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MHC and Mate Choice in Anolis sagrei

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MHC AND MATE CHOICE IN ANOLIS SAGREI

by

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DISSERTATION

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DEDICATION

This dissertation is dedicated to my family, Mom, Dad, Keane and David, whose enthusiasm for my work supports me every day. I also dedicate this work to Chris Eppig, whose daily guidance and support made this dissertation possible.

In Memory of Grandma, who passed during these years of study, and all the grandparents who sacrificed so much for their families.

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ABSTRACT

The study of sexual selection saw its intrepid inception when Charles Darwin observed the earnest with which many male birds must court a female before she will, but often not, mate with him. His idea of the "choosy female" was a radical one in the Victorian era when women were regarded as the "passive sex." In the century and a half that followed, sexual selection driven by female choice is now widely accepted and strongly supported. Today, with emerging technologies, the nuances and complexities of sexual reproductive are finally coming to light.

Female choice in two species is described here. First, the effect of familiarity was tested by allowing female sheepshead minnow, *Cyprinodon variegatus* to cohabit with a set of males, then replacing half of these males with new males. Spawnings with each individual male was recorded in both conditions. In the period in which females were with familiar and unfamiliar males, it was found that females spawned more with the familiar males.

In the second species, the brown anole, *Anolis sagrei*, sexual selection was investigated using a combination of behavioral, molecular, and statistical techniques. The

major histocompatibility complex (MHC) is a cluster of genes that encode receptors that are critical for adaptive immunity in jawed vertebrates. These genes have also been found to affect mate choice in many species, however, the genetic characterization of these genes is poor for non-avian reptiles. In order to examine the effect of the MHC on mate choice in the brown anole, a portion of this gene first needed to be described. As these data were gathered, courtship and mating behaviors were measured in the same animals. Using logistic regression to control for behavior, it was found in mating trials that females tended to mate with males that carried more genetic diversity.

TABLE OF CONTENTS

INTRODUCTION

For several decades, the study of female mate choice focused on male traits that were readily observable and measured. Using improved statistical techniques, combinations of male traits can be examined simultaneously to identify interactions while an integrative approach incorporates molecular and genetic data into the study of sexual selection. This dissertation examines these themes in two species, sheepshead minnow and the brown anole.

The first project tested if female sheepshead minnow, *Cyprinodon variegatus*, can become familiarized with individual males and if this recognition affects mating decisions. *Cyprinodon variegatus* females mate multiply over the mating season and must make multiple mating decisions over time. If high quality males have longer endurance on the lek and females track individual males' tenures, then familiar males are expected to have higher reproductive success than unfamiliar males. This prediction was tested in the laboratory by tracking the mating success of individual males. Groups of females were allowed to interact with four territorial males for two days. On the third day, two of the males were replaced with unfamiliar males and the mating responses were recorded for the next two days, as well as male traits: colour, territory size and aggressive behaviours. The differences in spawning success were found only to differ based on male familiarity rather than the characteristics of the males.

The goal of the second and third project was to combine genetic data with behavioral observations to test hypotheses of female mate choice for male genetic diversity in the brown anole lizard, *Anolis sagrei*. The Major Histocompatibility Complex

is a dense cluster of genes that function not only in immune defense, but also influence host-parasite ecology and behavioral ecology, and is important for conservation biology. In spite of its importance, very little characterization of the MHC has been published for squamates (snakes and lizards). We sampled diversity at the alpha-2 region of MHC-I genes and found a high degree of polymorphism as well as several conserved features of functional MHC molecules. These data were then incorporated with concurrent mating trials using the lizards that were sequenced. During binary choice tests, male behaviors were recorded as well as which of the two males in a trial copulated with the female. Logistic regression was used to analyze the behavioral data in conjunction with measures of genetic diversity to predict the likelihood of a male copulation with a female. This design was repeated in the lab at UNM and in situ in Florida where lizards were collected. The genetic influence on mate choice and environmental factors are discussed.

Chapter 1:

Effects of familiarity on mating preference in sheepshead minnow, *Cyprinodon variegatus*

Angela Hung, Astrid Kodric-Brown

ABSTRACT

Leks are display grounds where males gather to attract female mates. Sexual selection is driven by female mate choice in these systems. The variation in male displays on a lek may be low, thus females can gather more information by being attentive to a male's tenure on the lek. Defending a territory on a lek is energetically expensive and is therefore an honest signal of male quality. We tested if female sheepshead minnow, *Cyprinodon variegatus*, can become familiarized with individual males and if this recognition affects mating decisions. *Cyprinodon variegatus* females mate multiply over the mating season and must make multiple mating decisions over time. If high quality males have longer endurance on the lek and females track individual males' tenures, then familiar males are expected to have higher reproductive success than unfamiliar males. This prediction was tested in the laboratory by tracking the mating success of individual males. Groups of six females were allowed to interact with four territorial males for two days. On the third day, two of the males were replaced with unfamiliar males and the mating responses were recorded for the next two days, as well as male traits: colour, territory size and aggressive behaviours. Males that remained throughout the experiment had a higher mating success, females preferred to spawn with familiar males. Familiar males did not differ from unfamiliar males in behavioural traits (aggression and territory defence) or in breeding colour. Females were attentive to tenure on the lek instead of specific male behaviours. The ability to remain on a lek can be an indicator of good genes since defending a territory is energetically costly.

KEYWORDS

Familiarity, female choice, sheepshead minnow, *Cyprinodon variegatus*, pupfish, lek

INTRODUCTION

The lek mating system best exemplifies sexual selection for indirect genetic benefits. Males only provide sperm to females and no direct resource benefits (Borgia 1979). Females choose mates based on male displays and sometimes location on the lek, from which they must infer the quality of a male and his genes. In order for ornaments to persist, they must be condition-dependant signals that honestly reflect the suitability of a male's genes for a particular environment (Hamilton & Zuk 1982, Kodric-Brown & Brown 1984, Andersson 1994). Ornament expression is informative of the genetic quality of a male in terms of survivability, resource acquisition, and fertility. Females prefer high quality traits to pass on their underlying genetic components to offspring, increasing their survival and fitness. Males that are defending a territory on a lek must be in fairly good condition in order to compete.

However, if only the males with the best displays are on the lek, how can a female be assured of choosing the very best male? On many leks, maintaining a territory is energetically costly, thus, lek tenure is also an indicator of male quality. In ruffs, *Philomachus pugnax,* males do not feed while on the lek while they perform courtship displays (Hill 1991). In topi antelope, *Damaliscus lunatus*, males have lower body condition, are more susceptible to predators, and frequently fight during their tenure on a lek (Bro-Jorgensen & Durant 2003). In frogs, the major predictor of male mating success is chorus attendance (reviewed in Morrison et al. 2001). Because ornaments and displays

may not be highly variable in a lek and not entirely informative, females may add information about a male's quality by his persistence on a lek. In order to choose high quality mates, females must be able to recognize individuals in order to assess the duration of a male's tenure. Females may visit several leks repeatedly before copulating with a male.

Studies have shown that females prefer familiar males in non-lekking species (barnacle geese, *Branta leucopsis*: Choudhury & Black 1994; pygmy loris, *Nycticebus pygmaeus*: Fisher et al. 2003; wolf spiders, *Schizocosa spp*: Hebets 2003; Rutledge et al. 2010, cabbage beetle, *Colaphellus bowringi*: Liu et al. 2010; harvest mouse, *Micromys minutus*: Brandt & Macdonald, 2011; red deer, *Cervus elaphus*: Reby et al. 2011). Some proposed underlying mechanisms for preferring familiar males are maintenance of co-adapted gene complexes, intrasexual conflict. Prior experience with individual males, or particular male phenotypes, can also affect future mating decisions of females (Kodric-Brown 1995).

We examined female mating preferences for individual males in the sheepshead minnow, *Cyprinodon variegatus*, to determine if prior association affected subsequent female mate choice. If variation in male displays is low and tenure on a lek is an important, honest signal of quality, females should become familiar with high quality males and prefer them as mates. Unlike many other lekking species, female *C. variegatus* mate repeatedly throughout the breeding season and make a series of mating decisions

over time. This mating system particularly suited for the examination of temporal dynamics to determine if females can recognize individuals and adjust their mating preferences over time based on the familiarity of potential mates.

STUDY ORGANISM

Cyprinodon variegatus is a small fish (30 – 40 mm standard length) that is widely distributed in discrete subpopulations along the entire eastern U.S. coast (Haney et al. 2007). It is also found in streams and canals in Texas and New Mexico (Hassan-Williams & Bonner 2008). It is extremely hardy, tolerating a wide range of salinities (reviewed in Jordan et al. 1993) and temperatures (Bennett & Beitinger 1997).

Cyprinodon variegatus have a resource-based lek-polygynous breeding system. Female fish spawn throughout the breeding season with many males (Itzkowitz 1978). However, unlike classical lekking species, females lay eggs on male territories (Kodric-Brown 1977). Males establish and defend small territories in shallow waters with suitable oviposition sites. A lek may consist of as many as 20 territories with overlapping boundaries. Males develop distinctive breeding coloration, expressing bright, iridescent blue across the nape, an orange belly and orange pectoral and pelvic fins. Females are brown with a characteristic mottled pattern on the sides of their body. Courtship consists of a series of looping patterns performed by the male below the female and then they descend to the substrate. Females lay one egg at a time during a spawning event with a male which is easily observable by their distinctive body movement (Draud & Itzkovitz

2004). This allows direct quantification of an individual male's mating success. A pair may spawn several times in succession. There is no active parental care as the female leaves the male's territory after spawning, but eggs are defended as a consequence of being within the territory. Non-territorial males may be satellites around territorial males and sneak copulations (Kodric-Brown 1977).

Cyprinodon variegatus is an ideal system to study the role of familiarity in mate choice under experimental conditions because all fish can be individually marked to monitor behaviour and track pairings. They have a short life-span, and most males reproduce during a single breeding season. Individual males maintain territories for short periods of time – up to a couple of weeks (Kodric-Brown 1977). With such a high turnover of males on the lek during the breeding season, females can mate repeatedly with the same males or with males that have recently established breeding territories on the lek.

METHODS

Cyprinodon variegatus used in mate choice trials were maintained at the University of New Mexico, USA in 8-10ppt salinity at 26 degrees C. Fish experienced a 14:8 hour day: night cycle and were fed once a day between 1400 and 1600 hours on a mixture of 50% TetraMin Tropical Flakes® (Melle, Germany) and freeze-dried brine shrimp (San Francisco brand, Newark, CA, USA). We used sexually mature fish that

were the lab-reared descendants of a population collected from Lake Balmorhea, Texas (30°57.77′N 103°43.25′W).

All fish were individually marked with Visible Implant Elastomer® (Northwest Marine Technology, WA, USA). Fish were anaesthetized in 0.01% tricane methane sulphonate (MS-222) for 15 seconds, injected with a unique pattern of Elastomer® implants on one or both sides of the body alongside the dorsal fin, and placed in a clean, 38 litre recovery tank with strong aeration for at least 48 hours. Their behaviour was monitored during the recovery period. The implants did not affect the fish as they were observed to behave normally within a couple hours after the procedure.

For this study, only territorial males were used; sneaker/satellite males were excluded in order to get a more accurate quantification of female preference. Previous studies in a related species of pupfish, *C. pecosensis*, indicated that males that won in intrasexual competitions were likely to win in future contests (Kodric-Brown 1995). Territorial males were identified by placing three males in a 76 litre tank with four females and observing which males defended territories. This was the number of fish the tank could comfortably accommodate with room for each male to potentially defend a territory. Males that defended a territory in the tank were used in mate choice trials and housed in a separate tank. Losers of these contests were placed in one of four 79 litre tanks along with 15 to 20 females. Allowing females to cohabit with males more closely

approximates natural conditions and prevented indiscriminate spawning during mating trials. A total of 56 females and 30 males were used for mating trials.

Mate Choice Trials

Mate choice trials were conducted in outdoor fiberglass tanks, 1.8m in diameter holding 400 liters of water at a depth of 0.4m and a salinity of 8ppt, from 28 June to 18 August 2009. Each trial consisted of six females and four males. Four circular trays (diameter=20cm) with gravel were set equidistantly on the bottom of the tank to give the males a reference point for their territories and to provide a desirable oviposition substrate to defend.

Each trial lasted four days and six observations were taken over this period. On day one, six females were selected at random and added to the tank to acclimate for one hour. Then four males were added and allowed one to two hours to acclimate and set up territories (Fig.1). A ten minute observation began when the fish resumed normal activity (females foraging, males defending territories). The ten minute observation period was used because the fish will display all the activities within this time period. Observations were taken between 1200h and 1700h.

Three observations were taken over day one and two. On day three, all four males were removed from the mating tank to reduce the advantage of previous territory holding

experience. Two males were randomly selected from the initial four males in the mating tank and placed into a holding tank for 30 minutes. The other two were removed and replaced with two new randomly selected males from the tank housing territorial males (Fig. 1). All four males were simultaneously introduced into the mating tank. Fish were again acclimated for about an hour and once active, three more observations were taken over the two following days. For statistical analysis, we designated four categories of fish. "Out" refers to the two fish that began the trial and were removed on day three; "start" are the other two fish that began the trial and stayed in the tank for the second half of the trial, and are then referred to as "familiar" for the second half of the trial; "unfamiliar" are the new fish added on day three to replace the fish that were taken "out." Thus, "start" fish are the same as "familiars," but were categorized this way to facilitate comparisons and changes in behaviour within and between the two parts of each trial. Between trials, mating tanks were scrubbed clean, drained, refilled with fresh conditioned water, salt, and then aged overnight.

Because males were randomly selected and only checked to ensure that they had not been used in the same combinations previously, they were not size matched. We recorded and quantified the following behaviours -- number of chases between males, number of displays and fights, including the identity of the fish involved. In displays males face each other directly, perpendicularly or laterally and flare their fins without biting each other. In fights, males bite each other as they swim in fast, tight circles after each other. Because very few displays and fights occurred relative to chases, we

combined them and categorized them as male aggression for analysis. The sum of the three observation periods for each half of the trial was used for analysis. Territory size was estimated as the per cent area of the tank a male defended. A territory owner actively patrolled and chased males from this area. Boundaries could be determined by where the territorial male terminated the chase of an intruding male. The intensity of the breeding colour of the males was recorded on a scale from 0 to 1, where 0 is no blue/cryptic female mimic, 0.25 is light blue at the nape, 0.5 is bright blue, 0.75 is deep blue, and 1 is a nearly-black, iridescent blue and bright, orange fins. The average of the three observations was used for the value of the trial for territory size and colour for each two-day period. We also recorded the number of eggs a female laid with each male and the identity of the male that fertilized them; these were summed for each male for each two-day period. Spawnings were easily observed and are characterized by an S-shaped movement performed in tandem by a spawning pair. At the end of each trial, standard length was measured to the nearest 0.01mm with callipers and mass, to the nearest 0.01g. The residuals of length and mass were computed to get a single body size metric. Due to limited numbers, males were reused for different trials, but always in novel combinations. Thus, multiple mass and length measurements for individual fish were averaged for analysis. Eighteen trials were completed; one was excluded from analyses because a male died during the first half of the trial.

Statistical Analysis

Male fish were categorized into four roles for analyses: first two-day period: "start" and "out"; second period: "familiar" and "unfamiliar" (Fig. 1). T-test, blocked by trial, was used to compare the spawning success between "familiar" and "unfamiliar" fish. Matched pairs t-tests compared the colour, territory size, aggression and spawning during each half of the trials of the two males that stayed throughout the mating trial. Balanced and blocked ANOVAs were used to determine differences in male traits of all four roles between and within the two parts of each trial. For females that were observed to mate multiply, the number of eggs fertilized by unfamiliar males was compared to the number fertilized by familiar males with repeated measures t-test. A chi-square test determined the change in territory position of familiar fish. Unless noted, all tests were two-tailed. Data were analysed in JMP 9.0. This project has been reviewed and approved by the University of New Mexico institutional review board (IACUC protocol no. 07UNM037).

RESULTS

Fifty-six females (standard length 34.44mm ± 3.13mm; mean mass=1.42g±0.35g) and 30 males (mean standard length= 38.08mm±3.17; mean mass= 1.89g±0.42g) were used. While some of the males were reused for up to five different trials, all except four males were observed to spawn during the trial, excluding the possibility that certain males monopolized matings. Twenty six females were observed to mate.

The two familiar males in each trial fertilized significantly more eggs than the unfamiliar males (Fig. 2, blocked t-test: t_{50}=2.36, P=0.022). Familiar males spawned more during the second half of the trial than they did in the first half (one-tailed, repeated measures t-test: t_{34}=2.52, P=0.008). To determine if, by chance, males that stayed were more attractive to begin with, or if they had a mating advantage later, we compared the spawning success of males within the first half of the trial. There was no difference in the spawning success of the males that were removed (out) and those that became the familiar fish (start) in the second half of the trial (Student's t-test: t_{65}=0.54, P=0.56). Additionally, when familiar males were removed and reintroduced to the tank at the beginning of the second half of trials, they did not necessarily defend the same territories (X^2 test: X^2_2=1.71, P=0.19).

A t-test, blocked by trial, showed that there was no difference between the number of times females spawned during the first and second halves of each trial (t_{92}=1.50, P=0.14). Females that mated multiply during the second half of mating trials preferred familiar males (one-tailed, repeated measures t-test: t_{17}=2.47, P=0.012). There was no correlation between male size and spawning success (Pearson correlation: R=0.008, N=30, P=0.63).

We used analysis of variance (ANOVA), blocked by trials, to determine if familiar males had an advantage as a result of more time and experience in the tank. We compared the colour, territory size and aggressive behaviour of males at the beginning of the 17 trials (those that were removed and those that stayed) with males in the second half of the trials (those that were just introduced and those that stayed). Comparing these four

roles of males in one analysis gave us information about whether they differed within each half of the trial and between the two halves of the trial. We found that colour, territory size and aggressive behaviours were not significantly different between the four male roles (Table 1). Familiar males did not change in their traits or behaviour between the first and second part of the trial (Table 1), nor were the newly added males (unfamiliar) different from those that stayed from the first half (familiar).

DISCUSSION

Our results showed that familiarity affected the mating preferences of *C. variegatus* females, although individual recognition was not directly tested for. Females preferred to mate with males with which they had more experience. Since breeding colour, territory size or aggressive behaviour of familiar males did not change over the course of the experiment, or differ from other males, females were tracking male tenure on a lek, possibly as an indicator of male quality. Females recognized males by their individual characteristics rather than by the location of their territories, since these changed between the first and second parts of the trial. Selection for individual recognition is widespread and has been found in invertebrates (Odeen & Moray 2008; Gershman 2008) as well as fish (Zajitschek & Brooks 2008; Bierbach et al. 2011). In pygmy rabbits, *Brachylagus idahoensis*, familiarity between mating pairs increases reproductive success (Martin & Shepherdson 2012). Familiar signals and cues that

indicate the extended presence of high quality males are preferred by females (Reby et al. 2001; Fisher et al. 2003).

In leks with a high turnover of males, individuals that maintain a territory over an extended period of time should be of high quality, making tenure an important signal for females to be attentive to. The ability to shoulder the high energetic cost of defending a territory from intruders, while simultaneously courting females, is an honest signal of condition. In *C. pecosensis*, territorial males expressed nuptial coloration and were in better condition than non-territorial males (Kodric-Brown & Nicoletto 1993). In several lekking species, duration of lek attendance has been correlated with male reproductive success (reviewed in Hill 1991). Tenure on a lek predicted male reproductive success in tree frogs (Castellano et al. 2009), ruffs (Hill 1991) and sandpipers (Lanctot 1998). In tree frogs, males only attended leks when they were able to perform a competitive, energetically costly, courtship display. Thus, variation in male reproductive success was positively correlated with lek attendance (Castellano et al. 2009). In ruffs, male displays were energetically demanding due to long hours on the lek without feeding (Hill 1991). These studies show that in species where females visit leks repeatedly before copulating, they may be gathering information on male condition (Hill 1991).

Our results suggest that male traits are poor predictors of reproductive success. Males displaying on leks have similar competitive abilities and expression of secondary sexual traits – breeding coloration, leaving females to choose on other criteria, such as tenure on a territory. In ruffs and sandpipers, *Tryngites subruficollis*, male behaviours

also were unreliable predictors of mating success (Hill 1991; Lanctot 1998). A previous study of *C. variegatus* suggested that females mate randomly among territorial males irrespective of male quality (Draud & Itzkowitz 2004). These results may be a result of low variation in male traits on a lek because only males in good physical condition are in attendance.

The results of this study show that female choice is dynamic on an individual level because females spawned multiply during both parts of the trial. Although we did not detect any differences in male traits between preferred and non-preferred males, we showed that multiply mating females incorporate a time scale to assess lek tenure, and use this information for future reproductive decisions. Furthermore, in lekking birds, genetic analysis of clutches is revealing multiple paternities in broods (reviewed in Hess et al. 2012), showing that the integration of information about males that influence future mating decisions may be more widespread in lek species than previously thought. Although most studies offer a snapshot of female choice under certain conditions, some recent studies have measured female preferences over longer time scales: throughout a breeding season and over varying conditions (reviewed in Lehtonen et al. 2010). These studies found that females chose different male characteristics at different times (lark bunting, *Calamospiza melanocorys*: Chaine & Lyon 2008; house finch, *Carpodacus mexicanus*: Oh & Badyaev 2006; sand goby, *Pomatoschistus minutus*: Lehtonen et al. 2010).

FUTURE DIRECTIONS

The problem that arises if females consistently choose the same, or few, high quality male(s) is genetic homogenization of the population over several generations. Strong directional selection depletes genetic variance on a population over time, reducing the benefits of good genes choice for females. The paradox of the lek is then why do females continue choosing good genes (Kirkpatrick and Ryan 1991)? Using genetic data, it remains to be determined if the paradox in *C. variegatus* is resolved through female choice for heterozygous males (Neff and Pitcher 2008) or through genic capture (Rowe and Houle 1996), both of which are hypothesized to contribute to condition, and in this species, tenure on the lek. However, in *Cyprinodon* species, female multiple mating over the breeding season and male turnover on the lek may be enough to prevent reproductive skew by very few males in a population. Future work should examine turnover rates of males on leks and corresponding female preferences for individual males. Correlations between length of residency, female choice of males, and male reproductive success will provide insights into what types of information females gain and their subsequent decision rules, especially for species with a lek breeding system. It is likely that females, especially in fish with a promiscuous mating system, avoid depleting genetic diversity by mating with several, long-tenured males over the breeding season.

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FIGURE CAPTIONS

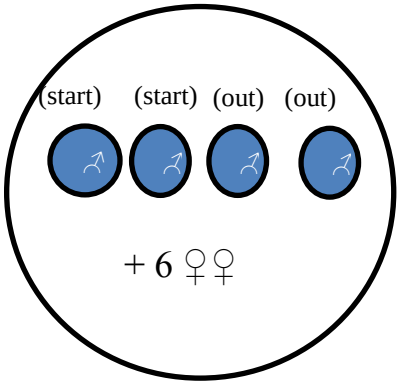
Figure 1. Experimental design. Trials were completed over four days. Part One: On the first day, four males and two females were placed into a mating tank and observed for three, ten-minute periods over two days. Part Two: On the third day, two males were removed and replaced with two new males. Three more observations were taken over days three and four.

Figure 2. Mean number of spawnings over three ten-minute observation periods with familiar and unfamiliar males (paired t-test blocked by trial: t_{50}=2.36, N=17 trials, P=0.022). Each error bar is constructed using one standard error from the mean.

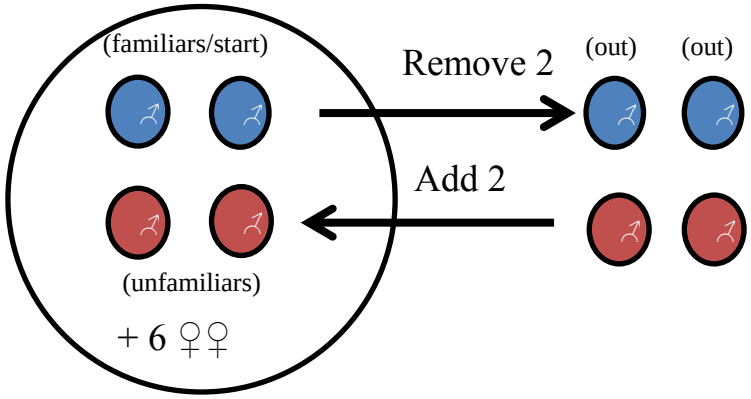
Table 1. Mean values of territory size, colour and aggression of each male type $\pm$ SE. Territory was recorded as the per cent of the tank defended by males, colour is scaled from 0 to 1, and aggression is the number of chases and fights between males for 17 trials. Blocked one-way ANOVA was used to compare the differences between the four male types of each of these variables.

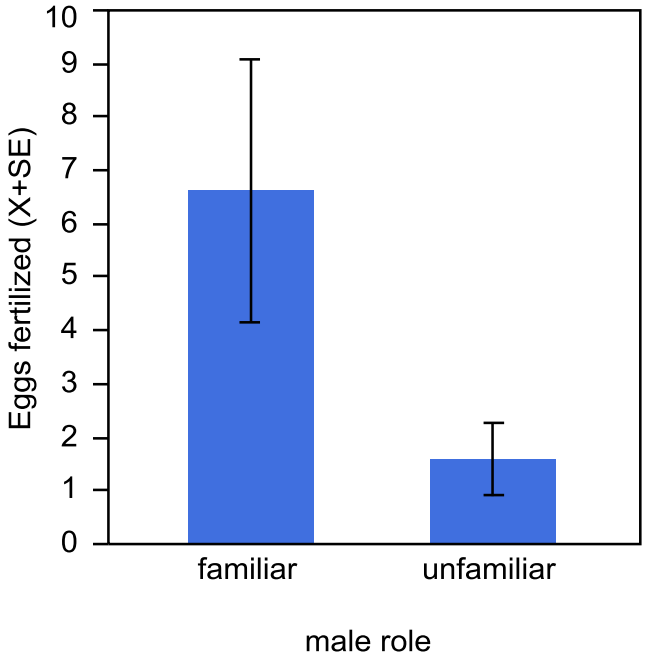
FIGURES

Part 1 – Day 1



Part 2 – Day 3




10
9
8
7
6
5
4
3
2
1
0
Eggs fertilized (X+SE)
familiar
unfamiliar
male role

	Male Role	Territory	Colour	Aggression
First half	Start	20.25±4.04	0.21±0.036	22.71±3.68
	Out	22.75±4.04	0.18±0.036	27.71±3.68
Second half	Familiar	24.91±4.04	0.24±0.036	22.97±3.68
	Unfamiliar	21.87±4.04	0.19±0.036	15.56±3.68
Blocked ANOVA		$F_{3,116}$=0.23, P=0.87	$F_{3,116}$=0.59, P=0.62	$F_{3,116}$=1.85, P=0.14

Chapter 2:

On the polymorphism of MHC class I loci in invasive populations of the brown anole, *Anolis sagrei*

Angela Hung, Robert D. Miller

ABSTRACT

The Major Histocompatibility Complex is a dense cluster of genes that function not only in immune defense, but also influence host-parasite ecology and behavioral ecology, and is important for conservation biology. In spite of its importance, very little characterization of the MHC has been published for squamates (snakes and lizards). Yet this information would be invaluable for understanding the evolution of the immune system in amniotes and, more specifically, for a basic understanding of reptilian immunology. Here, we present a study of MHC class I polymorphism in the brown anole, *Anolis sagrei*, a member of the most diversified lizard clade. We sampled diversity at the alpha-2 region in 116 lizards from three locations in Florida and found 136 nucleotide sequences and 108 peptide sequences. These sequences have several conserved features of functional MHC molecules. Individuals carried between 1-11 peptide alleles, indicating the presence of multiple class I loci with gene copy number variation. In a phylogenetic analysis of squamate MHC, *A. sagrei* grouped with iguanid lizards, however, one variant was more closely associated with iguanas than anoles. These data should begin to lay the groundwork for additional studies in non-avian reptile MHC, including more members of the *Anolis* genus.

INTRODUCTION

The Major Histocompatibility Complex (MHC) is a gene dense region unique to the genomes of jawed vertebrates that functions in immune defense. Many of the genes within the MHC encode cell-surface receptors that bind antigens and present them to T-cells, initiating responses to pathogenic infections (Klein and Sato 2007). There are two classes of MHC presenting molecules, designated class I and II. For the most part, class I genes are expressed widely throughout various tissues in the body and class I molecules present endogenously sourced antigens to $CD8^+$ cytotoxic T cells, while class II genes are expressed on professional antigen presenting cells and the molecules they encode present exogenous antigens to $CD4^+$ helper T cells (Kaufman et al. 1994). Both classes of molecules can bind and present short peptide antigens to patrolling T cells. The nature of the antigens that are presented by the MHC depends on the amino acid residues in the binding site of the receptor, which are encoded by the region of greatest genetic polymorphism (Klein and Figueroa 1986). In MHC class I molecules the binding site is encoded by exons-2 and 3 of the α chain gene (Kaufman et al. 1994). An enormous degree of polymorphism facilitates presentation of a wide array of antigenic peptides from the plethora of pathogens that organisms are typically exposed (Spurgin & Richardson 2010).

The MHC has been studied most thoroughly in humans and several (nonhuman) major taxonomic groups representing amphibians, fish, birds and mammals. Non-avian reptiles, however remain under-studied (Kelley et al. 2005). Sequences from a limited sampling of species including water snake (Grosserger and Parham 1992), Ameiva

(Grosserger and Parham 1992), Chinese soft shelled-turtle (GenBank accession no. AB185243), sphenodon (Miller et al. 2006), gecko (Radtkey et al. 1996), saltwater crocodile (Jaratlerdsiri et al. 2012), and iguanas (Glaberman and Caccone 2008) have been collected. As a major lineage of amniotes, non-avian reptile MHC data are needed to complete our understanding of the evolution of the MHC and comparative immunology of amniotes in relation to themselves and to other vertebrate classes.

Here, we use sequences encoding the alpha-2 region of MHC class I molecules from three populations of *Anolis sagrei* in Florida. The anoline lizards (order: Squamata) are a highly diversified genus found in tropical and subtropical regions. Their history of extreme diversification makes them a popular model for evolutionary studies. *A. sagrei* is a small lizard (40-65mm snout-vent-length) from the Caribbean that has been introduced and spread throughout the southeastern United States, where they are now an abundant invasive species (Campbell 1996). This species has also colonized Hawaii and Taiwan (Kolbe et al. 2004), proving themselves to be adept invaders.

METHODS

Tissue samples

Anolis sagrei were collected from three locations in Florida; Merritt Island (N = 77), Cape Canaveral (N=28) and Miami (N=11). Genomic DNA was extracted from tail clips using DNeasy Blood and Tissue spin column kits (QIAGEN) according to the manufacturer's protocol. These procedures have been been reviewed and approved by the UNM Main Campus Animal Care and Use Committee (#100359).

PCR, cloning and sequencing

Exon 3 of MHC class I genes were amplified by PCR using forward primer: 5' AGGAGAGACGGGAGCAAACCAGGGTA 3' and reverse primer: 5' AGCCAACTCCACGCASWYBTCCTCCA 3'. These primers were based on *A. segrei* sequences amplified using primers described in Radtkey et al (1996) (see Results). The 25 µl reaction mix included 20-30ng of genomic DNA, PCR buffer, dNTP mix, and *Taq* polymerase according to Advantage-HF2 pcr kit (Clontech, Mountain View, CA, USA) protocol and 0.1µM of each primer. Amplification occurred under the following conditions: one step at 94˚C for 45s, 33 cycles of 94˚C for 30s, 66˚C for 30s, and a final step at 68˚C for three minutes.

Due to the mixed population of sequences amplified, PCR products were cloned for sequencing using the TOPO Top10 Cloning Kit (Invitrogen, San Diego, CA). Twelve to 16 colonies containing the correct insert size were sequenced using the Big-Dye Terminator v3.1 Cycle Sequencing kits (Applied Biosciences). The resulting sequence chromatograms were edited in Sequencher.

Sequence analysis

Sequences were aligned with ClustalW. Substitutions that appeared in only a single individual at a position that was otherwise conserved in all other sequences were attributed to PCR error. Three sequences with premature stop codons were excluded from

further analyses. And were attributed to null alleles and likely not under selection and were also excluded.

Genetic distances and phylogenetic trees were calculated using MEGA 5.1 (Tamura et al. 2011). Bootstrap values showed that the best-fitting tree was a maximum likelihood tree constructed using the Tamura-Nei model, assuming a gamma distribution with invariant sites after 1000 bootstrap replications. Genetic diversity of individuals was calculated in Microsoft Excel 2010. Statistical analyses were completed using JMP 9.0 (SAS Institute 2010).

Phylogenetic reconstruction of squamates was completed using MHC-I, exon 3 nucleotide sequences from Genbank for the following species: green anole (*Anolis carolinensis*), accession XM_003229776; Galapagos marine iguana (*Amblyrhynchus cristatus*), EU604308; Galapagos land iguana (*Colonophus subcritatus)*, EU604316; Rhinoceros iguana (*Cyclura cornuta*), EU839669; geckos (*Lepidactylus lugubris, Lepidactylus spp.*), U58169, U58172, respectively; Ameiva (*Ameiva ameiva*), M81096; Balsas armed lizard (*Ctenosaura clarki*), EU839667; spiny-tailed iguana (*Ctenosaura defensor*), EU839666. Tuatara (*Sphenodon punctatus*), DQ145788, was used as an outgroup. These were found by Blast searches against three *A. sagrei* sequences (196c1, C8c2, 348c9).

RESULTS

Characterization of MHC class I, exon-3

Using degenerate PCR primers capable of amplifying a 176 bp region of MHC class I exon 3, first described by Radtkey et al. (1996), 12 unique sequences were isolated from eight lizards. Since these primers also amplified a number of non-MHC loci in *A. sagrei* the sequences isolated were used to design more specific primers that amplified 143bp product, excluding primers.

Using the *A. segrei* specific primers genomic DNA was used as a template to amplify and 10-16 independent clones from 116 lizards. Collectively these clones contained 136 unique nucleotide sequences encoding 108 peptide sequences. There was an overall average of 4.6 nucleotide sequences (range: 2-11) per individual and 4.5 peptide variants per individual (range: 1-11), indicating that at least six loci were amplified by our primers. PCR and cloning was repeated for individuals with fewer than three sequence variants, which identified up to three new sequences. Constructing a neighbor-joining tree could not resolve strong relationships between sequence variants and so could not be assigned to specific loci (not shown).

The region of exon-3 that was amplified encoded many of the conserved sites characteristic of functional MHC class I genes. Seven amino acids were conserved in all sequences (Fig. 1). Additionally, amino acid residues Y4, Y11, T31, R34, W35 are well conserved in *A. sagrei* (Fig. 1) and across species (Fig. 2). These sites correspond to pocket binding site F in mammalian class I molecules whose structure has been established. Pocket F anchors the termini of bound peptides and has remained fairly conserved across species (Kaufman et. al. 1994). Another conserved site, D7, is potential salt-bridge forming residue (Fig. 1).

The *A. sagrei* protein sequences differed by 1- 25 amino acids residues; positions 2 and 38-47 in particular were highly variable (Fig. 1), reflecting their potential role in binding peptide antigens. For comparison, residues 5, 25, 28, 29, 37, 38, 41, 42 and 45 are polymorphic binding residues in human HLA class I molecules.

Anolis sagrei *compared to other squamates*

Phylogenetic reconstruction was completed using MHC class I, exon-3 nucleotide sequences for squamates (Fig. 3). Bootstrap values were derived from 1000 replications. As expected, the analysis revealed that *Anolis* MHC-I exon-3 forms a clade within the iguanid lizards. One sequence grouped more closely with the green anole, which may reflect a relatively shallow divergence time. A second *A. sagrei* variant (196c1) clustered with the Galapagos and green iguanas and may be a trans-species polymorphism which are known to confound phylogenetic analyses (Klein et al. 1998). This sequence was fairly common, found in 50 out of 116 lizards sequenced. However, neither the relationship among the available *Anolis* sequences nor the *A. sagrei* in the iguana group is well supported, which is likely a consequence of the short sequence length and high polymorphism in the gene region.

Population differences

The average number of variants was not significantly different between Cape Canaveral and Merritt Island populations (Fig. 4a; Student's t-test: n=106, t_{114}=-0.15, p=0.88). The Miami population had fewer variants on average than both the Cape

Canaveral population (Fig. 4a; Student's t-test: n=39, t_{114}=-2.06, p=0.042) and the Merritt Island population (Fig. 4a; Student's t-test: n=99, t_{114}=-2.175, p=0.032). The average peptide diversity also differed between populations with Miami having significantly less diversity compared Cape Canaveral, but not Merritt Island (Fig. 4b; ANOVA: $f_{2, 113}$=3.26, p=0.042, n=114).

DISCUSSION

The Mhc class I, exon-3 region was sequenced from 116 *Anolis sagrei* lizards from three populations in Florida. Collectively, the gene sequences generated encoded 108 unique peptide sequences all with the characteristics of functional class I molecules such as a potential salt bridge forming residue and conserved pocket binding residues. There was a large amount of diversity in Mhc class I genes between individuals. This is consistent with the MHC being under positive selection and its role in being able to bind a variety of antigens and initiated responses to a diverse number of pathogens. This is functionally significant as the *Anolis* genus is common in the tropical and subtropical zones of the Caribbean where pathogens are diverse and abundant. Anoles have been found infected with helminthes (Bursey & Brooks, 2010), malarial parasites (in Florida, Perkins et. al. 2007), coccidia (Bui et. al. 1992), and even Chytridiomycosis fungus (Kilburn et. al. 2011), thus their immune system is under strong selection. The mechanisms maintaining this extraordinary diversity include heterozygote advantage,

frequency-dependent selection, balancing selection (Spurgin and Richardson 2010) and mate choice (Milinski 2006).

Class I genes are of particular interest in malarial infections due to the intracellular stage of the parasite. In birds, associations have been found between the prevalence of malaria in great reed warblers (*Acrocephalus arundiaceus*) and MHC class I characteristics (Westerdahl et al. 2005). A specific MHC class I allele has also been found to protect against severe malaria from *Plasmodium falciparum* infection in humans (Hill et al. 1991).

The high genetic diversity at MHC loci is also consistent with high diversity in mitochondrial DNA found in invasive populations of *A. sagrei* in Florida (Kolbe et al. 2004). Although *A. sagrei* is invasive to the U.S., and a reduced level of diversity might be expected as a result of founder effects, high levels of genetic diversity are generated as established populations have the opportunity to breed with individuals from subsequent introductions from source populations from the Carribbean. It is likely that additional sequences will be identified with additional sampling both within individuals and populations.

There were genetic differences between the populations that were sampled. The Miami population had a lower average number of alleles, than both the Merritt Island and Cape Canaveral populations, and lower amino acid diversity than the Cape Canaveral population. Genetic analysis of Florida lizards indicated that the source populations of Merritt Island and Cape Canaveral are from Western and East-central Cuba while Miami lizards are predominantly from Western Cuba (Kolbe et al. 2007). Although Miami was

the least sampled of the three populations, additional sampling will likely reveal many more variants but remain distinct from the Merritt Island and Cape Canaveral groups.

In a phylogenetic reconstruction of squamates, Iguanids formed a monophyletic group. However, the relationships between MHC sequences between the available *Anolis* sequences were not well resolved. Two possible trans-species polymorphisms, one shared between *A. sagrei* and *A. carolinensis*, and one shared between *A. sagrei* and the Galapagos marine (*Amblyrynchus cristatus*) and land (*Colonophus subcritatus*) iguanas, as well as the green iguanas, may account for the weak phylogenetic support. The high levels of sequence diversity within *Anolis* allowed for one *A. sagrei* sequence to group more closely with the iguana species, confounding the overall phylogeny. These weak relationships may be the consequence of using such a short, polymorphic region.

While there is much to learn about the MHC in lizards, continued study in the *Anolis* genus would facilitate a wide range of studies. Due to their extensive radiation throughout the Caribbean and South America into one of the most diversified vertebrate clades, these lizards already provide many classic examples for comparative studies in island evolution, dispersal, vicariance, divergence and adaptive radiation, niche partitioning, community ecology, and communication, among other topics (Losos 2009) . How has their diversification affected the structure and diversity of the MHC? Or did their MHC genes help facilitate their radiation and spread? Additionally, with complex visual displays and dominance interactions, it would be unsurprising to find some effects of MHC alleles in shaping morphological traits or behaviors as has been found in ring-necked pheasants, *Phasianus colchinus* (von Schantz et al. 1996), white-tailed deer,

Odocoileus virginianus (Ditchkoff et al. 2001), mandrills, *Mandrillus sphinx*, (Setchell et al. 2009) and sand lizards, *Lacerta agilis* (Olsson et al. 2005).

Furthermore, *A. sagrei* is an important system for research in colonization and invasion biology. Of the approximately 400 species of Anolis, *A. sagrei* stands out as an especially adept invader (Losos 2009). It is possible that the structure and diversity of the MHC in these species may have a role in a species' adaptability to new habitats.

As we increase our knowledge of the MHC and immune systems of non-avian reptiles, we will improve our foundations in comparative immunology and biology. More information is still sorely needed and efforts should continue to elucidate the genomes of reptiles. The information about immune evolution may support the use of diurnal Anoles and other reptiles as model organisms when nocturnal rodents are not appropriate, such as for understanding immunological effects of sunlight, UV radiation, UV activated-vitamin D dependent pathways, skin immunology in the absence of fur, skin microbiomes and contact transmission of pathogens. Not only is this relevant from an immunological perspective, but these data will be valuable for evolutionary studies, host-parasite interactions (Kubinak et al. 2012) as well as behavioral ecology (Milinski 2006) and conservation biology (Ujvari & Belov 2011).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE CAPTIONS

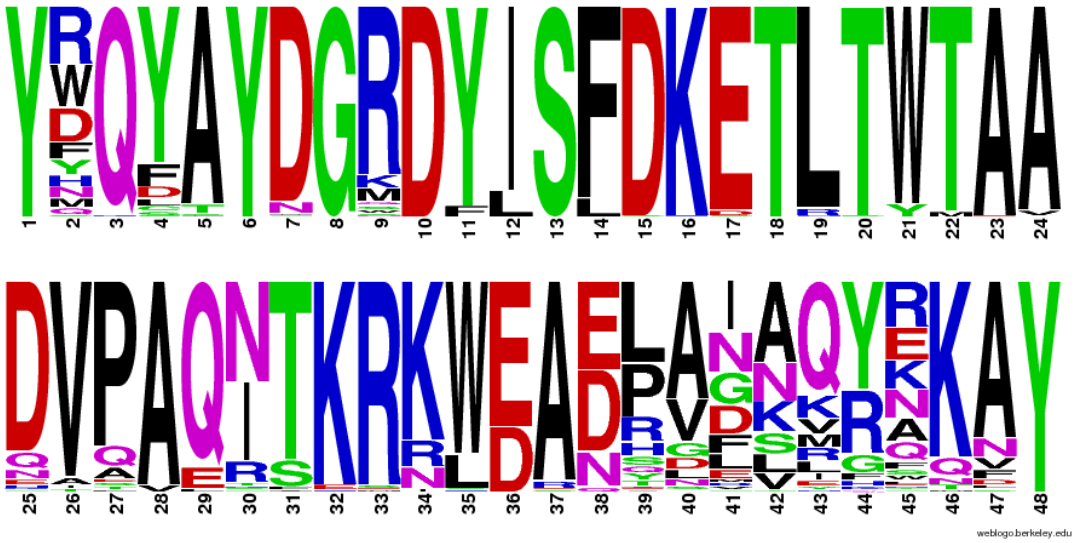
Fig. 1 WebLogo plot of amino acid frequencies at each position for *A. sagrei*, calculated using 108 sequences. Positions 5, 25, 28, 29, 37, 38, 41, 42, 45 are variable binding sites in human HLA class I molecules. D7 is a potential salt-bridge forming residue. Residues Y1, Y6, G8, D10, S13, T18, Y48 were conserved in all lizards

Fig. 2 Cross-species alignment including three *A. sagrei* alpha-2. Dashes represent gaps, dots indicate identity with the first sequence. *A. sagrei* sequences include translated forward primers. Positions are numbered to be consistent with Figure 1. Shaded sites indicate binding residues in pocket F

Fig. 3 Maximum likelihood tree with 1000 bootstrap replications of Squamate MHC class I, exon-2 nucleotide sequences. *Sphenodon punctatus* was used as the outgroup

Fig. 4 Average number of sequences (a) and average peptide diversity (b) found in each lizard for each population sampled. Miami lizards had fewer variants on average than both the Cape Canaveral population (Student's t-test: n=39, t_{114}=-2.06, p=0.042) and the Merritt Island population (n=99, t_{114}=-2.175, p=0.032). Line is the overall mean. b) Miami lizards were less diverse in their alpha-2 regions than Cape Canaveral and Merritt Island (ANOVA: $f_{2, 113}$=3.26, p=0.042, n=114). Line is the overall mean

FIGURES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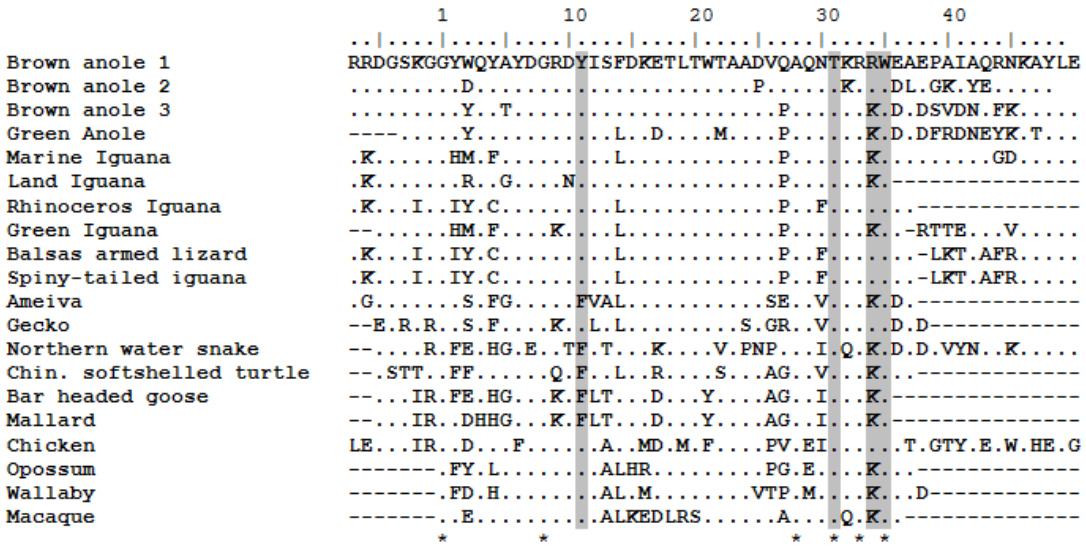
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Brown anole 3 .....Y..T.....P.....K.D.DSVDN.FK.....
Green Anole ----.....Y.....L..D....M....P.....K.D.DFRDNEYK.T...
Marine Iguana .K.....HM.F.....L.....P.....K.....GD.....
Land Iguana .K.....R..G....N.....P.....K.----------
Rhinoceros Iguana .K...I..IY.C.....L.....P..F.....----------
Green Iguana --.....HM.F....K....L.....P.....K..-RTTE...V.....
Balsas armed lizard .K...I..IY.C.....L.....P..F.....-LKT.AFR.....
Spiny-tailed iguana .K...I..IY.C.....L.....P..F.....-LKT.AFR.....
Ameiva .G.....S.FG.....FVAL.....SE..V...K.D.----------
Gecko --E.R.R..S.F....K..L.L.....S.GR..V.....D.D----------
Northern water snake --....R.FE.HG.E..TF.T...K....V.PNP...I.Q.K.D.D.VYN..K.....
Chin. softshelled turtle --.STT..FF.....Q.F..L..R....S...AG..V...K...----------
Bar headed goose --...IR.FE.HG...K.FLT...D...Y....AG..I...K.----------
Mallard --...IR..DHHG...K.FLT...D...Y....AG..I...K.----------
Chicken LE...IR..D...F.....A..MD.M.F....PV.EI.....T.GTY.E.W.HE.G
Opossum -------.FY.L.....ALHR.....PG.E....K...----------
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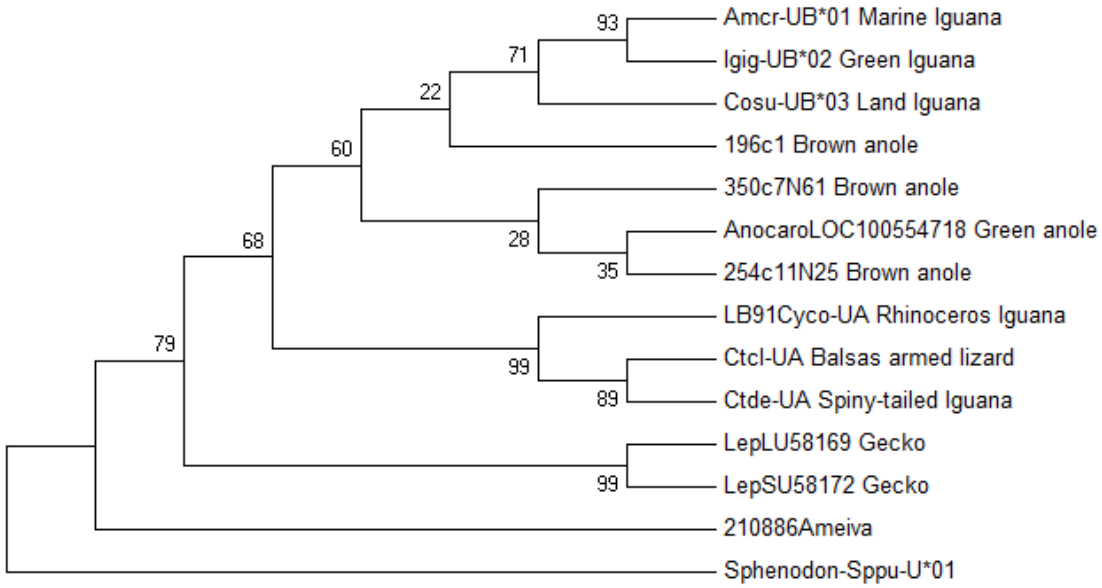
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LB91Cyco-UA Rhinoceros Iguana
Ctcl-UA Balsas armed lizard
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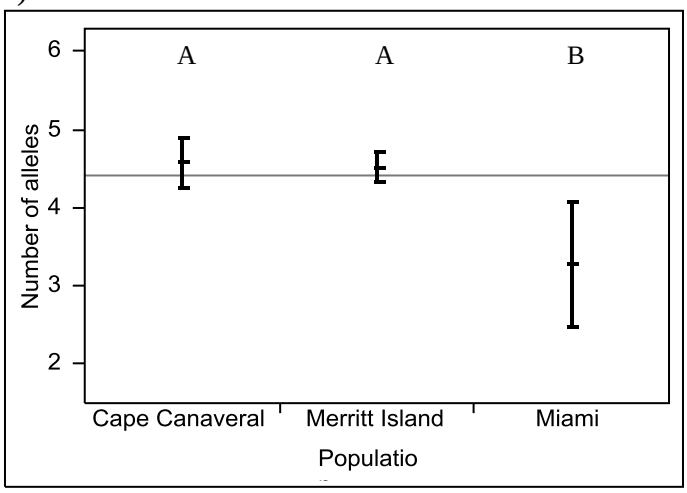
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Number of alleles
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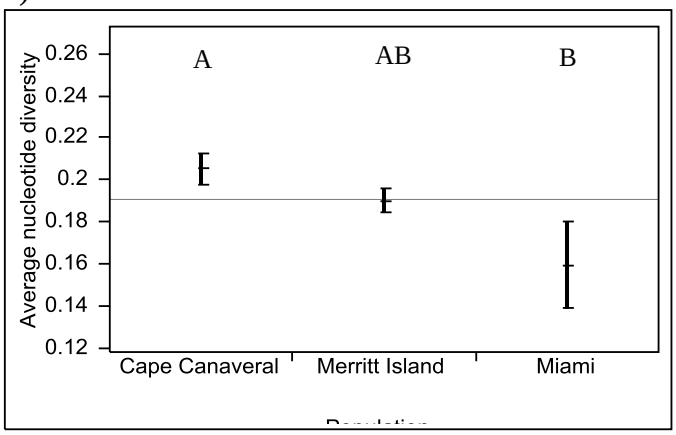
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Cape Canaveral
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Chapter 3:

The Major Histocompatibility Complex, Reproductive Success and Behavior in the Brown Anole, *Anolis sagrei*

Angela Hung, Astrid Kodric-Brown, Robert Miller

Introduction

The problem of mate choice for extravagant ornaments rather than tangible resources, such as food and shelter, has occupied behavioral ecologists for decades. Zahavi (1975) and Hamilton and Zuk (1982) have offered the most compelling arguments for the indirect benefits of genes. Genetic benefits, advertised through ornaments, are heritable and can increase fitness through progeny. Instead of gaining direct benefits from mates—food or protection to grow healthy offspring—mates are chosen to provide only the genes that build and maintain progeny. Females prefer genes that increase the vigor, survival (Zahavi 1975; Kuijper et al. 2012) or parasite resistance (Hamilton and Zuk 1982; Dunn et al. 2012; Prokop et al. 2012) of their young rather than the material benefits of paternal care. To maximize the benefits of parental investment and fitness returns through offspring, the more heavily investing sex, females in most species, select their mates carefully (Trivers 1972; Kuijper et al. 2012).

Recently, tests of mate choice for genes that produce highly immunocompetent offspring have gained traction (Bernatchez & Landry 2003; Drury 2010). The deleterious effects of parasites on development have been overlooked except in cases of major perturbations. Although the effects of parasites and pathogens may not prevent normal development, the energetic cost of fighting chronic, low-level infections is ultimately paid in reproductive success (Svensson et al. 1998). To circumvent these costs, females choose mates to produce young with strong immune systems, in order to clear infections more quickly with less energy expenditure, and thus can allocate more to reproductive effort. While choosiness for genetic traits is difficult to observe due to epigenetic and

pleiotropic effects on phenotypes, the major histocompatibility complex (MHC) presents a unique opportunity to examine selection for the indirect genetic benefits of parasite resistance.

The major histocompatibility complex is a group of genes unique to the genomes of gnathostomes (Klein and Sato 2000). Many of the genes within the MHC encode cell-surface receptors that bind antigens. The receptor-antigen complexes present self-peptides under normal conditions. When a cell becomes infected, some MHC molecules begin to present antigens from the pathogen. Detection of non-self antigen-MHC complexes by T-lymphocytes initiates an immunological response (Klein and Sato 2007). The binding groove of MHC receptors are antigen-specific, so an enormous degree of polymorphism is necessary to present peptides derived from the multitudes of pathogens that organisms are typically exposed to. Indeed, the MHC is the most polymorphic region of the genome (Klein and Figueroa 1986). The maintenance of allelic diversity is driven by natural selection through parasites (Spurgin & Richardson 2010), and by sexual selection through discriminating females (Piertney and Oliver 2006).

Since the seminal observation that the MHC influences mate preferences in laboratory mice (Yamazaki et al. 1976), a proliferation of research across taxa has followed suit (Piertney and Oliver 2006). These studies are framed by two general hypotheses of female choice for indirect genetic benefits: preference for "good" genes or for "compatible" genes (Neff and Pitcher 2005). "Good genes" selection predicts that specific alleles, or allele types in mates are preferred (Cutrera et al. 2012). Good alleles are preferred because they offer a specific fitness advantage to offspring (Mays and Hill

2005) and females are largely congruent in their preference of potential mates that carry such alleles (Neff and Pitcher 2005). Specific MHC variants of immune genes can be "good" when they have been successful in defending against a prominent parasite and females pass on the immunological advantage to their offspring. The genetic benefit to offspring derived from "compatible" genes selection depends on the genotype of the chooser (Neff and Pitcher 2005), which can be easily characterized at the MHC. The advancement of molecular tools has facilitated several specific predictions regarding compatible MHC genes. Diversifying selection drives preference for dissimilarity in genotype, or increased genetic distance. Dissimilar nucleotide sequences translate into amino acid substitutions in MHC molecules that can affect antigen binding. Females can choose mates based on the number of amino acid differences between her alleles and those of a male (Agbali et al. 2010; Setchell et al. 2010; Joula and Dearborn 2012). Alternatively, females can choose males with high individual diversity, where alleles vary widely from each other within a male and without reference to her own genotype (Miller et al. 2009). Alternatively, females can also choose males with different alleles, irrespective of the degree of sequence dissimilarity (reviewed in Penn 2002; Radwan et al. 2008; Setchell et al. 2010).

More recently, hypotheses have been proposed that do not conform to the good genes-compatible genes dichotomy, as preferences for heterozygosity and allele-counting have been identified (Drury 2010). Heterozygosity at MHC loci is predicted to be adaptive when parasites in the environment are numerous and highly variable because offspring can inherit the widest possible range of alleles against multiple pathogens

(Jeffrey and Bangham 2000; Spurgin and Richardson 2010). Broadly, high levels of genome-wide heterozygosity are associated with increased growth, survival, and developmental stability (Brown 1999) and reproductive success (Thoß et al. 2011). Another strategy is allele counting, where mate choice results in offspring with a particular number of MHC alleles (Reusch et al. 2001, Bos et al. 2009). A specific allele number is hypothesized to be favorable in order to carry sufficient MHC alleles for parasite defense, but an overabundance of alleles may increase the risk of autoimmune disorders. MHC molecules in the thymus are responsible for selection of T-cell populations before entering systemic circulation (Jeffrey and Bangham 2000). T-cells then enter the negative selection process in the thymus, which is also mediated by MHC molecules. An excess of MHC alleles may remove too many T-cells from systemic circulation for a robust, adaptive immune response during an infection.

The MHC also has phenotypic effects that permit evaluation of males by females. Receptors and their ligands can influence an individual's odors and odor perception. Individual odor profiles are developed when antigens are released from their MHC receptors as cells are destroyed and then excreted in urine and/or sweat. MHC ligands have been found to activate neurons in mouse olfactory tissue (Boehm and Zufall 2006) and brain regions in humans (Milinski et al. 2013). These peptides can potentially be bound by corresponding MHC receptors expressed in olfactory epithelium, several of which have been found in mice (Boehm and Zufall 2006). The receiver of such a chemical cue can infer information about the types of MHC the sender bears, providing a mechanism for recognition of what other individuals express on their cells. Olfactory

preferences that reflect MHC makeup are well documented in three-spined sticklebacks, *Gasterosteus aculeatus*, mice, humans, sand lizards, *Lacerta agilis* via femoral pore secretions (reviewed in Ziegler et al. 2005), and bank voles, *Myodes glareolus* (Radwan et al. 2008).

Finally, the MHC genotype can affect the expression of morphological traits or behaviors in some species. Specific sets of alleles in white-tailed deer, *Odocoileus virginianus,* were associated with more antler points (Ditchkoff et al. 2001). In ring-necked pheasants, *Phasianus colchinus,* males with a particular allele have longer spurs, (von Schantz et al. 1996), and the intensity of the red in mandrill faces, *Mandrillus sphinx,* is related to several MHC alleles (Setchell et al. 2009). The number of MHC alleles in male common yellowthroats, *Geothlypis trichas,* positively correlates with mask size and survival. In sand lizards, *Lacerta agilis,* the MHC genotype affects parasite loads, color expression and mate acquisition behaviors (Olsson et al. 2005). Any MHC genotype that is highly effective in parasite defense should increase the overall condition of males giving condition-dependent signals or high trait values of ornaments.

To date, the relationship between MHC and reproductive success has been studied in only two species of Lepidosaurs. A survey of mating pairs in a wild population of tuatara, *Sphenodon punctatus,* showed that while male body size was the main predictor of male mating success, there was evidence for disassortative mating based on MHC amino acid distances (Miller et al. 2009). In the lab, female sand lizards preferred mates with dissimilar MHC, but female choice can be confounded by male behavior and other

life history characteristics in the wild (Olsson et al. 2003). Males with a particular MHC genotype are more effective at acquiring mates (Olsson et al. 2005).

This study investigates the role of MHC genes in the mate choice of brown anoles, *Anolis sagrei*. Members of the *Anolis* genus are well known for their colorful dewlap displays, which are performed in conjunction with series of pushups and headbobs. These visual signals function in intrasexual (Orrell and Jenssen 2003; Simon 2011; Henningsen and Irschick 2012) and even interspecific communication (Leal 1999).To date, mate choice studies in several *Anolis* species have focused on visual displays. However, these behavioral and morphological traits and male mating success are not correlated (Tokarz et al. 2005; Lailvaux and Irschick 2006), except for high dewlap extension rates occurring in displays that did not result in copulation (Simon 2011).

This study addresses if MHC is a criterion of mate choice in *A. sagrei*. The role of visual displays in mate choice are re-examined with incorporation of MHC data to see if 1) MHC genotype or diversity affects mating outcomes, 2) there are correlations between observed MHC alleles and male display behavior. Parental investment theory predicts that female anoles should be choosy because they are investing heavily in reproduction and need to maximize genetic benefits from males (Trivers 1972). Females are continually gravid through the breeding season and pay a significant survival cost by carrying a large egg (Cox and Calsbeek 2009). The discovery of post-copulatory offspring sex-ratio bias by females based on male condition in *A. sagrei* is evidence of the ability of females to distinguish between males of differing quality (Cox et al. 2011).

We determine if females also use pre-copulatory mate choice for males with advantageous alleles which may enhance the immunity of their offspring.

We focus on MHC class I (MHC-I) molecules because of their widespread expression throughout the body on most nucleated cells. Notably, class I receptors have been found in mouse olfactory epithelium where they may mediate pheromone detection (Hegde 2003; Chamero et al. 2012). Exon 3 of MHC class I genes were analyzed to determine the amino acid diversity of male and female lizards. Most of the genes' sequence variability resides in exons 2 and 3 which encode the binding groove of the receptor (Kaufman et al. 1994). These regions are highly variable and polymorphic because the nature of the antigens that are presented by MHC-I is determined by the encoded amino acids in the binding site. Carroll et al. (2002) have shown that untrained mice can detect odor differences among class I variants, particularly when the variation occurred at peptide binding positions.

Anolis sagrei from three populations in Florida were used for mate choice trials. The amino acid diversity of male and female lizards was determined to test the good genes and compatible genes hypotheses of mate choice for indirect genetic benefits. We compared the behavior of males carrying common alleles to see if mating success based on display behavior was modulated by specific MHC alleles.

Methods

Study organism

Anolis sagrei is a small (snout-vent length: 40mm-65mm), subtropical lizard that has been introduced to the southeastern United States, where it is now abundant (Campbell 1996). *Anolis sagrei* is native to the tropic to subtropic zones of the Caribbean and are exposed to diverse parasites such as helminthes (Bursey & Brooks, 2010), malarial parasites (in Florida, Perkins et. al. 2007), coccidia (Bui et al. 1992), and even Chytridiomycosis fungus (Kilburn et al. 2011). These pathogens are exerting selective pressures on the immune system. Across taxa, MHC class I genes have been shown to offer protection against malaria (*Acrocephalus arundiaceus*: Westerdahl et al. 2005; *Homo sapiens*, Hill et al. 1991).

As a member of the trunk-ground ecomorph, *A. sagrei* is one of the more aggressively territorial and polygynous species of the *Anolis* clade (Losos 2009), and accordingly is sexually dimorphic. In Florida, the mating season runs from April through August where resource-defense polygyny is observed (Tokarz 1998). Females lay a single egg approximately every 10 days (Orrell and Jenssen 2003). Although *A. sagrei* is oviparous, these eggs are a significant portion of female body mass. Embryos complete a fifth of embryological development in the egg before being laid (Sanger et al. 2008). Young are autonomous and do not require additional parental care.

In 2010 and 2011, anole lizards were collected from three sites in Florida: Merritt Island (28˚22'N, 80˚42W), Cape Canaveral (28˚24'N, 80˚39W), and Miami (28˚36'N, 80˚19W), by picking individuals off branches at night or noosing during the day. These were transported to the University of New Mexico (UNM), Albuquerque, NM. Males were housed individually in one half of a 37.85 liter glass tank that was partitioned by

glass or plexiglass with companion females which were not used in experiments. Females used in experiments were housed in 75.71 liter tanks without dividers. Up to ten females shared a tank. Females were housed in tanks adjacent to, at most, four males. Males that were in visual contact with a female were not used in a trial with that particular female. Terrarium liners (Zilla) were used as substrate in all tanks. PVC pipes were provided as perches for each male while short, wood boards were provided to females. Lizards were fed crickets (*Acheta domesticus*, Fluker's Farm) *ad libitum* every one to two days and misted twice a day. Crickets were dusted with calcium powder (Zoo Med Repti Calcium) once a week. Lizards were housed at 27-30˚ C during the day and 24˚C at night on a 14:10 hour light:dark cycle. Heat was provided by 75 watt incandescent light bulbs and UVA and UVB were provided by fluorescent lights (Zilla Tropical 25 UVB) up to 10 hours per day.

In 2012, lizards were noosed from Merritt Island and Cape Canaveral. Lizards were collected weekly and up to 15 were kept together in a 62 liter plastic bin with coconut substrate (Zoo Med Eco Earth®) and wooden perches. These were fed crickets *ad libitum* and replenished daily. Lizards were kept at ambient temperatures (34-35˚C during the day, 27˚C at night) and misted every day. They were returned to where they were collected once their mating trial was complete. All lizards collected each year were toe-clipped for identification; up to one centimeter was clipped from tails for DNA extraction.

Since mating trials were conducted concurrently with molecular characterization, trials could not be set up based on MHC genotypes. In the absence of data on allelic

diversity, the three locations were used as a proxy of genetic distance. Anoles are extremely philopatric and do not disperse far from their natal territories (Calsbeek 2009), so populations are likely to be discrete throughout their range. Only females from the Merritt Island population were used in mating experiments.

Mate choice experiment

For mate choice trials, two males were placed in a one meter tall, circular plastic tank, 1.8m in diameter with their terrarium liners and perches placed across from each other. Two digital camcorders (Samsung SMX-C10) were placed on opposite sides of the tank; each recorded lizard behavior in one half of the tank. One male was from Merritt Island and the other was from either Cape Canaveral or Miami. The males were filmed for one hour, then a female was introduced in the center of the tank and recording resumed for an additional two hours. The number of headbobs, pushups, dewlap extensions and the amount of time each male spent displaying, were quantified from video recordings. The number of display bouts, successional combinations of headbobs, dewlap extensions and pushups, was also recorded. One display was considered complete when at least 10 seconds elapsed before the next movement, or when the lizard moved to a different part of the tank. Trials were conducted at the University of New Mexico from September-October in 2010 (7 trials), April-November 2011 (57 trials), and May 2012 (14 trials). Data recording was stopped when the female copulated with one of the males. Lizards in a trial that resulted in copulation were excluded from future trials. Trials in Florida followed the same procedure, but the mating arena was a 1.2m x 1.2m square and

only PVC pipe perches were provided for males during the trial. Twenty-nine trials were completed in June and July 2012. All interactions during the full two hours of footage were recorded following the addition of the female because lizards showed a much higher level of activity than those used in the UNM trials. The male that copulated first and the male that copulated the most were recorded separately as binary variables. Whereas many mate choice studies prevent intrasexual and intersexual interactions that may influence female behaviors, our design allowed all individuals to interact with each other. This design provided a more realistic setting to study mating behavior.

MHC Genotyping

DNA from all lizards used in mating trials was extracted from tail clippings using DNeasy Blood and Tissue kits (QIAGEN) following the spin-column protocol. The peptide binding region encoded by exon 3, MHC class I genes were amplified by polymerase chain reaction (PCR) using forward primer: 5' AGGAGAGACGGGAGCAAACCAGGGTA 3' and reverse primer: 5' AGCCAACTCCACGCASWYBTCCTCCA 3'. The 25 µl reaction mix included 20-30ng of genomic DNA, PCR buffer, dNTP mix, and *Taq* polymerase according to Advantage-HF2 PCR kit (Clontech, Mountain View, CA, USA) protocol and 0.1µM of each primer. Amplification occurred under the following conditions: one step at 94˚C for 45s, 33 cycles of 94˚C for 30s, 66˚C for 30s, and a final step at 68˚C for three minutes.

PCR products were cloned using TOPO Top10 Cloning Kits. Twelve to sixteen *Escherichia coli* colonies from ampicillin nutrient plates were chosen for colony PCR

amplification using M13 universal primers (Operon). The 25µl reaction mixture contained PCR buffer and 1.25U*Taq* polymerase (New England Biolabs), 0.1µM of each primer, dNTPs (Promega) and molecular grade water. The thermocycling profile consisted of an initial cell lysing at 94˚C for 5min, 26 cycles of 94˚C for 30s, 56˚C for 45s, and a final step at 68˚C for 5min. Clean-up was completed by combining 0.4µl of ExoSAP-IT (USB, Cleveland, OH USA) with 1.0 µl of PCR product and incubated according to manufacturer's protocol. The product was prepared for Sanger sequencing with Big-Dye Terminator v3.1 Cycle Sequencing kits (Applied Biosciences) and run in UNM Biology department's Molecular Biology Facility. The resulting sequences were confirmed to be MHC-I using NCBI's BLAST searches.

Sequences were aligned with ClustalW and edited in BioEdit v7.1.3. Sequences that appeared in a single individual with a non-synonymous nucleotide substitution at a position that was conserved in all other sequences were attributed to PCR error and were edited at that position to be consistent with other sequences. There were 18 such sequences. Three sequences with premature stop codons were excluded from analyses.

This project has been reviewed and approved by the UNM Main Campus Animal Care and Use Committee (#100359).

Statistical Analyses

Only trials with copulations and male displays in the presence of females were analyzed. Univariate and principal components analyses (PCA) were completed in JMP 9.0 and logistic regressions were run in SAS 9.2 using the glimmix procedure with "trial"

as a random effect. Male displays were highly correlated, so the first two principal components derived from the number of headbobs, pushups, dewlap extensions, number of displays, and duration of display were used. These data were not normally distributed and were log base 10 transformed for normality. PC1 accounted for 79.58% of the total variance and describes males that were active displayers; all display variables loaded positively with each other at between 0.41 and 0.49 level. PC2 accounted for 15.28% of the variance and separates males that used primarily headbob and pushup displays without dewlaps from males that used only dewlap displays (Table 1).

Genetic diversity was calculated in MEGA 5.0. Six variables were used to describe genetic choice based on the MHC: 1) the number of MHC peptide alleles found in each lizard. 2) The difference in number of alleles between lizards, which was calculated by subtracting the number of a male's alleles from the number found in the female he was tested with. If the difference was less than zero, the male carried more alleles; if the difference was greater than zero, the female had more alleles. 3) The number of MHC alleles that males shared with the female in each trial. One of the compatible genes hypotheses predicts a negative association with copulation probability if females sought males with different alleles. 4) Average distance to female (p-distance) is the average of all pairwise distances between the peptide variants found in each male-female pair in each trial. Pairwise distances were calculated as the number of amino acid differences as a proportion of all positions (48 amino acids). This tests the compatible genes hypotheses that females are choosing males with the greatest genetic distance from herself. 5) Individual diversity was determined by taking the average of pairwise

distances between MHC alleles within an individual. This tests whether females are choosing males with the most diversity so offspring will inherit MHC that recognizes a wide array of pathogen antigens. 6) Average diversity between pairs is the average of the individual genetic diversities of each possible male-female pair of each trial. This variable looks for an intermediate value of genetic diversity that is preferred by accounting for the amino acid diversity of each pair of lizards taken together, approximating the potential offspring genetic diversity. Unlike p-distance, the measure of females' individual genetic diversity is used to calculate this variable. The number of sequences, allele difference, and the number shared are gross descriptions of individual haplotypes while distance to female, individual diversity and average diversity between pairs quantify how much the amino acid sequences differ from each other. Except for the average diversity between pairs, these genetic variables have been used previously in mate choice studies (Setchell et al. 2010; Cutrera et al. 2012; Juola and Dearborn 2012).

Distance to female, individual diversity and average diversity between pairs were multiplied by 10 so that all data were scaled similarly for regression analyses. Male source was included as a categorical predictor in the models. Regression models were calculated for trials completed at UNM to predict whether or not a male will be the first to copulate. Two sets of logistic regression models were run for Florida trials: predicting whether or not a male will copulate first, and which male will copulate the most times.

MHC and Behavior

The relationships between genotypes and display variables were analyzed to test the possibility that the MHC affects display behaviors which in turn influence female choice. PC1 and PC2 values were tested against peptide alleles that appeared in four or more males to see if certain alleles had an effect on display behaviors. The PC1 and PC2 values were compared between lizards that carried each individual allele and those that did not. Student's t-tests were completed in JMP 9.0. Chi-square tests were used to identify alleles associated with male mating success.

Results

Mating trials-University of New Mexico

In mating trials conducted at UNM, copulations were observed in 16 out of 78 trials. Only trials where copulations were observed were used for analysis and all years were pooled. Due to the low sample size, Cape Canaveral (N = 7) and Miami (N = 9) males were pooled and classified as "non-local" males. Female preference trended towards non-local males; 13 copulations of the 16 were with non-local males (binomial test: one-tailed $p = 0.038$, two-tailed $p = 0.077$), six with Miami males and seven with Cape Canaveral males. Males from each population did not differ in display behaviors (Wilcoxon tests, Cape Canaveral: N = 7, Merritt Island: N = 16, Miami: N = 9, $p > 0.25$).

Mating trials-Florida

In Florida, copulations occurred in 23 out of 29 trials. Two of these trials included Miami males collected in 2011 and the remaining 27 used Cape Canaveral

males collected in 2012. The number of copulations by each male as well as which male mated first were recorded. The number of copulations by any one male varied from 0-2, with one male copulating four times and two males copulationg three times. Due to the low variation in the number of copulations obtained by males, "copulate most" was used as a binary variable in the regression analyses. There were no significant differences between non-local and local males in copulating first (binomial test: $p = 1$) or most (binomial test: $p = 1$). Cape Canaveral males were the first to copulate with the female in 11 out of 21 trials while Miami males did not copulate first in these trials (2 trials).

In six trials, Cape Canaveral males copulated more than the Merritt Island males and both Miami males copulated more than Merritt Island males. There was no mating preference by females (binomial test: $p = 0.21$). As with the males used in UNM trials, populations did not differ in the number of headbobs, pushups, dewlap extensions, but Merritt Island and Cape Canaveral males differed in display strategies identified by principal components analysis (Table 2). Cape Canaveral males had lower values of PC2, performing primarily dewlap-only displays.

MHC Sequence Analysis

Exon 3 of MHC class I genes were sequenced in study lizards (Merritt Island: $N = 77$; Cape Canaveral: $N = 28$; Miami: $N = 11$). We identified 136 unique nucleotide sequences that were 143 base pairs in length. These encoded 108 unique MHC peptides. Individuals carried one to eleven peptide variants, averaging of 4.5 overall. At most two alleles in an individual would be expected if a single locus was amplified, therefore at

least six loci were amplified by our primer pair. Alleles differed by 1- 25 amino acids residues. We were unable to assign alleles, or variants, to specific loci and so were unable to calculate the heterozygosity of lizards.

Logistic regression

Akaike information criterion (AIC) was used to identify the best fitting regression models according to the lowest AIC value (Table 3). In UNM trials (N = 16), the best model that predicted which male copulated first included display behaviors, the following genetic variables, and male source population. The significant predictors were: 1) the number of alleles shared, 2) the amino acid distance between males and females, 3) the genetic diversity of the male, 4) the averaged genetic diversity of each male-female pair, and 5) male source (Table 3). Consistent with univariate analyses, males from Merritt Island were less likely to copulate. In addition, the genetic diversity of a male was a positive predictor of copulation while allele sharing was a negative predictor. However, p-distance to the female had a weak, negative weight on copulation probability and the average diversity between pairs had a strong negative effect on copulation (Table 3). There was some multicollinearity among and between the genetic parameters. In the UNM regression analysis, the variance inflation factor (VIF) for number of alleles and allele difference between pairs were marginally high (number of alleles: VIF = 5.6; allele difference: VIF = 5.9; Table 3). However, VIF values over 10 are cause for concern that coefficient errors are overinflated due to collinearity of variables (Kutner et al. 2004).

In the Florida trials (N = 23), the best models included PC1 and male source to predict both the first copulation and the highest number of copulations; PC1 was the only significant predictor in both (Table 4) and was positively correlated to mating success in both cases.

Genes and behavior

The effect of MHC on display behavior in males was tested by comparing PC1 and PC2 values of lizards with and without certain alleles. Alleles that were identified in four or more individuals were analyzed (Fig. 1). PC1 was higher in lizards that carried peptides 8, 37, 39, and 76, and lower in lizards with peptide 36. PC2 was lower for lizards carrying allele 1, associating with dewlap-dominant displays (Student's t-tests, Table 6). The same alleles were also examined in relation to mating success in males. While there were no alleles that were associated with the most copulations obtained, all six males carrying peptide allele 44 were the first to mate in a trial (Chi-sq: $X^2_1 = 8.83$, $p = 0.003$). However, these relationships were not significant after Bonferroni alpha adjustment.

Discussion

MHC and Mating success in A. sagrei

The results of this study indicate that MHC genotype has some role in sexual selection in *A. sagrei* in a controlled lab environment. Support for both compatible genes selection for highly diverse males and intermediate MHC diversity were supported while

the possibility of good genes effects should be further explored. Individual male allelic diversity and non-local males predicted first copulation strongly and positively. Females chose sires that could pass on a wider variety of alleles to their offspring to protect them against a broad range of pathogens. The average diversity between pairs was a strong negative predictor. This variable incorporates the amino acid diversity of both the female and males in each trial to represent self-referent female choice to produce a specific genotype in offspring. Females with high MHC diversity avoided mating with males that were highly diverse in favor of males with lower diversity. This variable was used because the average number of alleles found across the three lizards in a trial varied widely between trials, making identification of an "optimal number" of alleles impossible. Allele sharing also negatively predicted first copulation, as females preferred males with different alleles from themselves.

The number of loci amplified could not be identified, so testing the preference for high levels of heterozygosity was not possible. The occurrence of 11 unique MHC peptide sequences in two individuals indicates that at least six loci were amplified, and as many as 11 loci are possible if the lizards were homozygous at all sites. If the number of alleles carried by an individual was considered as a proxy for heterozygosity, then these data do not support female preference for high levels of heterozygosity. Full characterization of *A. sagrei* MHC-I genes to include more coding regions is needed to identify the number of loci in the genome and to measure heterozygosity for a stronger conclusion.

The preference for MHC genotypes was not replicated in *in situ* trials. In the Florida trials, display behaviors positively predicted both the first copulation and the highest number of copulations in a trial, controlling for male source population. Males that performed many display bouts with all components (headbobs, dewlap extensions and pushups) had a higher probability of mating. These results seemingly contradict prior work on male anole displays and female choice. However, our study stands apart because we analyzed display behavior as bouts in addition to headbobs, pushups and dewlap extensions separately. This study also differs from most by permitting males to interact with each other. Males displayed at and circled each other but were not observed to interfere with matings. Without tethering males, females are free to assess male displays in different contexts, i.e. courtship and intrasexual competition, and to evaluate multiple males simultaneously to make direct comparisons. While many male behaviors may be intended for consexuals, females may evaluate the same signals across contexts to increase their information about males (Kuijper et al. 2012). *Anolis sagrei* territories are adjacent each other so several males are easily in view of females (pers. obs.).

There were several factors that influenced the outcomes of mating trials in the two locations. At UNM, copulation rates (16 copulations out of 78 trials) were very low which may have been a result of suboptimal conditions in the lab and season. Most copulations were observed between April through August. In particular, sufficiently high temperatures and humidity were difficult to maintain to artificially extend the breeding season. In addition, females were randomly selected for mating trials and may not have been sexually receptive at the time of the trial. The use of hormone injections was

avoided in case of behavioral side effects. Copulation rates were much higher in Florida where lizards were maintained in ambient conditions during the breeding season. Males were in captivity for less time and were probably in better condition and more vigorous than those housed long term at UNM. It appeared that matings were less likely to be initiated by females, in contrast to UNM trials (pers. obs.). Females were observed to run from males in the mating arenas, and being unable to escape, the setup may have affected the males' perception of receptivity in females. It is unclear how often forced copulations occur in the wild, since females are not confined and spend most of their time in a territory where intruders are excluded by the territorial male.

MHC and behavior

MHC genotype may be an underlying mechanism for male mating success. We compared PC1 and PC2 values in lizards carrying MHC variants that appeared in four or more males across all three populations. There were significant differences in carriers of six alleles. PC1, which was a positive predictor of male mating success in Florida, was higher in male carriers of four MHC peptide variants—carriers performed more of every display behavior. The four variants may be protective against disease and increase overall body condition, vigor and display rates. One variant decreased PC1 another was associated with low PC2 scores, or dewlap-only displays. Allele 36, which occurred with lower PC1 scores, may be a variant that pathogens have escaped from; males with this allele may have had lower immunocompetance and did not perform as well. These interpretations are highly speculative; information on parasite loads in these lizards that

allow direct tests of the relationships between MHC genotype, immunocompetance, condition, and behavior are needed. Alternatively, MHC class I genes may have a more direct effect on behavior that impact mating outcomes. Class I gene expression has been found in the central nervous system in trout (Fischer et al. 2005) and mammals (Elmer and McAllister 2012). Mouse strains differing in MHC type exhibit different fear and exploratory responses (Brown et al. 1999) and genetic polymorphism coincides with behavioral variation in trout (*Oncorhynchus mykiss*; Azuma et al. 2005).

Allele 44 may be a "good gene." All males with this variant copulated during mating trials. However, these data should be interpreted with caution as the allele was found in only six males, nor can we speculate on the underlying mechanism. For associations with MHC and both display and mating behaviors, gene expression needs to be confirmed.

Evaluating the MHC

This study does not address the mechanism(s) for evaluating MHC genotype. There are at least three possibilities. Although small, anoles have what appears to be a functional vomeronasal organ and olfactory bulb (Armstrong et al. 1953) and are able to discriminate flavors (Stanger-Hall et al. 2001). We included the substrate from each male's holding tank in UNM mating trials so females could access chemical cues. However, this was not possible in the Florida trials.

We show some support that MHC can influence display behavior, which may be an indicator of body condition. Anole pushup behavior has previously been identified as

an honest signal of body condition that correlates with running endurance (Leal 1999). If MHC genotype does influence display performance through increased immunocompetance/lower parasite loads, territory acquisition and defense and thus, reproductive success are affected. Genotype can also be signaled by light reflectance. The bright orange color of *A. sagrei* dewlaps is derived from pterin and carotenoid pigments (Steffen and McGraw 2007), with carotenoids having many immunological functions (Lozano 2004). Males with low parasite loads, as a consequence of a good MHC makeup, would rely less heavily on carotenoids for their immune system, allowing them to allocate the pigment to ornaments such as the dewlap (Lozano 1994). Carotenoids stored in the dewlap should be quickly accessible to individuals under stress and may reflect current body condition (Lozano 1994). *Anolis sagrei* dewlaps do not reflect strongly in the UV spectrum (Macedonia et al. 2000).

Anolis sagrei is capable of sperm storage (Calsbeek et al. 2007) and cryptic sex-ratio bias based on sire body size (Cox and Calsbeek 2010) and body condition (Cox et al. 2011). While pre-copulatory choice based on MHC was only partially supported by our data, there are opportunities for post-copulatory female choice to be mediated by the MHC. Multiple paternity has been reported to be as high as 80% in a population of *A. sagrei* (Calsbeek et al. 2007), thus paternity analyses and tracking offspring survival would further clarify if there is an optimal level of MHC diversity that offers the most benefit to offspring. The fitness of particular MHC alleles could also be measured by tracking individual survivorship and reproductive success.

Future Directions

It is likely that the preference for good genes and compatible genes are not mutually exclusive. Theoretically, the potential to pass on genes that demonstrably confer disease resistance *in addition to* diverse genes suited to various contingencies ought to have higher fitness outcomes than producing young with only one or the other genotype. Field data are needed to reconcile the preference differences between the UNM and Florida trials to determine the relative roles of MHC, male display behavior, and MHC modulation of behavior. Another factor in *A. sagrei* reproduction to be resolved in the field is the potential for mutual mate choice. Tokarz (2008) has shown that males preferentially court and mate with unfamiliar, nonresident females. Females exhibit polymorphism in dorsal patterning that covaries with immunocompetance (Calsbeek et al. 2008). It should be explored whether the pattern-associated variation in immunocompetance is related to MHC genotypes and if males are preferring good quality females.

Anole lizards and the MHC can potentially yield numerous insights into the evolution of behavior and the vertebrate immune system. *Anolis sagrei* offers many more research opportunities in areas of invasion biology, tropical ecology, disease ecology etc. In conjunction with other *Anolis* species and lizard genera, comparative analyses of the MHC are needed to understand the evolution of the vertebrate immune system, the potential for parasites to drive speciation and other ecological principles. With only three species of lizards, including the current study, to represent the clade, much work remains

in lizard mating behavior and their MHC. Occupying the phylogenetic space that separates the well-studied birds and mammals, these data will facilitate more representative comparative studies on vertebrate evolution. In a final note, the holistic approach of behavioral immunogenetics is critical for insights on disease control, outbreaks in wild populations, disease spread as climate changes and the dynamics of zoonotic diseases. For an enhanced perspective on these issues, we need to characterize the ecology of pathogens and host-parasite interactions as they coevolve with host immune systems and the reciprocal impacts of behavioral responses.

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Figure Captions

Table 1. Principal components analysis of male behaviors. Data were log-transformed for normality.

Table 2. Display behavior of males by source population. Values are reported as $\overline{X} \pm$ SE (median), with comparisons of each behavior between populations in Florida trials (Wilcoxon tests, *$p<0.01$).

Table 3. Logistic regression models identifying characteristic of males copulating first (FL copulation and NM copulation) or males that copulate the most times (FL copulate most) during each trial. ΔAIC from the best model (ΔAIC=0, in bold) are shown for each model. Asterisks indicate models with significant predictors. Genetics included the following MHC characteristics: number of sequences, difference in number of peptide alleles between males and female in a trial, number of peptide alleles shared, p-distance to female of trial, genetic diversity of individual, and averaged genetic diversity of each male-female pair. N/A indicates where sample sizes were too low to complete the analysis.

Table 4. Best fitting logistic regression models to predict if a male will copulate first in a trial (NM and FL copulate first) and if a male will copulate the highest number of times (FL copulate most). Trial was entered as a random factor.

Figure 1. Number of males carrying each peptide variant across sampled *A. sagrei* populations (Cape Canaveral, Merritt Island, and Miami, FL). Colored bars indicate alleles with significant effects on behavior in carrier males compared to non-carrier males (Student's t-test). Increased PC1 corresponds to high levels of all male displays; decrease in PC2 corresponds to an increase in dewlap displays and a decrease in headbob and pushup behaviors. Only variants found in four or more males are shown. Variant numbers were arbitrarily assigned to each unique peptide sequence.

Table S1. Specific allele effects of PC1 and PC2 of display behaviors in carrier males. Increased PC1 corresponds to high levels of all male displays; decrease in PC2 corresponds to an increase in dewlap displays and a decrease in headbob and pushup behaviors (Student's t-test).

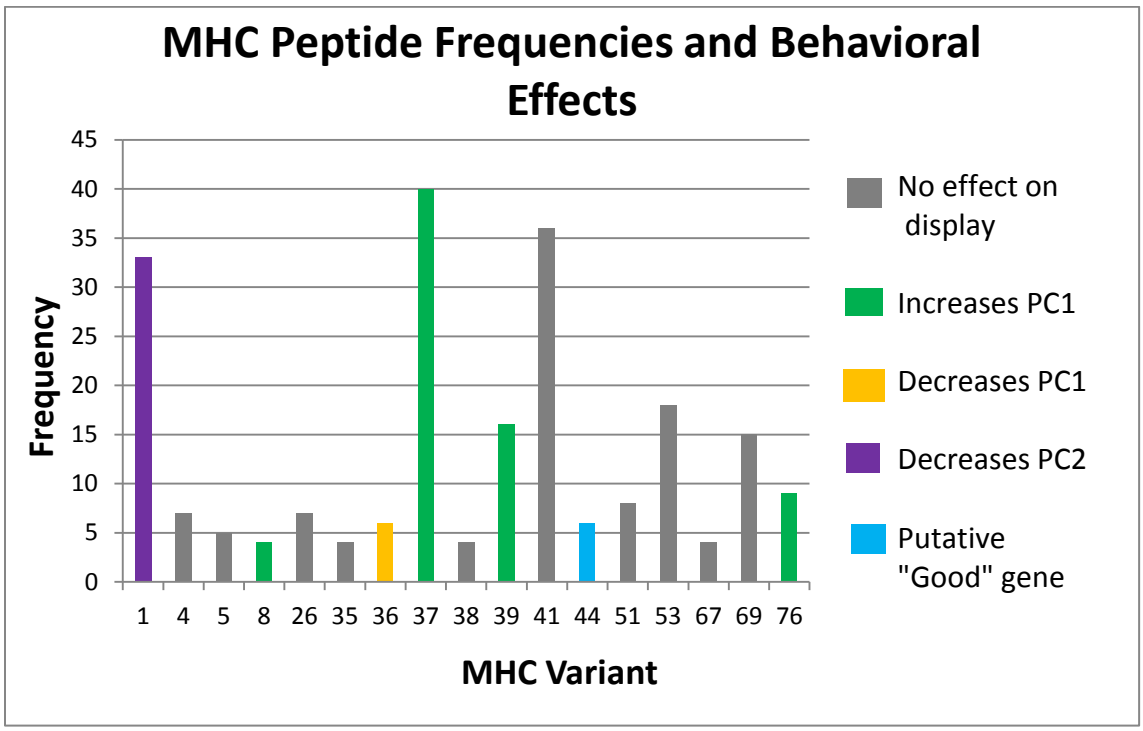
Figures

Variable	PC1	PC2
Number of displays	0.48	-0.27
Headbobs	0.41	0.54
Pushups	0.41	0.57
Dewlaps	0.43	-0.52
Display duration	0.49	-0.21
Eigenvalue	3.98	0.76
Percent of variance	79.58	15.28

Population	Displays	Headbobs	Pushups	Dewlaps	Display duration (sec)	PC1	PC2
Cape Canaveral N=20	50.43±31.9 (53)	37.43±75.1 (2)	70.04±172.0 (0)	117.67±88.0 (96)	405.38±412.2 (298)	1.13±1.1 (1.08)	-0.42±0.9 (-0.50)
Merritt Island N=22	31.43±32.0 (23)	47.74±82.5 (12)	56.61±121.8 (3)	73.61±72.3 (57)	305.61±356.7 (183)	0.97±1.4 (1.09)	0.17±0.6 (0.07)
Miami N=2	11.5±3.5 (11.5)	3±0 (3)	0±0 (0)	11±12.7 (11)	25.5±10.6 (25.5)	-0.18±0.3 (-0.18)	-0.33±0.3 (-0.33)
Between and among populations (χ^2)	5.49	3.08	4.81	5.65	4.26	2.32	9.44*

Predictors	Model ΔAIC: NM copulate first	Model ΔAIC: FL copulate first	Model ΔAIC: FL copulate most
Male source	19.2	0.5	-2.9
Display	14.6*	-2.1	1.1
Male source+display	2.3*	**0***	**0***
Male source x display	9.2	4.1	3.7
Genetics	34.8	9.5	5.0
Genetics+male source	29.8	11.2	-0.2
Genetics x male source	N/A	17.6	N/A
Display + genetics	22.1*	8.3	9.1
Display + genetics+male source	**0***	11 .0	2.5

Analysis	parameters	estimate	VIF	p-value
UNM model: copulate first	PC1		1.39	NS
	PC2		1.38	NS
	Number of alleles		5.60	NS
	Allele difference between pairs		5.91	NS
	Number shared	-344	1.46	<0.0001
	p-distance to female	-39	1.80	<0.0001
	Diversity of individual	1788	3.57	<0.0001
	Average diversity between pairs	-1914	3.59	<0.0001
	Male source	2087 (Cape Canaveral)	1.76	<0.0001
		-4389 (Merritt Island)	1.40	
Florida model copulate first	PC1	0.52	1.12	<0.0001
	PC2		1.36	NS
	Male source		1.26	NS
Florida copulate most	PC1	0.44	1.12	<0.0001
	PC2		1.36	NS
	Male source		1.26	NS


MHC Peptide Frequencies and Behavioral Effects
Frequency
45
40
35
30
25
20
15
10
5
0
1 4 5 8 26 35 36 37 38 39 41 44 51 53 67 69 76
MHC Variant
No effect on display
Increases PC1
Decreases PC1
Decreases PC2
Putative "Good" gene

Allele	Behavior	Number of males carrying (out of 76)	t-ratio$_{df}$	p-value
36	Decreased PC1	6	$-3.02_{6.27}$	0.022
8	Increased PC1	4	$2.82_{9.16}$	0.020
37	Increased PC1	40	$1.99_{64.57}$	0.051
39	Increased PC1	16	$2.08_{56.08}$	0.042
76	Increased PC1	9	$2.26_{11.40}$	0.044
1	Decreased PC2	33	$-2.13_{63.25}$	0.037