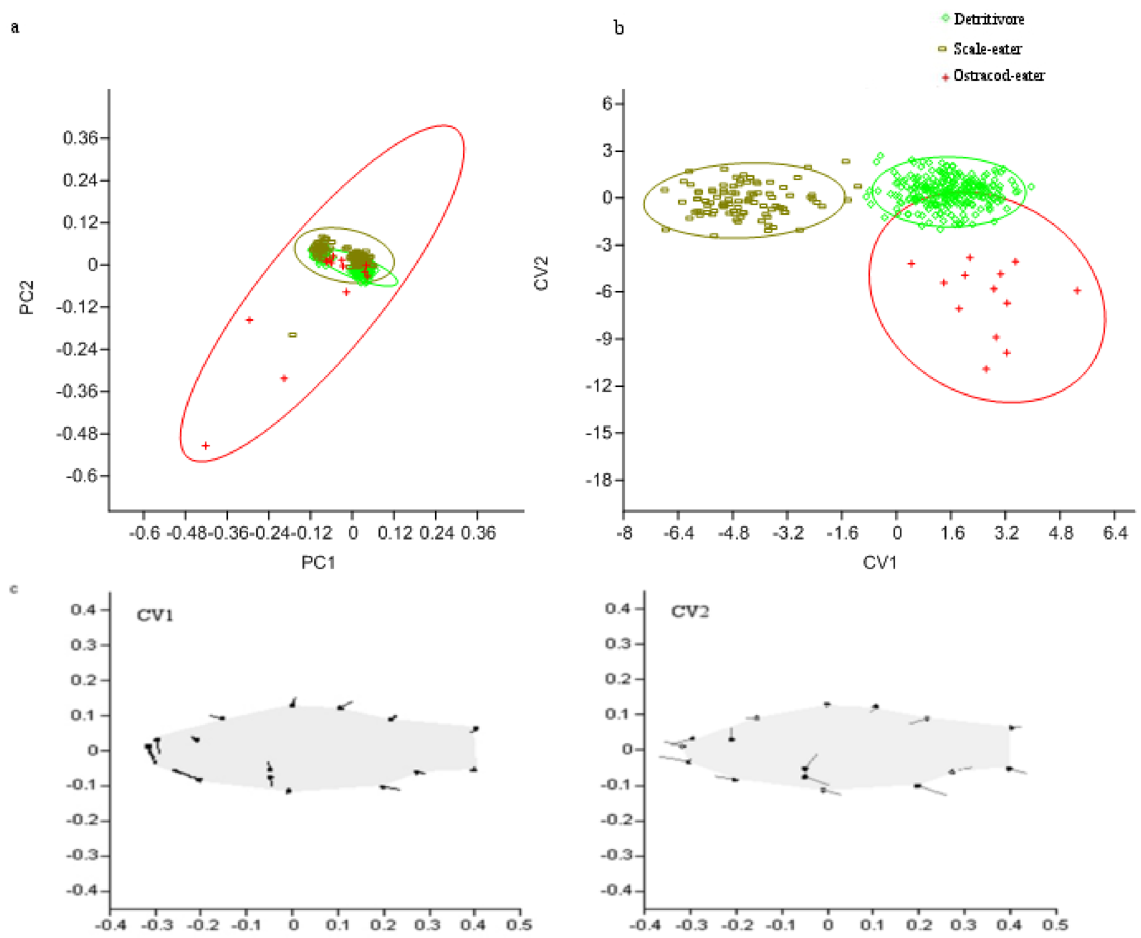

a
b
Detritivore
Scale-eater
Ostracod-eater
PC2
0.36
0.24
0.12
0
-0.12
-0.24
-0.36
-0.48
-0.6
PC1
-0.6
-0.48
-0.36
-0.24
-0.12
0
0.12
0.24
0.36
CV2
6
3
0
-3
-6
-9
-12
-15
-18
CV1
-8
-6.4
-4.8
-3.2
-1.6
0
1.6
3.2
4.8
6.4
c
CV1
CV2
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.4
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5

Figure 3

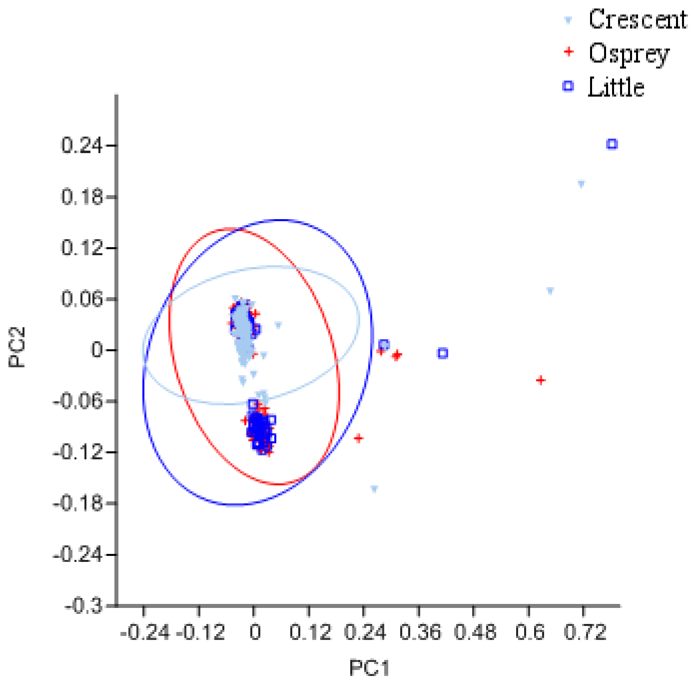

Crescent
Osprey
Little
0.24
0.18
0.12
0.06
0
-0.06
-0.12
-0.18
-0.24
-0.3
PC2
-0.24 -0.12 0 0.12 0.24 0.36 0.48 0.6 0.72
PC1

Figure 4a

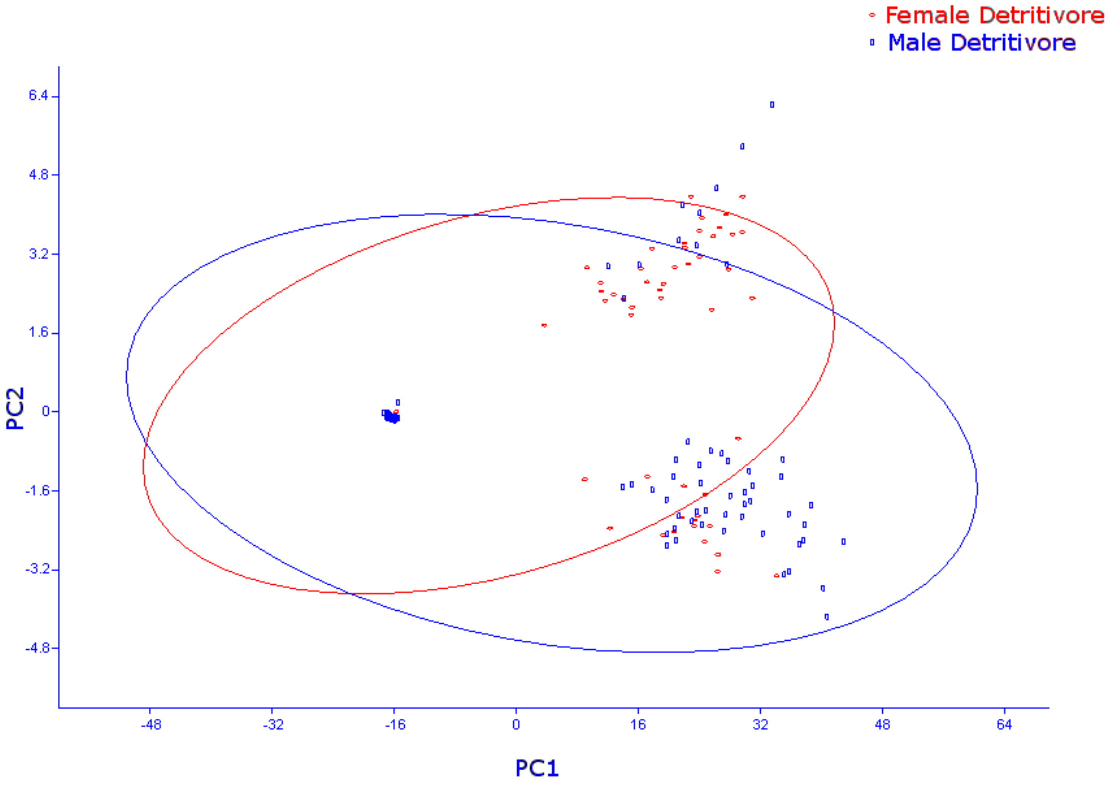

Female Detritivore
Male Detritivore
6.4
4.8
3.2
1.6
0
-1.6
-3.2
-4.8
PC2
-48
-32
-16
0
16
32
48
64
PC1

Figure 4b

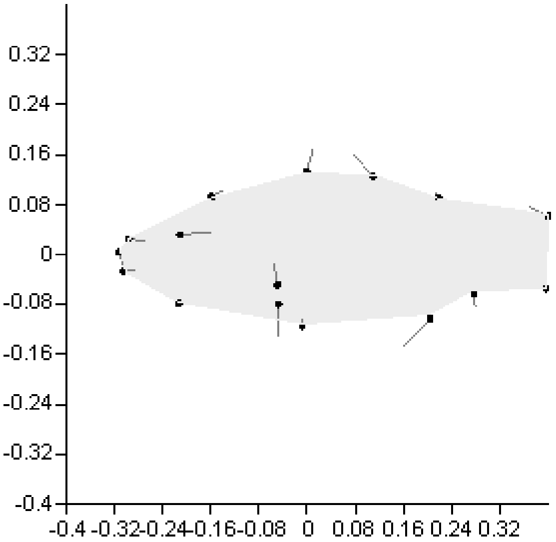


Figure 5a

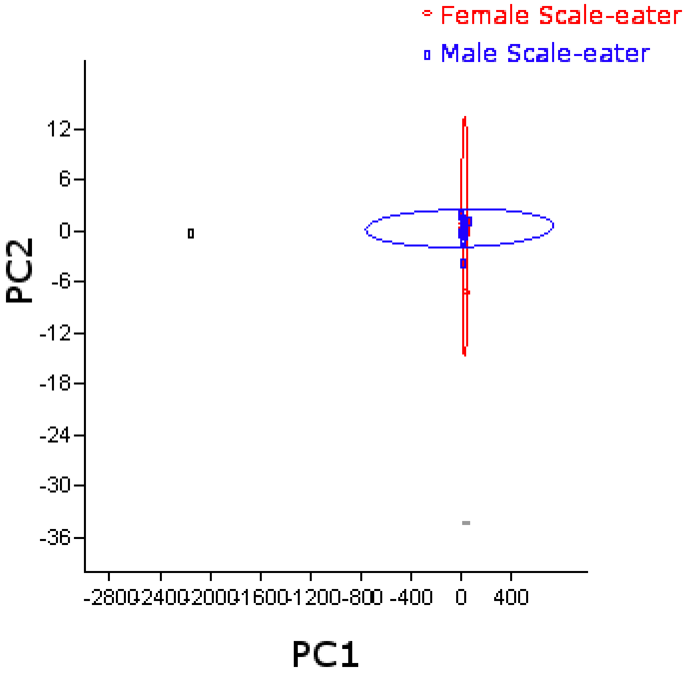


Figure 5b

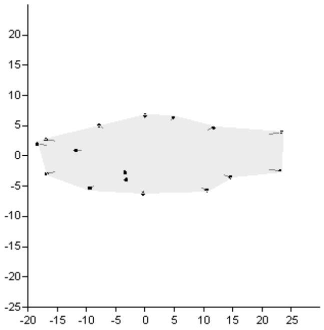
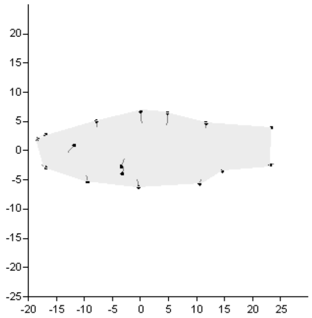


Figure 5c


20
15
10
5
0
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-20
-15
-10
-5
0
5
10
15
20
25

Chapter 3: Mutual mate choice maintains reproductive isolation in a sympatric flock of pupfish (*Cyprinodon spp*) in the Bahamas

Rhiannon J.D. West and Astrid Kodric-Brown

Abstract

Female mate choice reinforces reproductive isolation in sympatrically speciated species. Male mate choice, while not as strong a selective pressure as female mate choice, could also act as a reproductive isolation mechanism in sympatric species. Using a binary choice design, we examine the importance of visual and olfactory cues in female mate choice and focus on the ostracod-eater, the least abundant of the three sympatric species of pupfish (*Cyprinodon spp*). We also examine male mate choice in the scale-eater and the detritivore. Females use visual and not olfactory cues and prefer conspecific males. Males also preferentially associate and court conspecific females. Thus, mutual mate choice acts a strong premating isolation mechanism in these sympatrically speciated pupfish.

Introduction

Female mate choice stemming from unequal parental investment (Trivers 1972) is common in most sexually reproducing systems (Andersson 1994). Sexual selection is a powerful process that can facilitate sympatric speciation (Ritchie 2007) particularly when the divergence is based upon invasion of a new ecological niche (Maan and Seehausen 2011) and is a signal of localized adaptation (van Doorn, Edelaar and Weissing 2009). Generally, sexual selection in conjunction with ecological niche expansion results in prezygotic isolation that maintains species boundaries (Coyne and Orr 2004; e.g. cichlids: Baylis 1976, Elmer et al. 2010, Mexican pupfish: Strecker and Kodric-Brown 2000; Palms: Savolainen et al. 2006).

While female mate choice is the classic paradigm of sexual selection, male mate choice has also been shown to shape the evolution of female traits. Where males invest in offspring, male mate choice is common (as reviewed in Gwynne, 1991; e.g. beetles: Chen, Salcedo and Sun 2012; fish: Myhre et al. 2012). Recently a growing literature has shown that male mate choice can exist in the absence of paternal care (as reviewed in Edward and Chapman 2011). Male guppies (*Poecilia reticulata*), haplochromine cichlids (*Astatotilapia flaviijosephi*) and swordtails (*Xiphophorus malinche*) preferentially court larger females (Dosen and Montgomerie 2004; Werner and Lotem 2006; Tudor and Morris 2009). Males can also adjust the amount of effort they invest in courtship (Werner and Lotem 2006; Lihoreau et al. 2008; Hoefler et al. 2009) or male-male competition and mate guarding (Jormalainen, Merilaita, and Tuomi 1993). Males can gain fitness advantages by being choosey (Bonduriansky 2001; Dosen and Montgomerie 2004;

Werner and Lotem 2006; Reading and Backwell 2007; Tudor and Morris 2009 but see Barry and Kokko 2010; Bierbach et al. 2011).

Both visual and olfactory cues are often used in female mate choice within and between species living in sympatry (reviewed in Candolin 2003; Smadja and Butlin 2009).). Indeed, many cues are used in mate choice as multiple messages to identify conspecifics and differentiate between males of differing quality (Candolin 2003). In the Mexican sympatric species flock of pupfish (*Cyprinodon*) both visual and olfactory cues are important in maintaining prezygotic isolation (Strecker and Kodric-Brown 2001).

Mutual mate choice, where females and males preferentially mate with phenotypically similar individuals would strengthen sympatric speciation through strong premating isolation. Here we examine both female and male mate choice in a sympatrically speciated flock of pupfish (*Cyprinodon spp*). Specifically, we test the use of visual and olfactory preferences in both female and male mate choice.

The system

Three sympatrically speciated and as yet undescribed species of pupfish occupy three saline lakes on San Salvador Island, The Bahamas (Holtmeier 2001,Turner et al. 2008, Martin and Wainwright 2011). The species rapidly evolved (Martin and Wainwright 2011) in the last 6,000 years, when the lakes on San Salvador Island formed (Pacheco & Foradas, 1986; Hagey & Mylroie, 1995; Milliken et al. 2008). The species are comprised of a scale-eater who preys upon the other two species, a detritivore and an ostracod-eater (Holtmeier 2001; Turner et al. 2008; Martin and Wainwright 2011). The species differ mainly in trophic level, morphology related to trophic specialization (mouth morphology:

Martin and Wainwright 2011), and body shape (West and Reade*, in process*). Species isolation is likely maintained by prezygotic isolation as the three species readily hybridize in the laboratory (Holtmeier 2001; Martin and Wainwright 2013) but exist in sympatry with overlapping habitats. The detritivore and scale-eater have a polygynous mating system with males defending contiguous territories. Females mate with multiple males and deposit several eggs on a male's territory during a spawning sequence. Males defend territories thus provide incidental protection of eggs from predation. We have observed males chase away females who are attempting to forage on the territory. Scale-eaters territories are smaller than those of detritivores, are usually located in shallower water and are confined to small, rocky outcrops near the shoreline. Ostracod-eater breeding behavior is unknown.

In wild populations, females use visual cues to discriminate between scale-eater and detritivore males (Kodric-Brown and West, *in press*). Here we examine the cues used in assortative mating and species recognition that function in premating isolation in all three species. To determine if mate choice is symmetrical and is present in males as well as females, we also examine male mate choice. .

Materials and Methods

Female mate choice

We examined the strength of premating isolating mechanisms in F1 populations of detritivores, scale-eaters and ostracod eaters from Crescent Lake on San Salvador Island, The Bahamas. Laboratory raised offspring from wild caught parents were used to lessen potential trapping impacts on all three species, but especially on the rare ostracod-eaters.

The founding Ostracod-eater population consisted of 15 individuals. The scale-eater and detritivore parental generation consisted of 20-30 individuals per species. The parental fish were housed and bred in large (1.89 kl) stock tanks with a 14:10 light:dark schedule and salinity at 35 ppt. Offspring were raised to maturity and used in all trials. For both visual and olfactory trials we used 20 females of each species and 20 males of each species in novel combinations.

Visual cues

A small pool (91 cm in diameter) was filled to a depth of 10cm with water at 35ppt salinity, consistent with salinities in Crescent Lake. Three clear Lee's Kritter Keeper® (30.23 x 19.81 x 20.57 cm) tanks were placed in the pool and spaced roughly equidistant from each other and from the pool wall. Two males of each species were size matched within 5mm of each other and each pair was placed in one of the three tanks. A female of one of the three species was then introduced into the center of the pool. Ten minute trials began once she had started to explore the pool and visited each set of males. The time the female spent within 5cm of each tank, either facing or swimming parallel to the tank was recorded. The female was removed and replaced with a female of one of the other species, until females from all three species had been used. The males were then replaced with a novel combination of males and a new set of females and the trials were repeated. The water from the male tanks was collected, pooled together by species and used in subsequent olfactory trials.

Olfactory cues

The olfactory trials were performed to test whether ostracod-eater females discriminated between the odors of conspecific and heterospecific males and whether detritivore and scale-eater females reacted to the scent of ostracod-eater males. Since we have shown that scale-eater and detritivore females from Crescent Lake did not respond to olfactory cues from males (Kodric-Brown and West, *in press*), we predicted that females of these two species also would not respond to olfactory cues from ostracod-eater males.

We used a binary choice design to measure female responses to olfactory cues. Following Kodric-Brown and West (*in press*) female ostracod-eaters were placed in a 22 L aquarium (PETCO Bookshelf aquarium, 60 cm long x 20.65 cm wide) filled with 20 L of water at 35ppt. Two lines were drawn on the front of the aquarium to delineate three "compartments." The two outside compartments were 17 cm wide and the middle one was 26 cm wide. One L capacity intravenous bags (Tyco Healthcare "Kendall Kangaroo" IV) were suspended from the ceiling over each end of the aquarium. One intravenous bag was filled with conspecific male water and the other was filled with one of the two heterospecific species' male water. The water was dripped into each end of the tank at 1drip/sec. The amount of time the female spent in each of the end compartments was recorded for 10 minutes. The female was then moved into a new aquarium and the procedure was repeated with male water from the other species. We randomized both the side the male conspecific water was dripped into and the order the heterospecific male water was presented.

Male mate choice

A binary-choice design tested whether male detritivores and scale-eaters discriminated between conspecific and heterospecific females. Because of insufficient numbers of ostracod males, their preferences could not be determined. A 20 gallon aquarium was equally divided into 3 20x30 cm compartments with two clear plastic dividers. Two female detritivores were placed into one outside compartment of the tank and two female scale-eaters were placed into the other outside compartment of the tank. A male of one species was placed in the center compartment. Ten minute trials began once all five fish were active. We recorded the time the male spent swimming parallel to or directly facing a compartment. After 10 minutes the male was replaced with a male of the other species and data was recorded again. The dividers were not sealed and the trials did not test specific cues used in male mate choice. Thus, these trials only tested male preference and did not exclude olfactory cues. A total of 24 males of each species and 24 females of each species were used in novel combinations. No female pairs were used more than once.

Statistical analyses

Female mate choice

A normality test on the visual preferences times indicated they were not normally distributed. Normality assumptions on an Anderson-Darling test were met with a transformation raising female preference times to the 0.2 power ($P = 0.08$ $AD = 0.667$). A MANOVA with female and male species and female by male interaction terms was performed on the transformed time data. A post-hoc Tukey’s HSD examined significant differences between groups. Olfactory preferences also were not normally distributed and did not match any particular distribution, thus Bonferroni adjusted Kruskal-Wallis tests were used to examine differences in female preferences between conspecific and heterospecific males.

Male mate choice

Male preference times were not normally distributed. A distribution test indicated a Johnson Transformation would be appropriate (*AD* =0.261, $P = 0.703$). Transformed data were then analyzed with a one way ANOVA comparing male preferences for conspecific versus heterospecific females. All analyses were performed with Minitab 16 software.

Results

Female mate choice

Visual cues

Females of all three species spent significantly more time with males of their own species ($F_{8,171} = 10.51$ $P < 0.000$). The time spent with conspecifics did not differ between species although female detritivores showed the most variation in time spent with conspecifics. The time females spent with heterospecific males did not differ between species (fig. 1).

Olfactory cues

Female ostracod-eaters did not discriminate between conspecific and heterospecific males (scale-eater males: $H = 0.39$, $DF = 1$, $P = 0.534$; detritivore males: $H = 0.39$, $DF = 1$, $P = 0.534$).

Male mate choice

Males of both species preferred to associate with conspecific females (fig. 2: Detritivore males: $F_{1,46} = 7.37$, $P = 0.009$; $d = 0.784$; $r = 0.365$; Scale-eater males: $F_{1,46} = 4.99$ $P = 0.030$, $d = 0.644$; $r = 0.307$).

Discussion

Species recognition in the sympatric species flock of Bahamian pupfish is based on visual (fig. 1) and not olfactory cues in all three species (Kodric-Brown and West *in press*). This indicates that premating isolating mechanisms are maintained in all three species. These results differ from those on the only other known sympatric species flock of Mexican pupfish who use olfaction in species discrimination (Strecker and Kodric-Brown 1999). The two systems are superficially similar and have both speciated in less than 6-8,000 years (Martin and Wainwright 2011). However, in the Mexican flock, females of the most basal species, *C. beltrani,* a detritivore, do not discriminate between conspecific males and those of two other species, a mollusk feeder (*C. labiosus*) and a piscivore (*C. maya*). In the Bahamian flock all three species show preferences for conspecifics based on visual cues. Avoidance of predatory scale-eaters likely reinforces selection on the detritivores and may have accelerated speciation (Kodric-Brown and West, *in press*).

When males are forced to choose between conspecific and heterospecific females, both detritivores and scale-eaters prefer their own species (fig. 2). We previously showed that during binary female choice visual trials with field-caught fish from all three lakes where they occur, detritivore males courted females of both species, whereas scale-eater males preferentially courted conspecific females (Kodric-Brown and West, *in press*). There are several explanations for these seemingly contradictory results. Selective pressures on the formation and maintenance of premating isolation may differ for each species due to frequency dependence, Detritivores are very common and comprise > 95-99.5% of the community. Thus the selective pressures acting on males of the two species are asymmetric. The low abundance of scale-eater females places a strong selective pressure on scale-eater males to prefer and expend more energy courting conspecifics (fig. 2;

Kodric-Brown and West, *in press*). Detritivore males do not pay such high fitness costs, since they most often encounter conspecific females. When forced to choose, they preferentially court conspecifics but otherwise court every passing female.

The parental populations of the fish used in this study were obtained from Crescent Lake. Crescent Lake differs in size and composition from the other two lakes where these species flocks occur. It is the smallest lake and has a substrate consisting of flocculent material that is unsuitable for egg laying and thus male territories. It is s the only lake where detritivore and scale-eater males defend small, contiguous territories. We have previously shown that visual cues are important species isolating mechanisms in both detritivore and scale-eater females in Crescent Lake. This differs from the other two lakes where only scale-eater females have significant visual preferences (Kodric-Brown and West, *in press*). Premating isolating mechanisms in detritivore males are likely reinforced by competition for limited breeding ground which causes substantial overlapping with male scale-eater territories. Territorial detritivore males in this lake continuously encounter females of both species (pers. observ.), and may be forced to choose between courting conspecific versus heterospecific females. This has likely directly selected for choosey males in Crescent Lake. Male mate choice should be examined in the other two lakes to determine what factors affect male choosiness and thus reinforce species isolation.

Morphometric studies of the three species showed that they differ primarily in jaw structure (Martin and Wainwright 2011). We have shown that scale-eaters are much more fusiform than detritivores and ostracod-eaters. Additionally males of the three species differ in body shape, specifically the height of the nuchal hump, but females do not (West and Reade *in prep*). Thus, male secondary sexual traits likely function as visual cues in female preference. Further studies of intraspecific variation in morphometric trait and color preferences may provide insight into what visual cues are important in species discrimination and mate choice. It is likely that males have preferences for gravid females (Sargent, Gross, and Van Den Berghe 1986) and thus use visual cues to detect females of high fecundity. Differences between species may be sufficient cues for male mate choice. Males may be making courtship decisions based on female activity, which we did not quantify. While most models of sympatric speciation require ecological invasion or strong sexual selection, models of mutual mate choice suggest that it is sufficient to create and maintain reproductive isolation (Almeida and deAbreu 2003). Mutual sexual selection (Almeida and deAbreu 2003; Pierotti et al. 2008; Puebla, Bermingham and Guichard 2011) together with ecological niche invasion (Nosil 2012) and the direct selective pressures of predators (Albert and Schluter 2004) is likely a powerful selective pressure (*sensu* Maan and Seehausen 2011) for premating isolation in this system.

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Figures

Figure 1:

Females prefer visual cues from males of their own species. 95% confidence interval with median lines and mean symbol (circle with cross). Means that share letters are not significantly different from each other at the α=0.05 level.

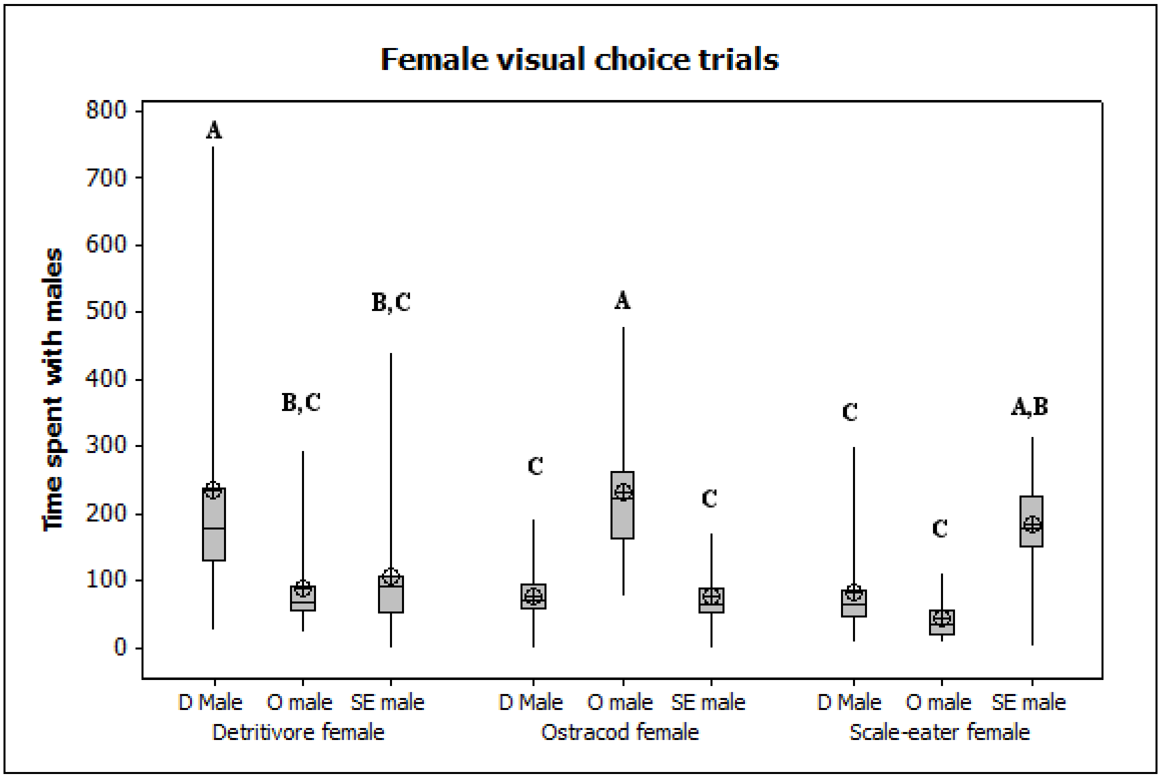
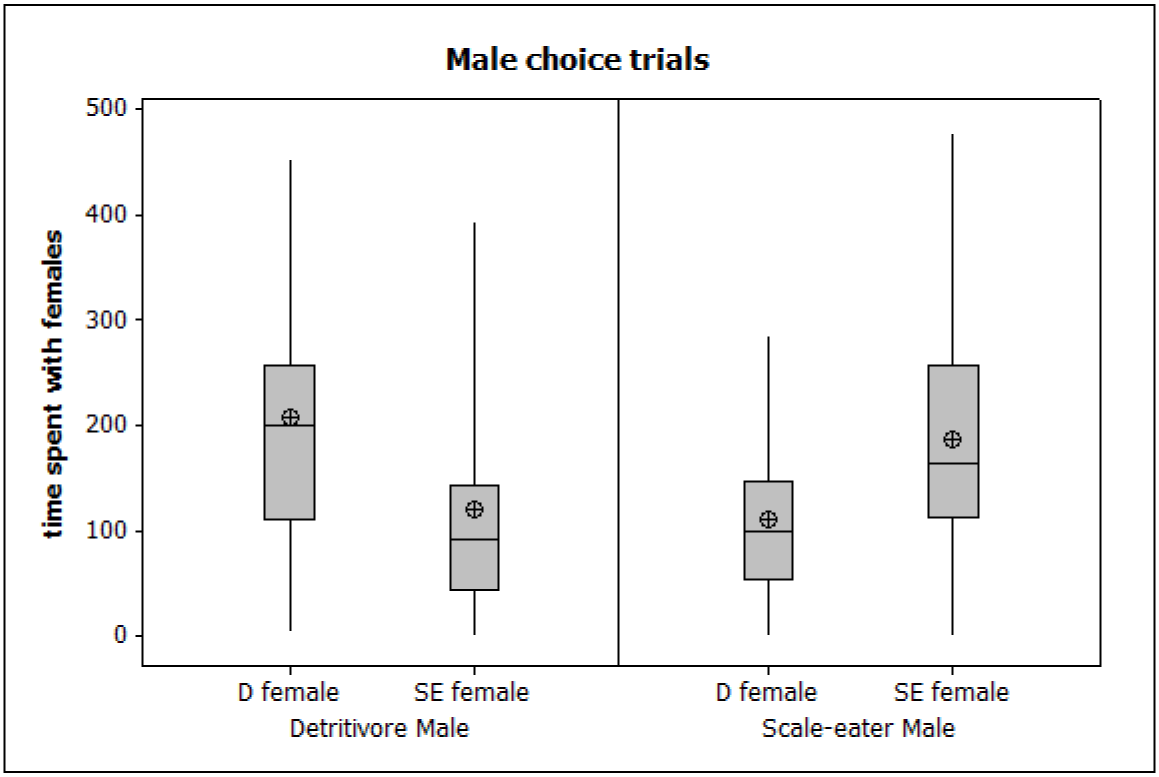


Figure 2:

Males of each species prefer to associate with conspecific females (Detritivore males: $F_{1,46}$=7.37, p=0.009; Scale-eater males: $F_{1,46}$=4.99 0.030; 95% confidence interval with median lines and mean symbol {circle with cross}).



Chapter 4: Role of parasites in sympatrically speciated pupfish (*Cyprinodon*)

Rhiannon J.D. West, Scott LaPatra, and Irene Salinas

Abstract

Recognition and behavioral avoidance of parasitized conspecifics or individuals with slightly different immunities, either due to differences in genotype and/or regional parasite load, would be favored by natural selection to maintain a healthy phenotype. This avoidance of parasitized conspecifics may play a role in species differentiation in two ways: 1) rapidly changing genes that encode for immune function, such as the Major Histocompatibility Complex may lead to small, localized differences in immune function. If small differences in immune function and parasite load drive behavioral avoidance of conspecifics, then reproductive barriers within a population are effectively formed through non-random mating. If such nonrandom mating barriers persist, they may lead to isolation of gene pools and subsequently, sympatric speciation; 2) Changes in parasite resistance may be tightly coupled with trophic differentiation as parasitic infection often occurs when organisms are foraging. To address this question, we performed two experiments to examine how parasites affect female choice in sympatrically speciated pupfish (*Cyprinodon spp.*). We infected and sham infected male *C. labiosus* and *C. maya* with *Ichthyophonus hoferi.* In a series of binary choice trials, we presented female *C. labiosus* and *C. maya* with olfactory cues from infected and non-infected conspecific males. Then we presented the females with odors from infected conspecific males and non-infected heterospecific males. Results indicate that infection was successful in both species but varied in infectivity. Our findings show that females preferred non-infected conspecific males and suggest that reproductive barriers may be formed by female preference for healthy male conspecifics. Additionally, reproductive barriers between species are strong and prevent mating with heterospecific males.

Introduction

Sympatric speciation is the splitting of one evolutionary lineage into two or more lineages in the absence of geographic isolation (Coyne and Orr 2004; Coyne 2007). Competition for diverse resources can cause disruptive, frequency-dependent natural selection which leads to assortative mating that culminates in genetically distinct species (reviewed in Bolnick and Fitzpatrick 2007; Vamosi 2005). There is agreement about the importance of ecological processes such as trophic differentiation to exploit different diets, with corresponding ecological, morphological, and genetic divergence (Barluenga et al. 2006; Elmer et al. 2010a, b; Schliewen et al. 1994; Schliewen et al. 2001). The mechanisms that underlie and promote premating isolation in sympatric species have been understudied (Maan and Seehausen, 2011), despite the abundance of theories on speciation mechanisms and research on model systems such as sticklebacks (Schluter 2000), cichlids (Barluenga et al. 2006; Schliewen et al. 2001; Seehausen 2006) and arctic char (Gislason et al. 1999; Jonsson and Jonsson 2001). The role of sexual selection in sympatric speciation has been heavily debated, as assortative mating by itself does not necessarily result in speciation (e.g. Bolnick and Kirkpatrick, 2012; Arnegard and Kondrashov 2004; Wilson et al. 2000 but see Lande 1989; Turner and Burrows 1995; Chapman et al. 2003; Ritchie 2007). When assortative mating is based on ecological traits such as diet, size, or habitat (e.g. color morphs in Midas cichlids: Elmer et al. 2009; ‘roundfin’ sailfin silversides: Herder et al. 2008) sexual selection may facilitate (Maan and Seehausen, 2011) or even accelerate (Eizaguirre et al. 2009) differentiation.

Since Hamilton & Zuk (1982), the role of parasites in mate choice and sexual selection has received considerable attention (see reviews by Møller 1990; Clayton 1991). Changes in behavior towards parasitized conspecifics has been shown across a variety of taxa (trout: Karvonen et al. 2004; mice: Kavaliers et al. 2004; humans: Fincher & Thornhill 2008; and reviewed in Barber et al. 2000). High parasite loads can drive reproductive isolation in populations via behavioral modifications that increase avoidance of conspecifics who do not share an adaptive immunity to parasites (Thornhill & Fincher 2008; Eizaguirre et al. 2009). Parasites may act as a diversifying selective agent in two ways: 1) Due to parasite-host interactions, genes encoding immune function change rapidly compared to the rest of the genotype. In some sympatric species, reproductive isolation is based solely on a diverged trait and changes in immune function (e.g. color and major histocompatibility complex in African cichlids; Blais et al. 2007). If small differences in immune function and parasite load drive behavioral avoidance of conspecifics, and these selective pressures persist over time, then reproductive barriers are effectively formed through non-random mating of individuals in a population. If such nonrandom mating barriers persist, they may lead to isolation of gene pools and subsequently, sympatric speciation. 2) Alternately, if niche expansion is the primary selective agent in an adaptive radiation, parasites may serve as secondary reinforcement to divergent selection. If trophic invasion results in an adaptive radiation, parasites associated with different microhabitats or diets may accelerate speciation. This has been shown in three-spine stickleback (*Gasterosteus aculeatus*) where lake and stream ecotypes prefer the scent of conspecifics, differ in parasite community and load, differ at the MHC and in which genes are expressed upon exposure to parasites (Eizaguirre et al. 2011; Lenz et al. 2013).

Here we examine the role of female mate choice in maintaining reproductive barriers in parasitized sympatric pupfish, *Cyprinodon labiosus* and *C. maya*. They are trophic specialists that occupy different trophic niches. *C. labiosus* feeds primarily on hard-shelled mollusks and *C. maya* is a piscivore. The mating behavior of these fishes has been well studied and indicates that scent is an important component of female mate choice (Kodric-Brown & Strecker 2001). The fish used in this study are laboratory bred descendants from populations in Lake Chichancanab, Yucatan, Mexico. We test female preference for olfactory cues of (1) parasitized and non-parasitized conspecifics to determine if discrimination of a novel parasite is based on odors and (2) parasitized conspecifics and non-parasitized heterospecifics to test the strength of premating isolating mechanisms.

Methods

Males of each species (n=32 *C. maya;* n*=32 C. labiosus*) were size matched within 3mm (27mm-30mm) as *C. labiosus* are smaller than *C. maya.* They were separated by species and held in eight 10gal tanks. Semi-permeable barriers divided each tank in half with 4 fish in each half. The environment was maintained at 6ppt salinity and 20-22 ^{0}C.

Infection experiment

Males were infected with an endoparasitic protist, *Ichthyophonus hoferi. I. hoferi* causes acute and chronic infections in over 80 different marine and anadromous fish species (Ramaiah 2006). The heart and liver are the primary infection sites for *Ichthyophonus sp*. in salmonids, clupeids, and cottids (Rand and Cone, 1990; Jones and Dawe, 2002; Kocan et al., 2004). The pupfish were fed either infected or uninfected minced rainbow trout (*Oncorhynchus mykiss*) heart and liver tissues once per day for 3 weeks. The rainbow trout viscera were obtained from either naturally infected or uninfected trout at a Clear Springs Foods, Inc., trout farm in Buhl, ID. Rainbow trout infection was verified at Clear Springs Foods. Additionally, fish were lightly supplemented with fish flakes and dried brine shrimp (Tetra®). Pupfish preferentially consumed the trout over fish flakes and dried brine shrimp. To control for scent effects of the parasite and differences in diet between species, at the end of the three weeks, the males were moved into new tanks and fed only fish flakes and dried brine shrimp for one week prior to the olfactory choice trials.

We verified the infection or the lack thereof by performing explant cultures of the heart and histological examination of heart, liver and gut. Explants were cultured in a standard *Ichthyophonus* media (5 mL of tris-buffered Eagle's Minimum Essential Medium (Sigma, St Louis, MO, USA), supplemented with 5% foetal bovine serum (Hyclone), 2 mm l -glutamine, 100 IU mL-1 penicillin, 100 l g mL-1 streptomycin and 100 l g mL-1 gentamycin (Gibco BRL).

Following the behavioral trials, males were killed with an overdose of buffered tricaine methane-sulfonate (MS-222) and necropsied. The heart and liver were aseptically removed from both exposed and control males. A portion of the heart (~1mm) was removed and cultured in *Ichthyophonus* growth media (McVicar, 1982; Spanggaard et al., 1994; Kocan et al., 2004), incubated at 17 C for 14 days and then examined under bright field microscopy for growth of *I. hoferi.* Additionally, to determine the extent of parasite presence and damage to the tissues, heart, liver and gut, samples were fixed in 10% formalin and embedded in paraffin. Three 5-um thick sections were obtained from each tissue sampled and stained with Hematoxylin and Eosin (H&E).

Olfactory cues

Olfactory trials tested whether females of each species discriminated between water born odors of (1) infected and non-infected conspecific males and (2) infected conspecific and non-infected heterospecific males. Each trial lasted 15 min. A 22 L aquarium (PETCO Bookshelf aquarium, 60 cm long x 20.65 cm wide) was filled with 20 L of water (at 6ppt). Two lines were drawn on the front of the aquarium, delineating 3 "compartments." The two outer compartments were 17 cm wide and the middle one was 26 cm wide. A one L capacity (Tyco Healthcare "Kendall Kangaroo" IV) intravenous bag was suspended from the ceiling over each end of the aquarium. In the first part of the trial, one of the IV bags was filled with 200 mL of infected conspecific male water and the other with non-infected conspecific male water. This water was obtained by mixing equal amounts of water from all relevant male tanks (n=32 per treatment). These bags allow accurate calibration of the drip rate of the 'male stimulus' water and are disposable. The IV bags were calibrated to deliver 1 drop per s (45 mL during each trial). A female was introduced into the central 'compartment' of the tank. A trial began when the female started to swim and explored all parts of the aquarium. Females of each species (*C. maya* n=27; *C. labiosus* n=19) were first presented with water from infected and non-infected conspecific males. The time the female spent in each compartment was recorded. At the end of the first trial the female was moved into another tank, allowed to acclimate as in the previous tank, and given a choice between water from infected conspecific males and water from non-infected heterospecific males (Fig. 1). The position of the IV drip that delivered water from conspecific males was changed between the first and second part of the trial to control for any potential side bias.

Statistical analysis

Female preference times were tested for normality and then ANOVAs were used to determine if mean association times differed between treatments. Differences in preference times between species were analyzed with a MANOVA.

Results

Growth media

Culture of heart samples in growth media allowed the recovery of spore or hyphae in 5 out of 32 total infected *C. labiosus* specimens examined (not shown). In two samples, spores exited the heart tissue and filled the plate 21 days after explant culture. We did not see similar results in the *C. maya* tissues.

Histology

No spores consistent with *I. hoferi* morphology were observed in any of the sections examined. However, infected specimens from both species showed histopathological signs indicating the success of the experimental infection. In the case of *C. labiosus*, both the heart and the gut were severely affected. The gut of infected *C. labiosus* showed tissue damage, detachment of the epithelial layer from the lamina propria and large accumulation of eosinophilic granular cells (EGCs) (Fig 2B). EGCs were observed in control gut samples but in much lower numbers (Fig 2A). Extensive signs of necrosis were present in the cardiac muscle fibers of the heart compared to control specimens (Fig 2C-F). In the case of *C. maya*, the heart of infected specimens was characterized by accumulations of lymphocytes not observed in the control group (Fig 2G-H) indicating an inflammatory response, but there were no signs of necrosis. Thus, overall *C. labiosus* tissues were more heavily affected by the infection.

Mortalities

C. labiosus were more susceptible to the parasite than *C. maya*. During the course of the three weeks, four C. *labiosus* males died. There were no mortalities in *C. maya* males.

Behavioral

Females of both species preferred non-infected conspecific males over infected conspecific males (figure 2a,b; *C. maya*: $F_{1,52}$=6.91, p= 0.011; *C. labiosus*: $F_{1,50}$=18.77, p<0.000). Neither *C. labiosus* nor *C. maya* females discriminated between infected conspecific males and non-infected heterospecific males (figure 2c,d; *C. maya*: $F_{1,52}$=1.64, p= 0.21; *C. labiosus*: $F_{1,50}$=0.00, p=0.98). *C. maya* females showed a trend in preference for non-infected heterospecific males. *C. maya* and *C. labiousus* females did not differ in strength of preference (MANOVA Wilk's λ=0.933 $F_{4,41}$=0.729, p=0.577).

Discussion

Females of both Mexican species have significant olfactory preferences for males of their own species (Kodric-Brown & Strecker 2001). Females of both species differentiated between infected and non-infected conspecific males. Interestingly, females did not discriminate between infected conspecifics and non-infected heterospecifics. These results suggest that infection (a) makes conspecifics as unattractive to females as heterospecifics and (b) strong premating isolating mechanisms that are not reversed by the condition of heterospecific males. *C. labiosus* were more severely affected by infection than *C. maya;* however, there was no difference in choosiness between species. If females were choosing males based on severity of infection, one would expect to see a stronger avoidance of infected conspecifics in *C. labiosus.* These results indicate that females are discriminating on infection status rather than severity of infection.

Ichthyophonus hoferi causes infections in a wide range of fish hosts. Affected organs are characterized by recurrent episodes of necrosis and inflammation. Although presence of granulomas is a diagnostic feature in *I. hoferi* infected hosts, we observed lymphocyte infiltrations in the heart of *C. maya* infected specimens instead. Histopathology was more advanced in *C. labiosus;* infected individuals had extensive signs of necrosis in the cardiac muscle fibers pointing to a greater susceptibility of this species to *I. hoferi* compared to *C. maya*. The gut was also affected and the presence of high number of EGCs is a common sign of parasitic infections in the teleost gut (Salinas, 2011). The variation found in the present study between both pupfish species is in agreement with previous work that has identified inter- and intraspecific differences in response to *I. hoferi* (Rahimian, 1998). As *C. maya* is piscivorous there may be differences in immune function between the species that allows *C. maya* to avoid infection from its feeding niche. Moreover, it is possible that *I. hoferi* produces a different histopathological effect in pupfish compared to natural hosts such as rainbow trout. It is unlikely that the salinity of the water affected the rate of infection as *I. hoferi* is found in marine, brackish, and freshwater fishes (reviewed in Hershberger et al., 2002) This protist grows at temperatures between 3°C and 20°C, with 10°C being the optimum. Thus, pupfish holding temperatures of 20-22 ^{0}C are not ideal for pathogen growth and may explain the decreased infectiveness in pupfish compared to salmonid fish. Nevertheless, our results clearly point to tissue damage in the cardiac muscle fibers of infected *C. labiosus* and *C. maya* and in the gut of *C. labiosus*. Overall, the tissue damage could have led to improper nutrient absorption and deficient circulatory performance. Performance studies in rainbow trout have demonstrated that swimming stamina, measured as time-to-fatigue, was reduced by approximately two-thirds in rainbow trout experimentally infected with *Ichthyophonus* (Kocan et al. 2006). In the present study, we observed significant differences in the mate choice of pupfish based upon male infection status. It is possible that swimming performance of pupfish was affected by parasitic infection although this was not measured and females did not see the males at any point in the experiment.

This study adds to the growing literature on the importance of parasite load in promoting behavioral isolation. Juvenile sticklebacks preferentially school with non-ectoparasitized groups (Dugatkin, FitzGerald and Lavoie 1994). Various populations of three-spine sticklebacks have been shown to prefer the scent of conspecifics (Rafferty and Boughman 2006), differ in parasite community (Maccoll 2009), load (Eizaguirre et al. 2011; Lenz et al. 2013), at the MHC, number of MHC alleles (Matthews *et al.*, 2010), and in which genes are expressed upon exposure to parasites (Eizaguirre et al. 2011; Lenz et al. 2013). Sympatric Icelandic three-spine stickleback lava and mud morphs mated assortatively (Ólafsdóttir et al., 2006), differed in parasite community, and number of MHC alleles (Natsopoulou, Pálsson, and Ólafsdóttir, 2012). However, the parasite/MHC results were not consistent in all lakes in which sympatric morphs were found. Sympatric cichlid species show marked divergence at MHC class II alleles, significant differences in parasites between species (Blais et al., 2007), use olfactory cues in their mate choice (Plenderleith et al., 2005), differ primarily in diet, coloration, and females assortatively mate on color (Maan et al. 2008; 2006). Norwegian whitefish (*Coregonus lavaretus*) morphs differed in ectoparasite composition although their behavioral and mate preferences are unknown (Knudsen, Amundsen, and Klemetsen 2003). Combined with our study these results indicate that parasites 1) can lead to differences in localized immune function and nonrandom mating based on these differences and, 2) are linked to trophic differentiation in species living in sympatry whatever the cause of initial genetic divergence.

To thoroughly test the parasite mediated sympatric speciation hypothesis, multiple modes of inquiry are required. Field data of actual parasite loads between and within species are needed to address the ecological relevance of our tests of the effects of parasite load on female mate choice. Unfortunately, the Mexican sympatric species flock has collapsed and thus such a study is not possible. The only other sympatrically speciated (Turner et al. 2008) pupfish flock may shed light on this issue. Fitness could be measured in offspring to determine if female mate choice affects parasite resistance of offspring (Maan and Seehausen 2011). To accomplish this, lab-bred species and hybrids could easily be placed in natural exclosures and fitness, mating preferences, and parasite loads could then be compared. Additionally, explicit tests of whether parasite load prevents introgression need to be performed. Parasites clearly reinforce reproductive isolation. Whether they can create premating isolation without other ecological factors is less clear. The evidence from color morphs of Victoria Lake cichlid species indicates that this may be the case; however alternative hypotheses such as whether the two color morphs are occupying different niches need to be clarified. Long-term, evolutionary studies are needed to address the feasibility of this form of divergence.

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Figures and legends

Figure 1 – Experimental design of olfactory trials

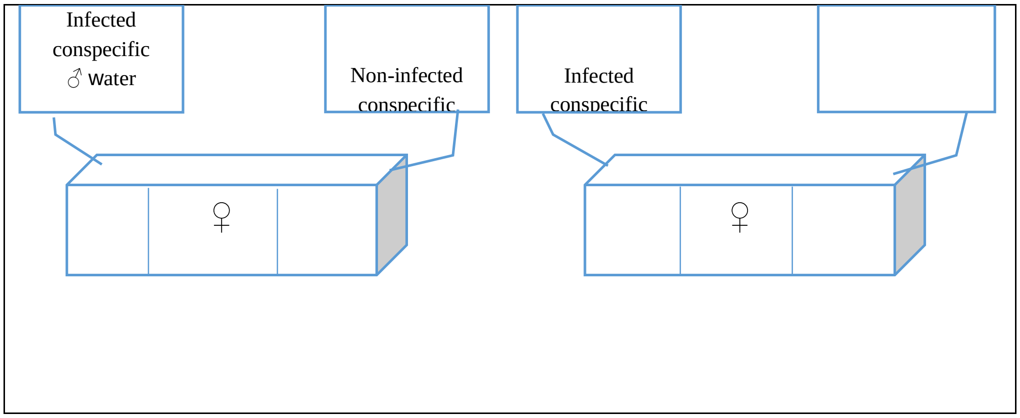
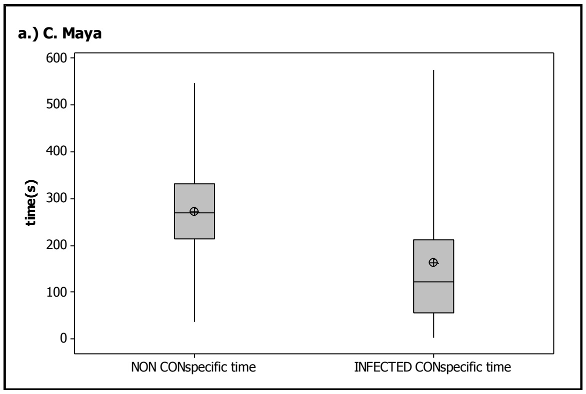
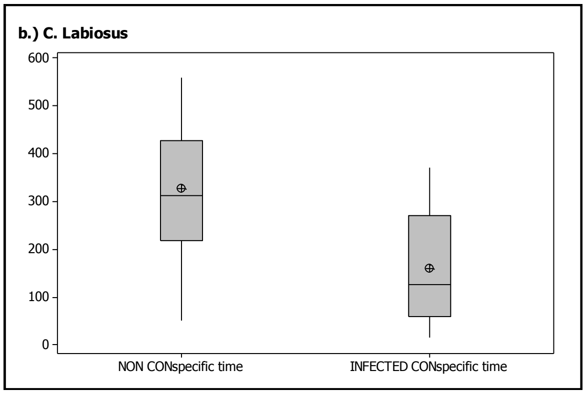
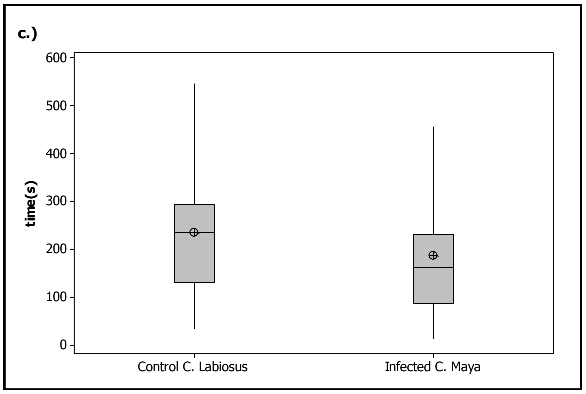


Figure 2 a) Females prefer control over infected conspecific males (a. *C. maya:* $F_{1,52}$=6.91, p= 0.011; b. *C. labiosus*: $F_{1,50}$=18.77, p<0.000). There is no difference in preference between non-infected (control) heterospecifics and infected conspecifics (c. *C. maya*: $F_{1,52}$=1.64, p= 0.21; d. *C. labiosus*: $F_{1,50}$=0.00, p=0.98)


a.) C. Maya
time(s)
600
500
400
300
200
100
0
NON CONspecific time
INFECTED CONspecific time


b.) C. Labiosus
600
500
400
300
200
100
0
NON CONspecific time
INFECTED CONspecific time


c.)
time(s)
600
500
400
300
200
100
0
Control C. Labiosus
Infected C. Maya

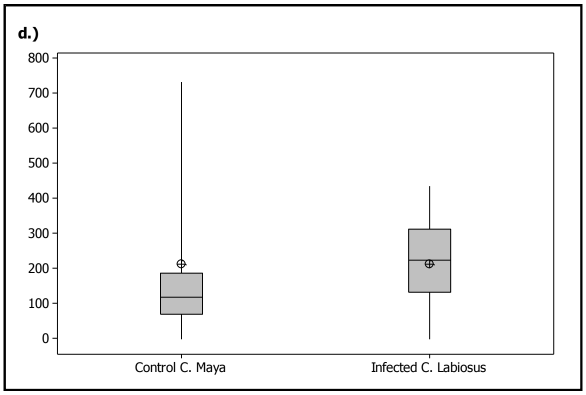
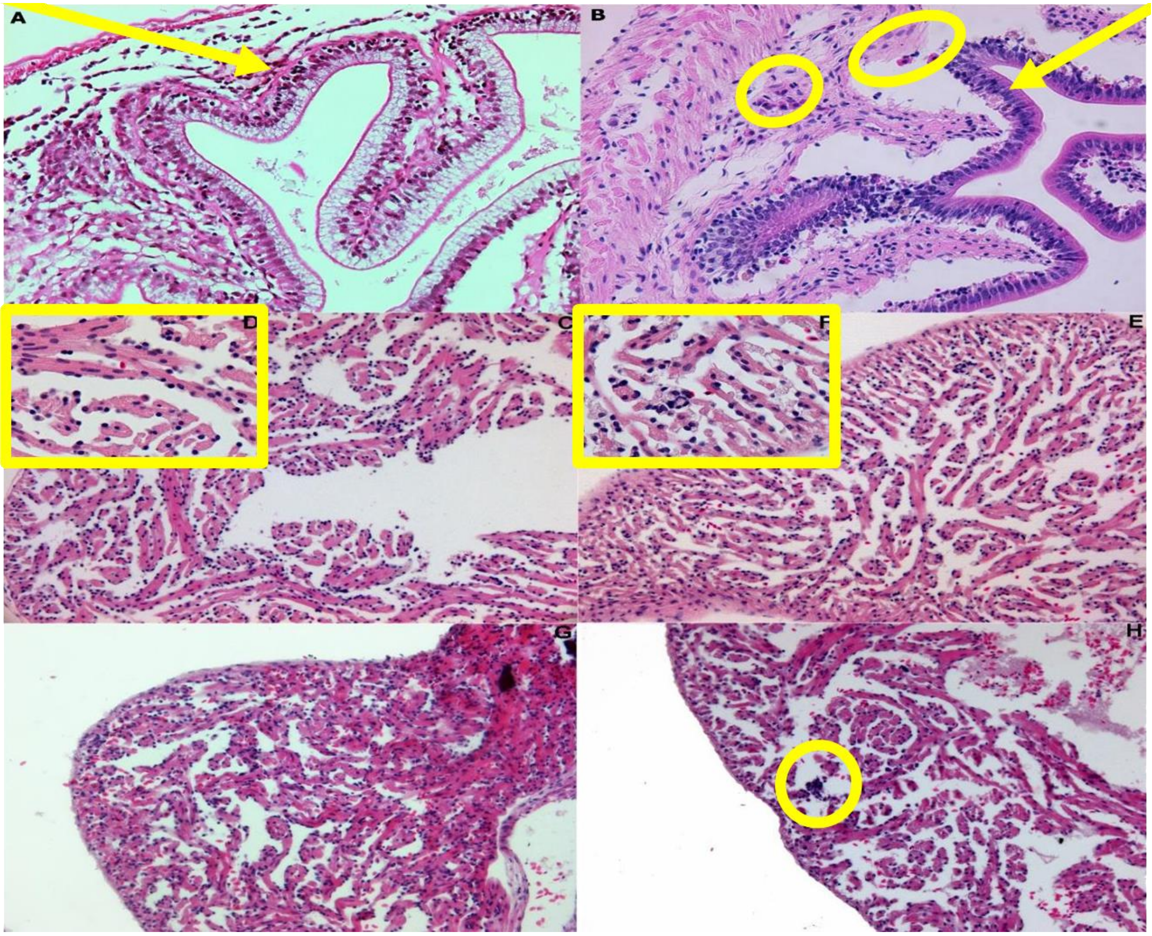

d.)
800
700
600
500
400
300
200
100
0
Control C. Maya
Infected C. Labiosus

Figure 3 – Bright field microscopy images from H&E stained explants. Controls are in the left hand column and infected in the right. A) Control gut sample from *C. labiosus* B) The gut of infected *C. labiosus* showed tissue damage, detachment of the epithelial layer form the lamina propria and large accumulation of eosinophilic granular cells (EGCs) C and D) control heart explants from *C. labiosus,* E and F) infected heart explants showed extensive signs of necrosis in the cardiac muscle fibers. G) control heart explant of *C.* maya; H) infected heart explant from *C. maya* was characterized by accumulations of lymphocytes not observed in the control group indicating an inflammatory response but was lacking signs of necrosis.



Summary

Multiple models have demonstrated the feasibility of sexual selection in sympatric speciation but not process itself (see Ritchie 2007 for a review). Single trait related to both ecological divergence and sexual selection, so-called “magic-traits” (Gavrilets 2003), have been thought to act as the sole character underlying reproductive isolation. In this dissertation, I and my collaborators have shown that multiple characters differ between species and traits under sexual selection differ from ecologically divergent traits. In the Bahamian system strong mating preferences based on visual traits suggest a role of sexual selection in the evolution of sexually-dimorphic traits that differ between species. Also, there is avoidance of heterospecifics due to predation of the scale-eating sister-species (Kodric-Brown and West, *in press*). Therefore, sexual selection, predator-prey dynamics, and niche use are thus likely reinforcing reproductive isolation between species. The last chapter of my dissertation shows that parasite load affects female mate choice but does not remove female antipathy towards heterospecifics. Future studies should examine the interaction between ecological characters, reproductive characters, mate choice, and habitat use to disentangle the evolutionary history of sympatrically speciated species.