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Mate Choice in a Sexually Dimorphic Marine Bird, the Great Frigatebird (Fregata minor)

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MATE CHOICE IN A SEXUALLY DIMORPHIC MARINE BIRD, THE GREAT FRIGATEBIRD (*FREGATA MINOR*)

By

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Darwin's theory of sexual selection explains the existence of sexual dimorphism, or within-species sex differences in shape, color, size, and behavior. In some cases, sexually dimorphic traits, especially extravagant male ornaments, seem maladaptive and thus in opposition to natural selection. The crux of Darwin's theory was that sexual selection arises from individual differences in reproductive success that result from competition for mates. In this dissertation, I investigated several aspects of sexual selection and the evolution of female mating preferences and male ornaments in the great frigatebird (*Fregata minor*). Frigatebirds as a group (family Fregatidae) are the most ornamented of any seabirds, and are among the most ornamented of any animal group. Their most prominent ornament is a gular (throat) pouch which becomes red in males during the breeding season, and which is inflated and displayed to females during courtship. Male courtship display also includes a warble vocalization and extension and trembling of the wings. I investigated the following issues concerning sexual selection and ornamentation in great frigatebirds: 1) the source of ornamental coloration in male great frigatebird gular pouches. I determined that this was a carotenoid-based color display; 2) the relationship of male mating success to gular pouch size and coloration. I determined that

mating success was not related to the size or color of this ornament; 3) the relationship between male vocal display traits and female preferences. Again, I found no relationship between vocal display traits and female preferences, and finally, 4) the role of a major histocompatibility complex (MHC) locus in female mate choice. The MHC is a highly polymorphic multi-gene family associated with immune defense and has been proposed to play a role in mate choice. I found a significant disassortative mating pattern amongst mated pairs compared to random pairings based on MHC genotypes. In summary, I found no evidence for female mating preferences based on visual or auditory display traits associated with male ornamentation. However, I did find evidence for female mating preferences based on genetic dissimilarity at an MHC locus.

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Chapter 1

Introduction

The theory of natural selection (Darwin 1859) provides a mechanistic explanation for the existence of species diversity on earth and for most of the features that living species currently exhibit. Natural selection, however, falls short of providing an explanation for the existence of within-species sex differences in shape, color, size, and behavior. In some cases, sexually dimorphic traits, especially extravagant male ornaments, seem maladaptive and thus in opposition to natural selection. The theory of sexual selection (Darwin 1859, 1871) was put forth to explain the existence of sexual dimorphism. The crux of Darwin's theory was that sexual selection arises from individual differences in reproductive success that result from competition for mates.

Competition as it related to sexual selection was defined broadly by Darwin to mean any mechanism by which males contend, directly or indirectly, for opportunities to mate with females (Andersson 1994). We now recognize two forms of sexual selection. Intrasexual selection refers to direct competition between individuals (usually males) for access to mates (usually females). Intersexual selection refers to indirect competition for mates through mate choice (usually by females), even in the absence of antagonistic encounters between competing individuals (Andersson 1994).

In order for female mate choice to evolve, the fitness benefits of being choosy must outweigh the costs associated with mate choice behavior. Fitness

benefits to females can be categorized as either direct benefits or indirect benefits (Kirkpatrick and Ryan 1991). Direct benefits refer to benefits females acquire from mating that increase their own survival or fecundity. Such benefits include access to high quality territories, nuptial gifts of food, or superior male parental care. Indirect benefits refer to genetic benefits that females acquire from a mate that ultimately result in increased fitness of their offspring. There are at least two classes of hypotheses related to indirect (genetic) benefits of female choice: Fisher's runaway model (Fisher 1930), and good genes models (Trivers 1972). Fisher's runaway model stipulates that a female preference becomes correlated with a particular male trait. The male trait is arbitrary and does not necessarily convey information about genetic quality of the male. Good genes models are based on the premise that male traits are indicators of genetic quality. Females that select males based on a trait that conveys good genes will be passing superior genes to their offspring, thereby improving their survival (Andersson 1994).

Darwin (1871) was also the first to distinguish between primary and secondary sexual traits. Primary sexual traits are those directly associated with the act of reproduction, such as the copulatory organs. Secondary sexual traits are physical attributes other than the sexual organs that distinguish males from females at sexual maturity, but which have no direct role in the mechanics of insemination (Andersson 1994). These secondary sexual characters may have no advantage from a natural selection perspective, and indeed may hinder survival in some cases. They are solely the product of sexual selection that in

some cases is strong enough to have overridden any countering effect of natural selection. A secondary sexual trait that is primarily used in display to the other sex rather than in intrasexual competition is often termed an ornament. It has been shown in some cases that females prefer to mate with males that exhibit the most elaborate ornamentation (Andersson 1994).

Elaborate male characters are often observed in species with polygynous mating systems. In such systems, where some males mate with multiple females while others do not mate at all, competition for mates is critical to male fitness. This can lead to strong selection for highly developed secondary sexual characters (Andersson 1994). Sexual selection may also drive the evolution of elaborate characters in monogamous species, particularly in species in which the sex ratio is male biased, so that some males must fail to find a female with which to mate (Darwin 1871, Fisher 1958).

In good genes models, females choose on male ornaments that act as indicators of male quality. The existence of such indicators poses a puzzle: what prevents all males from displaying high quality signals regardless of their actual quality? Individuals of inferior quality should “cheat” the system by way of false advertising in order to maximize their fitness. To overcome this problem, it has been postulated that sexual ornaments used as quality indicators must be costly to produce or maintain, thus preventing males of lower quality from developing and displaying high quality traits (Zahavi 1975, Grafen 1990). Zahavi (1975) termed such costly ornaments “handicaps.” The most healthy, fit males are able

to produce the most elaborate ornaments in spite of their cost, and are therefore more attractive to females.

Another problem for good genes models is why additive genetic variance for male quality is maintained, when both natural and sexual selection are continually acting to lower that variance. Hamilton and Zuk (1982) proposed one solution: that coevolutionary cycles between hosts and parasites maintain genetic variance in male fitness. Hamilton and Zuk (1982) went on to propose that bright colors associated with male secondary sexual characters are indicators of a male's ability to resist parasitism. Hosts that possess resistance to particular parasites are healthier and are therefore better able to produce high-quality ornaments. Females benefit indirectly from a preference for males with high-quality ornaments because their offspring will inherit the resistance to parasites that such ornaments indicate.

Another mate choice strategy that escapes the problem of low heritability of fitness related characters is mate choice based on genetic compatibility. One mechanism that has been suggested for choosing genetically compatible mates is mate choice based on the major histocompatibility (MHC) genotypes. The MHC is a highly polymorphic gene assemblage that plays a critical role in the development and activation of both the T-cell mediated and humoral arms of immune defense (Klein 1986, Penn and Potts 1999). MHC genotypes may be externally identified because it affects body odor (Leinders-Zufall et al. 2004, Boehm and Zufall 2006).

In my dissertation, I investigated several aspects of sexual selection and the evolution of female mating preferences and male ornaments in great frigatebirds. Frigatebirds as a group (family Fregatidae) are the most ornamented of any seabirds, and are among the most ornamented of any animal group. Great frigatebirds are typical frigatebirds in this regard. Their most prominent ornament is a gular (throat) pouch which becomes red in males during the breeding season, and which is inflated and displayed to females during courtship (Nelson 1975). Male courtship display also includes a warble vocalization and extension and trembling of the wings (Nelson 1975). I investigated the following issues concerning sexual selection and ornamentation in great frigatebirds: 1) the source of ornamental coloration in male great frigatebird gular pouches, and in particular whether or not this was a carotenoid-based color display; 2) the relationship of male mating success to gular pouch size and coloration and to other morphological and quality indicator traits such as body size and body condition 3) the relationship between male vocal display traits and female preferences, and finally, 4) the role of a major histocompatibility complex (MHC) locus in female mate choice.

This study was conducted on Tern Island, in the northwest Hawaiian Islands (23°45' N, 166°17' W). Tern Island is one of 10 small islands in the French Frigate Shoals atoll. The island is administered by the U.S. Fish and Wildlife Service as part of the northwest Hawaiian Islands national monument. Great frigatebirds are large seabirds, with wingspans averaging just less than 2 meters. They range widely throughout the Indo-Pacific region (Harrison 1985).

Great frigatebirds spend most of the non-breeding season foraging pelagically over large areas, and congregate on oceanic islands to breed (Harrison 1985, Dearborn et al. 2003).

Roughly 4,000 adult frigatebirds come to Tern Island during the breeding season (Dearborn and Ryan 2002). Great frigatebirds in this study population are size dimorphic with adult females being 24.4% larger than adult males in mass, 14.6% larger in culmen length, and 4.4% larger in wing chord (Juola and Dearborn, 2007). Males perform their complex courtship display typically in dense aggregations (Nelson 1975, Dearborn and Ryan 2002) beginning each year in January (Dearborn et al. 2001). Males display while perched on shrubs, as females fly above. A female will land beside a male, and appear to inspect him more closely. In most cases the female eventually leaves, but in some cases she remains perched in close proximity to the male for several days and then begins building a nest. The time from initial pair formation to nest building and egg laying is typically 1-2 weeks. Egg laying typically begins in early February and lasts through May. Clutch size is invariably one, and incubation is approximately 55-57 days. The immediate operational sex ratio, defined as the ratio of mate-attracting males to mate-searching females, is male-biased in this population, with approximately 5.5 males per female (Dearborn et al. 2001). Frigatebirds in this study population form socially and genetically monogamous pair bonds within a breeding season, and pair members share in all aspects of parental effort (Dearborn 2001). The duration of parental care by nesting frigatebirds is approximately 1 year, among the longest for all bird species. Great

frigatebirds are a long-lived species: there are individuals on Tern Island that are currently at least 44 years old (Juola et al. 2006).

Chapter 2

Carotenoids and throat pouch coloration in the great frigatebird (*Fregata minor*)

Summary

Carotenoid pigments are a common source of red, orange, and yellow coloration in vertebrates. Animals cannot manufacture carotenoids and therefore must obtain them in their diet to produce carotenoid-based coloration. Male great frigatebirds (*Fregata minor*) display a bright red inflated gular pouch as part of their elaborate courtship display. The basis of this coloration until now has not been investigated. Using high-performance liquid chromatography (HPLC), we investigated the types and concentrations of carotenoids that great frigatebirds circulate in their plasma and whether male gular pouch coloration was carotenoid-based. Great frigatebird plasma collected during the breeding season contained three carotenoid pigments in dilute concentrations--tunaxanthin, zeaxanthin, and astaxanthin-- with astaxanthin accounting for nearly 85% of the carotenoids present. Astaxanthin was the only carotenoid present in gular pouch tissue, but the concentration is the highest reported for any carotenoid-pigmented avian tissue. Throat pouch reflectance curves were measured with a UV-VIS spectrophotometer, revealing a complex pattern of one UV peak (approx. 360 nm), two absorption valleys (approx. 542 and 577 nm), followed by a plateau at approx 630 nm. The reflectance curve suggests a role for additional pigments, in particular hemoglobin, in the production of color in this ornament.

Background

Carotenoids are natural pigments that produce yellow, orange and red coloration in animals and are known to be an important source of ornamental coloration in fish, lizards, and birds (Endler 1980, Kodric-Brown 1989, Hill et al. 2002, McGraw et al. 2004). Animals cannot manufacture carotenoids, which therefore must be obtained through the diet (Fox 1979). In birds, carotenoid-based colors are common in feathers and bare parts (McGraw 2005), and in some species, it has been demonstrated that color intensity is a direct product of the overall concentration of carotenoid pigments present (Saks et al. 2003). Furthermore, experimental evidence indicates that consumption of carotenoids enhances coloration (Hill et al. 2002, Blount et al. 2003, McGraw and Ardia 2003, Alonso-Alvarez et al. 2004).

In addition to their role in color production, carotenoids serve a variety of important physiological functions including multiple stimulating effects on the immune system (Bendich 1989, Olson and Owens 1998, Hill 1999a). Empirical evidence in birds has demonstrated an association between carotenoids and immune activity (McGraw and Ardia 2003 and 2005, Alonzo-Alvarez et al. 2004). The dual functions of color production and immunostimulation suggest that carotenoid-based coloration could convey honest information about individual quality by way of a physiological tradeoff between expression of ornamental color and health status (Lozano 1994). Hypotheses on the costs associated with carotenoid-based coloration, however, remain controversial because so little is known about their intake (Lozano 1994, Grether et al. 1999, Hill 1999a, Hill et al.

2002, Godin and McDonough 2003). Recent advances in spectrophotometric technology allow for more objective and quantitative assessment of color while considering the range of avian visual perception (Andersson and Prager 2006).

In sexually dichromatic bird species, in which males exhibit brighter, carotenoid-based colors, males typically circulate higher levels of carotenoids in their blood than do females (Hill 1995, Bortolotti et al. 1996, Figuerola and Gutierrez 1998, Negro et al. 1998, McGraw and Ardia 2005). Seasonal patterns of carotenoid concentrations have been documented in both sexes, with carotenoid concentrations in blood typically being highest during the breeding season when the expression of colorful traits is most pronounced (Hill 1995, Negro et al. 1998).

A majority of studies investigating the significance of ornamental coloration in birds have focused on colorful species from the order Passeriformes(reviewed in Hill 1999b, and Hill and McGraw 2006). More recent studies are beginning to shed light on ornamental coloration in non-passerine birds (Negro et al. 2002, Massaro et al. 2003, Blas et al. 2006, Bortolotti et al. 2006, Velando et al. 2006, Mougeot et al. 2007). The great frigatebird (*Fregata minor*) is a large marine bird from the order Pelecaniformes that exhibits a high degree of sexual dimorphism. Males possess conspicuous sexual ornaments, including a bright red gular pouch that is inflated during elaborate courtship displays (Nelson 1975). These courtship displays are conducted in dense aggregations where females can assess many potential mates at the same time (Nelson 1975). Great frigatebird males exhibit dramatic seasonal variation in

gular pouch appearance. At the onset of the breeding season, pouches become bright red and extend down greater than 5 cm from the throat (F. Juola pers. obs., Fig. 2.1B). Red coloration remains intense while males actively display over the course of weeks or months (Fig. 2.1A and 2.1B). Once a male secures a mate, the gular pouch retracts into the throat (Fig. 2.1C) and the color begins to fade rapidly from red to orange (Fig. 2.1D). This decline in ornamentation occurs over the course of several weeks (F. Juola pers. obs.). Throughout the non-breeding season, male gular pouches remain small and dull orange in color. The rapid shift in coloration after mating suggests that development and/or maintenance of this bright red color is costly.

This striking ornament is assumed to be an example of sexual selection by way of female choice (Nelson 1975, Dearborn and Ryan 2002, Madsen et al. 2007a). The goal of this study was to investigate the source of red coloration in male great frigatebird gular pouches in hopes of shedding light on the possible signaling functions of this color ornament. Specifically, we tested whether male gular pouch coloration was carotenoid-based through identification and quantification of what, if any, carotenoids were circulating in blood and deposited in gular pouch tissue. In addition, we tested whether differences exist in circulating carotenoid levels between males and females of three differing physiological states: courting, incubating, and non-breeding. Integumentary coloration is predicted by levels of carotenoids in plasma for at least six bird species (reviewed in McGraw 2006). In dichromatic bird species that exhibit carotenoid-based coloration, colorful males commonly circulate higher levels of

carotenoids than do females (Bortolotti et al. 1996, McGraw et al. 2003, McGraw and Ardia 2005). In addition, females may circulate higher levels of carotenoids during the physiological state prior to egg-laying, in order to divert carotenoid pigments into egg yolk (Blount et al. 2000).

Finally, we describe the spectral curve of the male gular pouch and investigated whether any spectral properties of this curve might provide further insight into the basis of this colorful ornament. Pigments absorb light according to characteristic absorption functions, the patterns of which can provide insight into the types of pigments present in animal tissues (Andersson and Prager 2006). In particular, carotenoids absorb light in the blue-green wavelengths (430-500 nm) and consequently produce distinct reflection peaks in the yellow, orange, and red wavelengths (McGraw et al. 2007).

Materials and Methods

We collected blood and tissue samples for this study on Tern Island, French Frigate Shoals, in the northwestern Hawaiian Islands. There are roughly 2,000 nesting attempts by great frigatebirds on this 14-ha island each year (Dearborn and Anders 2006). One hundred and twelve adult great frigatebirds of known breeding status (61 displaying males, 10 incubating males, 11 non-breeding males, 10 searching females, 10 incubating females, 10 non-breeding females) were captured, either by hand or with a hand net, between the hours of 0900 and 1400 from February–March 2006. Breeding status was determined by behavior and plumage. Displaying males exhibit bright red inflated gular pouches and

freshly molted black plumage, while non-breeding males exhibit retracted, dull-orange pouches and an overall faded brownish-black plumage color. Females seeking mates fly low around the breeding colony and appear to be visually inspecting males with inflated throat pouches. These females also exhibit freshly molted black plumage; plumage of non-breeding females is also faded brownish-black in color. At the time of capture, approximately 1 ml of blood was collected from the brachial vein of each bird. Plasma was separated from blood by centrifugation of blood samples at 10,000 rpm for 5 minutes. Centrifugation was completed within 4 hours of blood collection, and all plasma samples were then stored at -20 C until carotenoid analysis approximately 4 months later. Eight gular pouch tissue samples were collected from adult male great frigatebirds found dead (within ~ 24 hrs) in the breeding colony from 1999 - 2006. Pouch samples were stored at -20 C until analyzed.

The types and amounts of carotenoids in plasma and gular pouch tissue were analyzed using high-performance liquid chromatography (HPLC). Plasma analysis followed previously published methods (McGraw et al. 2002; 2006), with the exception of using 50 µl of plasma from each sample for carotenoid extractions (because pigment concentrations were so low). Briefly, reverse-phase chromatography was run with a Waters Alliance 2695 autosampler instrument (Waters Corporation, Milford, MA) equipped with a Waters YMC Carotenoid C-30 5 um column (4.6 x 250 mm) that was heated to 30 degrees C with a built-in column heater. We used an isocratic solvent system (42:42:16, methanol:acetonitrile:dichloromethane, v/v/v), at a flow rate of 1.2 ml/min for 12

min, to analyze polar carotenoids after initial tests revealed that no non-polar carotenoids (i.e. cryptoxanthins or carotenes) were present. The detection limits of our system were 0.01 ug/ml for plasma analyses and 0.01 ug/g for pouch analyses.

For each gular pouch, a 0.5 g cube of flesh was sectioned and processed as follows: The gular pouch tissue was ground in presence of 2 mL hexane:TBME (1:1) for 5 min. The solvent was transferred to a new tube, while the grinding jar with rinsed with 1 mL hexane: MTBE to recover any residual pigment. The solvent was then centrifuged at 3000 RPM for 5 min. and transferred to a new tube where it was evaporated to dryness under nitrogen, then resuspended in 1 mL 0.02 M KOH in order to saponify (attempts to analyze carotenoids from unsaponified material were unsuccessful). It was then placed in darkness at room temperature for 4 hours, after which 1 mL saturated NaCl was added. The solution was vortexed, 2 mL ddH2O was added, and the solution was again vortexed. Next, 2 mL MTBE was added and the solution was shaken vigorously for 1 min. This solution was then centrifuged at 3000 RPM for 5 min., and finally evaporated to dryness. Standard HPLC methods (McGraw et al. 2006) were then followed exactly as for the plasma samples. Reported carotenoid concentrations in pouch tissues refer to dry weight of the starting material.

During the mating season of 2007, reflectance curves were obtained from 10 displaying male and 10 incubating male great frigatebirds using a UV-Vis spectrophotometer (USB4000, configured range 250-850nm, Ocean Optics Inc.)

with a pulsed xenon lamp (PX-2) as a light source. Using a black rubber sheath that excluded ambient light, we held a bifurcated micron fiber-optic probe 3 mm from and perpendicular to the pouch tissue. All measurements were conducted indoors, in a dark room. Each curve was generated relative to a white standard (WS-1, Ocean Optics). Using SpectraSuite software (Ocean Optics), we averaged 20 sequential spectra for each of five arbitrarily selected locations on each pouch sample. To obtain an average spectral curve for displaying males and for incubating males, the median percent reflectance for each 10 nm increment was averaged for the 10 individuals from each category, across the wavelengths relevant to the avian visual spectrum (300 – 700 nm).

Statistical Analysis

We used SYSTAT 11 (Systat Software, Inc.) for all statistical analyses. We used Mann-Whitney U tests to compare differences in the carotenoid concentrations and percent composition of each carotenoid present in the plasma of male and female great frigatebirds. We used a two-way ANOVA to compare differences between sex and three time periods. We conducted post-hoc analyses of differences between individual groups using Fisher's least significant difference test.

Results

Great frigatebird blood plasma contained three carotenoid pigments: astaxanthin, tunaxanthin, and zeaxanthin (total = 1.06 ± 0.06 μg/ml; mean ± SE). Astaxanthin

accounted for 84.5 ± 0.010% of the total plasma carotenoids (0.87 ± 0.04 µg/ml). Zeaxanthin and tunaxanthin accounted for 2.8 ± 0.003% and 12.7 ± 0.010% of the total plasma carotenoids, respectively (0.04 ± 0.004 µg/ml; 0.16 ± 0.02 µg/ml). A representative 2-dimentional HPLC chromatogram of plasma is presented in Fig. 2.3. Males had significantly higher astaxanthin concentrations in their plasma than did females, when individuals from all breeding stages were combined (Table 2.1). The relative proportion of astaxanthin was significantly higher in males compared to females, while the relative proportions of tunaxanthin and zeaxanthin did not differ between males and females (Table 2.1). Total carotenoid concentrations in plasma differed significantly between the sexes (two-way ANOVA, sex: $F_{1,106} = 36.08$, $P < 0.001$; Fig. 2.2), but not in relation to breeding category (two-way ANOVA, $F_{2,106} = 0.77$, $P = 0.47$; Fig. 2.2). However, there was a significant interaction between sex and breeding category (two-way ANOVA, sex * category interaction: $F_{2,106} = 18.976$, $P < 0.001$; Fig. 2.2). Post-hoc comparisons showed that non-breeding males circulated significantly higher carotenoids compared with courting males (Fisher's least-significant difference, $P < 0.001$), courting females ($P = 0.002$), incubating females ($P < 0.001$), and non-breeding females ($P < 0.001$). In addition, incubating males circulated significantly higher levels compared with courting males ($P = 0.004$), incubating females ($P <0.001$), and non-breeding females ($P < 0.001$). Courting males circulated higher levels than incubating females ($P = 0.007$) and non-breeding females ($P = 0.006$). Finally, courting females circulated higher levels than incubating females ($P = 0.003$) and non-breeding

females (P = 0.003). Astaxanthin was the only carotenoid present in gular pouch tissue. Pouch tissue concentrations (1268.8 ± 282.5 µg/g; mean ± SE) were extremely high compared to total plasma concentrations. One gular pouch had an astaxanthin concentration exceeding 3,000 µg/g (3668.0). A representative 2-dimentional HPLC chromatogram for pouch tissue is presented in Fig. 2.4.

The average reflectance curve for gular pouches of displaying males (n=10; 5 spectra averaged for each) showed a distinctive pattern containing an initial peak in the UV range at approximately 360nm. In addition, small absorption valleys occurred at approximately 542nm and 577nm, followed by a plateau at approximately 630 nm (Fig. 2.5). The average reflectance curve for gular pouches of incubating males (n=10, 5 spectra averaged for each) was similar to that of displaying males. There was an initial peak in the UV range at approximately 360nm, and the final plateau again appears at approximately 630nm. However, the absorption valleys present in the displaying male pouch spectra at 542nm and 577nm are not present in the incubating male pouch (Fig. 2.6).

Discussion

Carotenoids are a known source of ornamental coloration in a variety of bird tissues, including feathers (Stradi 1999, McGraw et al. 2001, McGraw and Hardy 2006), bills (Bennett et al. 1996, McGraw and Ardia 2005), and legs (Bortolotti et al. 1996, Casagrande et al. 2006). Far less attention has been given to colorful fleshy parts in birds (e.g. wattles, combs, eye-rings, pouches) and the potential

role that carotenoids play in the expression of these colors (but see Negro et al. 2006, Mougoet et al. 2007). Because the concentration of astaxanthin, a red carotenoid, in male great frigatebird throat pouch tissue was so high, it is likely that astaxanthin contributes substantially to the red color of these ornaments. This has implications for the information content of this display signal, as astaxanthin is a known stimulant of the immune system and a powerful antioxidant (Hussein et al. 2006, McGraw 2006). Previous work on this population demonstrated variation in displaying male pouch color (Dearborn and Ryan 2002). Males capable of displaying astaxanthin-rich pouch tissue may be advertising superior fitness if only excess carotenoids beyond what are needed for basic physiological functions is available for diversion into throat pouch tissue. If females select mates based on coloration of their throat pouches, selection of males with the most astaxanthin-rich pouches could potentially result in direct benefits to females. Perhaps the most intensely colored males are in better condition and are more efficient foragers, able to provide superior parental care. In addition, females could gain indirect benefits if the ability of males to acquire or assimilate carotenoids is a genetic trait that could be inherited by their offspring.

High concentrations of carotenoids have been reported in other avian tissues, such as in the feathers of American goldfinches (McGraw and Gregory 2004). One throat pouch tissue analyzed in our study exceeded 3000 μg/g, and two additional pouch tissues exceeded 2000 μg/g; these to our knowledge are the highest reported concentrations of carotenoids in any bird tissue. Astaxanthin concentration in throat pouch tissue was remarkably high compared

to the total carotenoid concentrations found in blood plasma. In addition, of the three carotenoids circulated in great frigatebird blood plasma, only astaxanthin was deposited in throat pouch tissue. It is rare that pure astaxanthin is deposited in avian tissues, as it is almost always accompanied by other 4-oxo-carotenoids (Stradi 1999). This study, to our knowledge, is the third demonstration of pure astaxanthin deposition in bird tissue and the first in bare skin. Interestingly, the two previous examples may both be the result of human introduction of astaxanthin into the environment (Negro and Garrido-Fernandez 2000, McGraw and Hardy 2006). Astaxanthin present in frigatebird blood plasma suggests that it is acquired directly from a food source, rather than being manufactured from another precursor. The dietary source of astaxanthin in great frigatebirds on Tern Island is not well understood. Great frigatebird diet has been reported to consist of primarily flying fish and squid (Metz and Schreiber 2002). Initial analyses of diet samples revealed moderate levels of astaxanthin in some fish (F. Juola and K.J. McGraw pers. obs.). Astaxanthin is commonly acquired through aquatic food sources such as fish and crustaceans (Goodwin 1984), and some squid species are highly enriched in astaxanthin (K. McGraw, M. Hipfner, and J. Dale, pers. obs.), making this a likely source for astaxanthin accumulation in great frigatebirds.

Carotenoid concentrations in great frigatebird blood plasma were similar to those reported for other large, non-passerine birds, and lower than those reported for small passerine birds (Tella et al. 2004). Carotenoid concentrations in blood plasma differ widely across avian species (Parker 1996, Tella et al.

1998, Slifka et al. 1999, McGraw 2005), with phylogeny being a significant predictor of circulating carotenoid levels (Tella et al. 2004). Additional factors such as diet, (Tella et al. 2004), occurrence of ornamental coloration (Tella et al. 2004), and efficiency in assimilating consumed carotenoids into the blood system (McGraw 2005) have been used to explain higher levels of circulating carotenoids in passerine birds. In addition, body size may play a role. In vertebrates, food consumption is proportional to body mass raised to the 3/4 power (Peters 1983). The increase in blood volume is directly proportional to the increase in species body mass. Thus, larger animals eat less food, and presumably fewer carotenoids, per body mass unit than smaller species, and incorporate relatively fewer carotenoids into their proportionally equal blood volume (Tella et al. 2004, McGraw 2005).

As has been found in other dichromatic species (Hill 1995, Bortolotti et al. 1996, Negro et al. 1998, McGraw and Ardia 2005), male great frigatebirds circulate higher concentrations of carotenoids then do females. Dietary differences or differences in the absorption capacities of males and females could mechanistically account for this difference in carotenoid levels. Interestingly, incubating and non-breeding males exhibited higher levels of circulating carotenoids compared with displaying males. A prediction of higher circulating carotenoid levels in more colorful males (while displaying) seems intuitive if the assumption is that males flood their system with more carotenoids during times of peak color production. However, circulating carotenoids diverted into tissues such as skin, beaks, or feathers may deplete carotenoid levels

circulating in the blood. For this reason, predictions of either higher or lower circulating carotenoid levels in more colorful males seem plausible. Lower carotenoid levels in displaying males lend support to the idea that carotenoids are depleted by diversion into throat pouch tissue. Courting females had higher circulating carotenoid levels than incubating and non-breeding females, a pattern similar to that reported in American kestrels (Negro et al. 1998). It's possible that females circulated higher carotenoid levels during courtship in preparation for egg laying, as carotenoids are a known component of egg yolk (Fletcher 1992, Royle et al. 1999).

The average spectral curve presented here is the first reported for any frigatebird throat pouch. A spectral curve derived solely from astaxanthin-based red tissue would predict a single absorption valley at approximately 470 nm, followed by a steeper upslope and plateau at around 550 nm. However, gular pouch tissue is highly vascular. Blood filled capillaries are visible in inflated pouches (F. Juola pers. obs.) The presence of oxyhemoglobin produces a characteristic spectral pattern of absorption bands at 542nm and 577nm (Zonios et al. 2001). Great Frigatebird throat pouches exhibited absorption bands at these locations. This suggests that increased blood flow and the presence of hemoglobin may contribute substantially to the overall coloration and spectral properties of these throat pouches during the display period. This notion is supported by the rapid fading of pouch color from red to orange once a male has secured a mate, something that could be accomplished through diversion of blood flow. Furthermore, one key difference between the spectral curves of

displaying males and those of incubating males was the lack of absorption bands expected with the presence of hemoglobin in incubating males. This supports the notion that gular pouch coloration is produced through a combination of carotenoid deposition and increased blood flow.

A secondary peak appeared in the UV-range of the pouch reflectance spectrum. UV-signaling in birds has been the focus of a number studies in recent years, since the advent of more sophisticated analytical methods. It has been demonstrated that females of some species use UV-reflecting feathers to assess male quality and prefer those males that exhibit greater UV reflection (Bennett et al. 1996, Hunt et al. 1999). UV-reflectance in avian skin parts is not well documented. Some examples have been reported in passerine species (Hunt et al. 2003, Jourdie et al. 2004) and in the orbital combs of the red grouse (*Lagopus lagopus scoticus*; Mougeot and Redpath 2004). Interestingly, UV brightness in the red orbital combs of the red grouse was negatively correlated with parasite infection rates (Mougeot et al. 2005), suggesting that UV reflectance could serve as a reliable signal of parasite resistance. It has been demonstrated that feathers with carotenoid-based colors create such UV spectral patterns by absorbing light from a structural white tissue that in which the carotenoids are embedded (Shawkey and Hill 2005). Frigatebird pouch tissue was comprised of two tissue layers, an inner tissue layer that was pink or white, and an outer dermal layer that contained the astaxanthin pigments (K. McGraw pers. obs.). It is possible that, as in grouse (Mougeot et al. 2007), the pouch tissue gets its UV reflectance from the white background layer.

Table 2.1 Comparison of plasma carotenoid concentrations and percent composition of each carotenoid present in male and female great frigatebirds.

	Males (n=82)	Females (n=30)	Z	P
Concentration (µg/ml)				
Astaxanthin	0.97 ± 0.05	0.59 ± 0.07	-4.69	<0.001
Tunaxanthin	0.18 ± 0.03	0.13 ± 0.02	-0.83	0.41
Zeaxanthin	0.04 ± 0.004	0.03 ± 0.01	-0.53	0.60
Total Carotenoids	1.18 ± 0.07	0.75 ± 0.49	-3.86	<0.001
Percent Composition (of total plasma carotenoids)				
Astaxanthin	82.00± 0.01	78.88± 0.02	-2.10	0.04
Tunaxanthin	14.94± 0.01	16.71± 0.02	-1.70	0.90
Zeaxanthin	3.06± 0.003	4.41± 0.01	-0.86	0.39

Mann-Whitney U tests were used to compare differences between males and females (Z-statistic). Mean ± SE are given for each group.

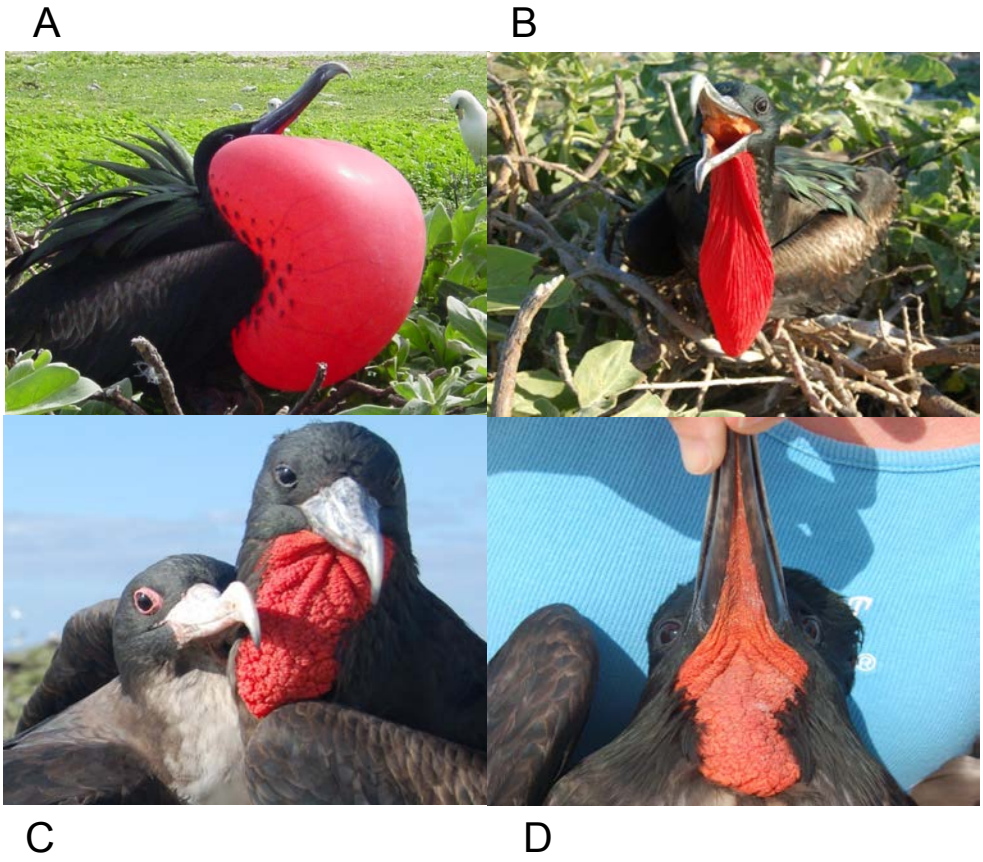

Figure 2.1 (A) Upper left: Male great frigatebird (*F. minor*) displaying a bright red, inflated gular pouch on Tern Island, French Frigate Shoals. (B) Upper right: Between bouts of displaying, gular pouch is extended from the throat and remains bright red. (C) Lower left: After pair formation, the gular pouch is deflated and begins to retract into the throat. (D) Lower left: Within days of pair formation, pouch coloration begins to fade from red to orange.

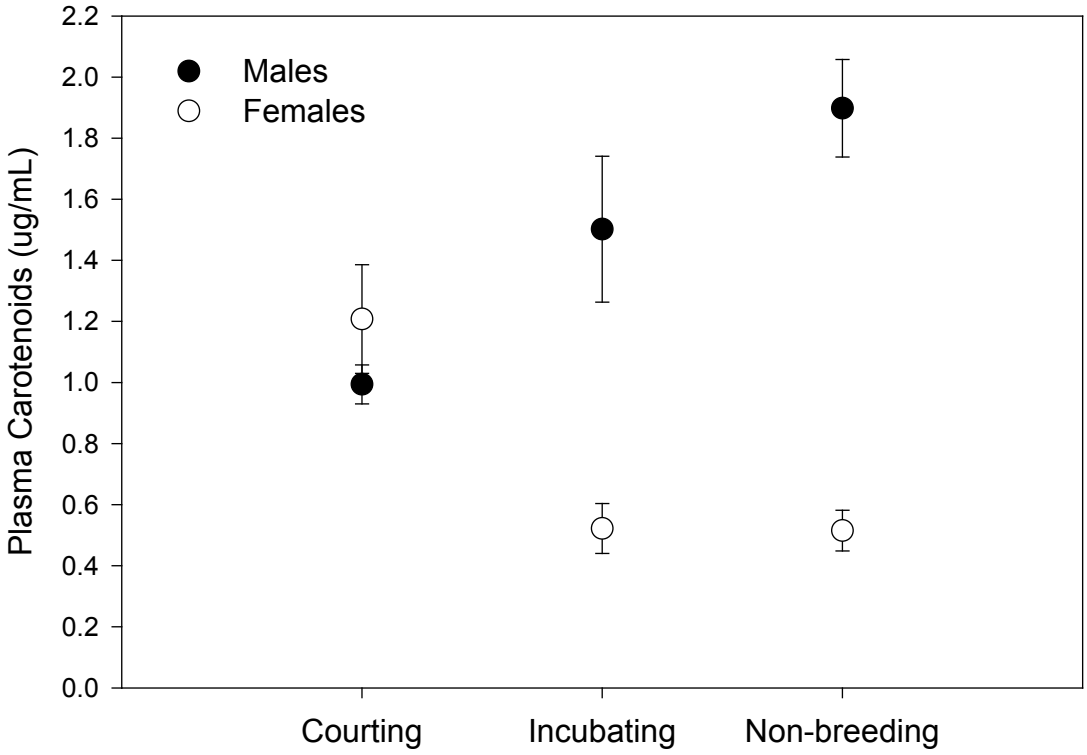

Figure 2.2 Great frigatebird plasma carotenoid concentrations by sex and breeding status.Sample sizes were courting males (61), courting female 10), incubating males (10), incubating females (10), non-breeding males (11), non-breeding females (10). Error bars represent standard errors of the means.

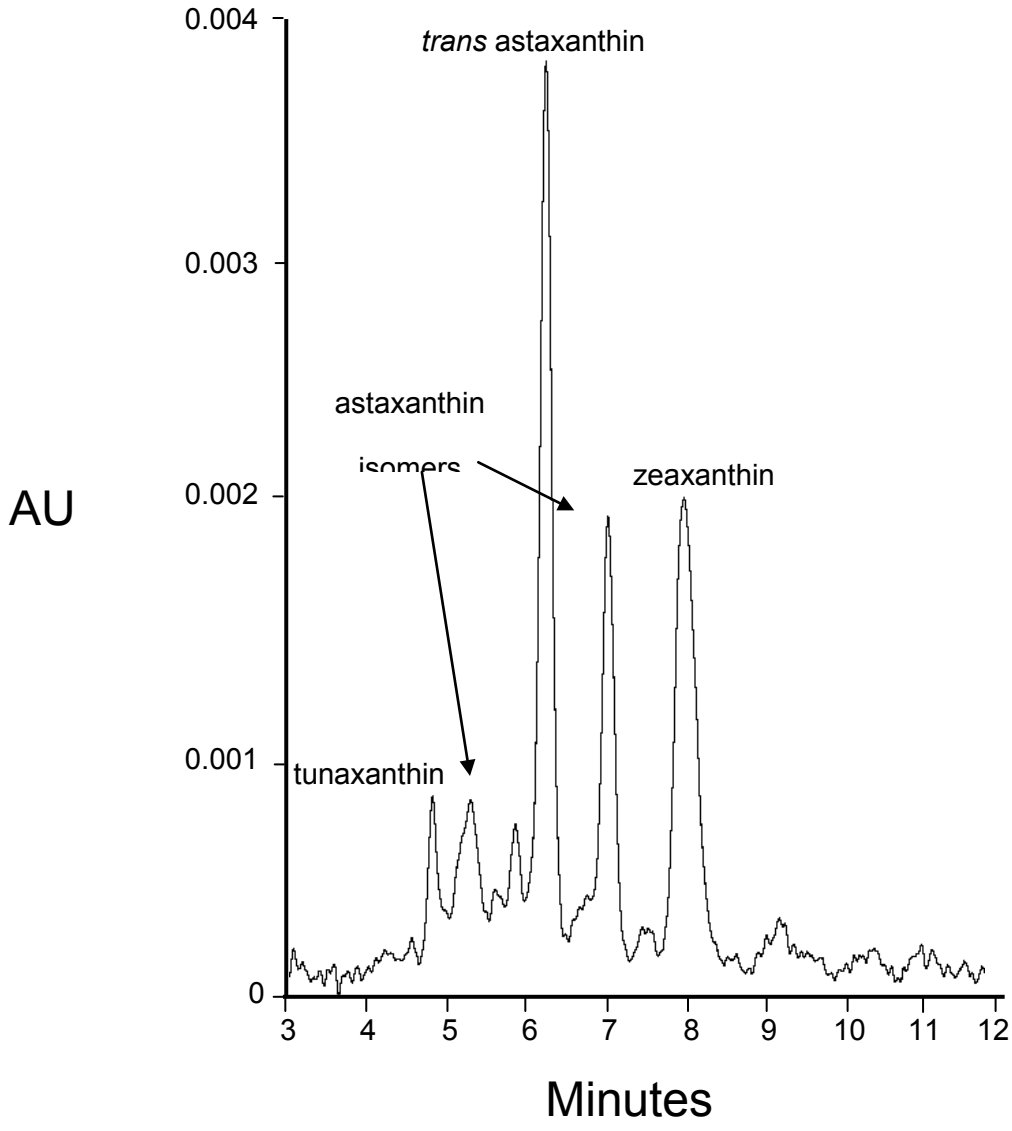

Figure 2.3 Representative two-dimensional HPLC chromatogram depicting carotenoids detected in the plasma of frigatebirds.

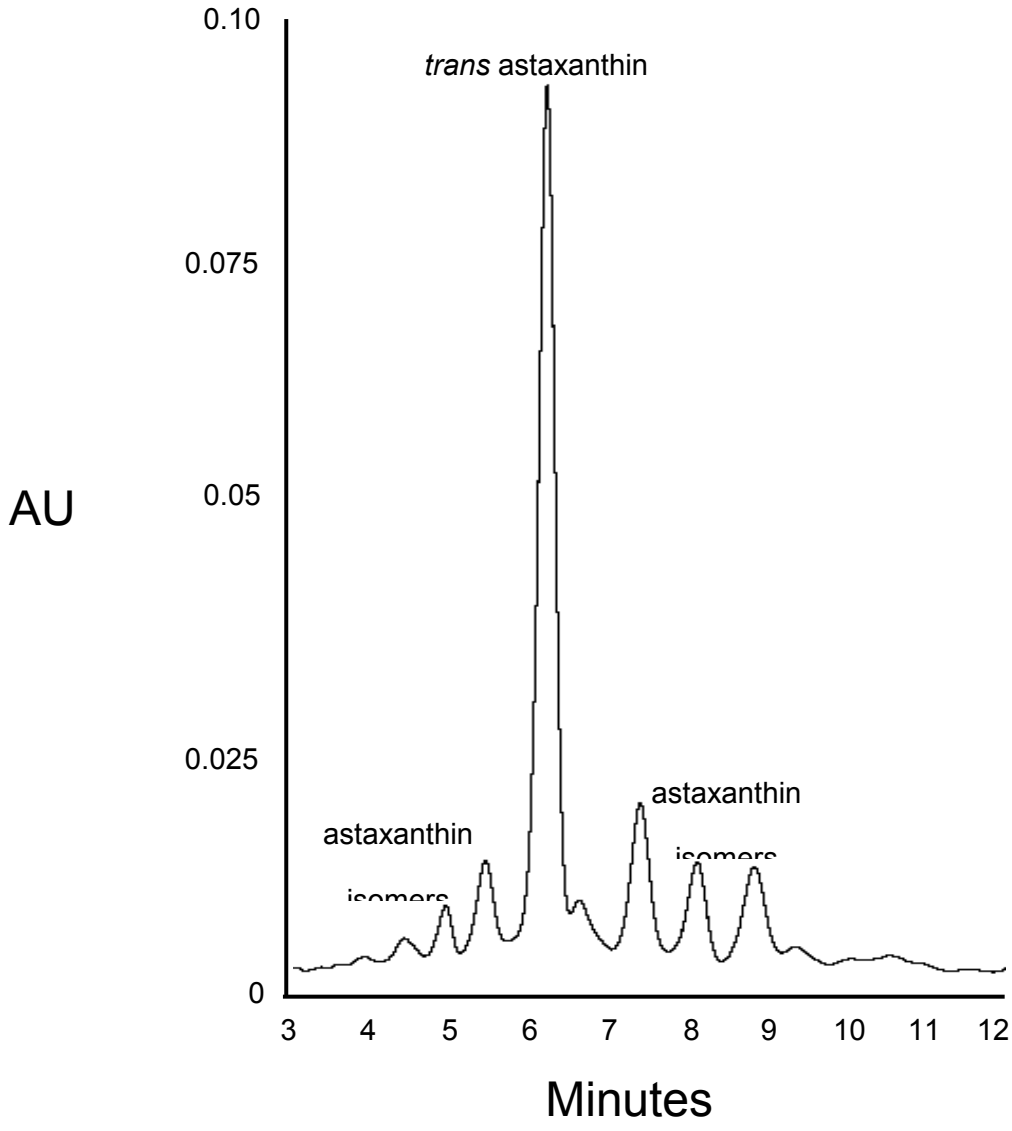

Figure 2.4 Representative two-dimensional HPLC chromatogram depicting carotenoids detected in the pouch tissue of frigatebirds.

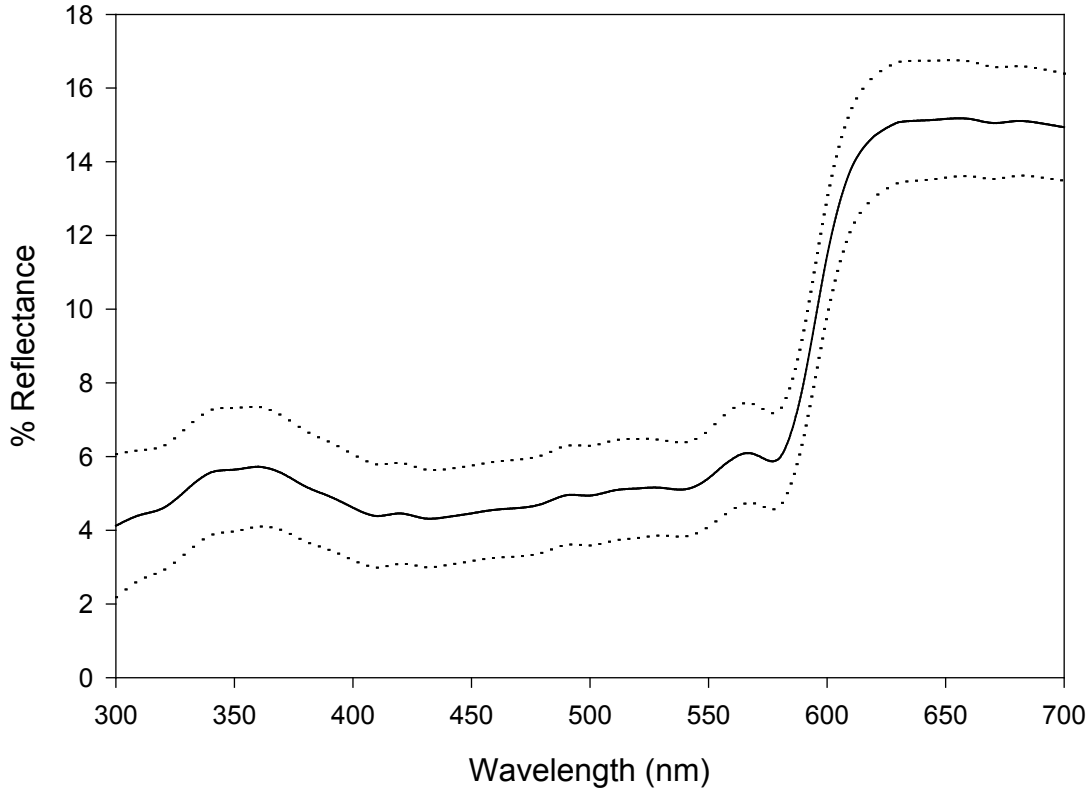

Figure 2.5 Mean spectral reflectance curve of the throat pouch of displaying male great frigatebirds (n=10). Measurements were averaged from 5 readings taken from the non-inflated portion of skin under the bill. Solid line represents the mean reflectance curve. Dotted lines represent the upper and lower 95% confidence limits.

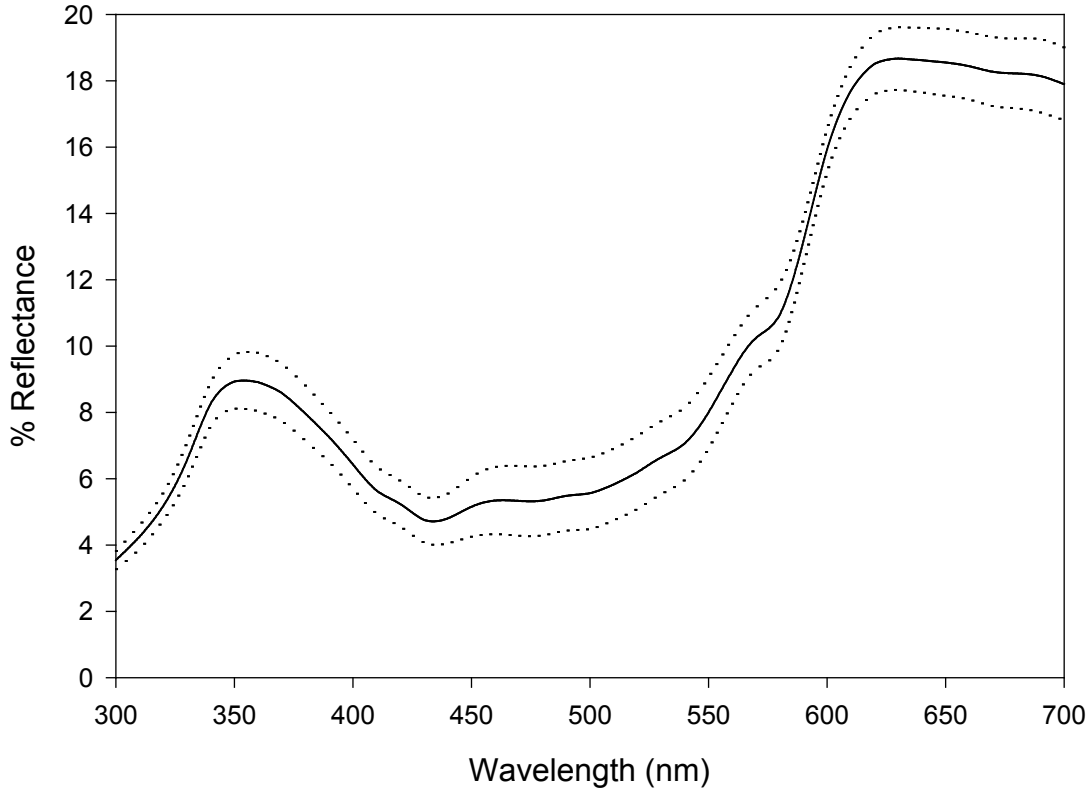

Figure 2.6 Mean spectral reflectance curve of the throat pouch of incubating male great frigatebirds (n=10). Measurements were averaged from 5 readings from the skin patch under the bill. Solid line represents the mean reflectance curve. Dotted lines represent the upper and lower 95% confidence limits.

Chapter 3

Ornamentation, color and size: Morphology and pairing success in the great frigatebird (*Fregata minor*)

Summary

Colorful ornaments are central to the study of mate choice and sexual selection. The size and intensity of male ornaments may provide honest information about individual quality, and thereby influence mate choice. In particular, ornamental coloration produced by carotenoid pigments may reveal information about the signaler's health due to carotenoids' immune-enhancing effects. Great frigatebird males inflate a red gular pouch as part of their pronounced courtship display. Gular pouch tissues are known to harbor extremely high concentrations of the red carotenoid pigment astaxanthin. We measured astaxanthin concentrations of blood plasma, coloration of gular pouches, and a number of morphological features including gular pouch size, body size, and body condition in displaying male great frigatebirds. We investigated the relationships between plasma astaxanthin concentration, gular pouch size, gular pouch coloration, body size, and body condition. In addition, we investigated what influence these variables had on pairing success. Plasma astaxanthin concentrations in displaying males significantly predicted red chroma of gular pouches and overall body condition. Body size and body condition were not good predictors of gular pouch size. Only one variable, body condition, significantly predicted pairing success, though this relationship was weak. Our findings suggest that astaxanthin contributes to gular pouch coloration and is related to size-adjusted

mass. The finding of no relationship between ornamental size or coloration and mate choice concurs with other recent investigations of mate choice in frigatebirds. Morphological traits including gular pouch color and size and body size may perhaps play some composite or age-dependent role in mate choice, but individually these factors were poor predictors of pairing success in great frigatebirds. The courtship display of great frigatebirds is an amalgamation of complex signals including visual and auditory cues and perhaps chemical cues as well. These signals may act in concert such that identification of one or several key factors that predict pairing success in this highly dimorphic species remains elusive.

Background

Darwin (1859, 1871) proposed the theory of sexual selection to explain the evolution of secondary sexual characters (Darwin 1859, 1871). According to the theory, male ornaments and other sexually dimorphic traits have evolved because of their effects on variation in reproductive success resulting from competition for mates. Competition may include not only physical contests between males, but male displays that facilitate female choice (Andersson 1994).Past studies of a number of animal groups have supported sexual selection as the explanation for the evolution of a wide variety of ornamental traits (Andersson 1994). Here we test the theory with respect to one of the most striking visual ornaments found in any animal: the red gular pouch of great frigatebirds (*Fregata minor*).

Models of sexual selection, as well as empirical evidence, have shown that ornamentation may develop in proportion to phenotypic quality or condition, thus serving as honest signals (Andersson 1994, Cotton et al. 2004, Alonso et al. 2010).For colorful ornaments to serve as indicators of male quality there must be a cost associated with their production (Zahavi 1975, Grafen 1990). The most healthy, fit males are able to produce the most elaborate ornaments in spite of their cost, and are therefore more attractive to females. If directional selection drives the evolution of elaborate ornaments, then over time the additive genetic variance of such traits should diminish, and with it, the benefits of being choosy, a phenomenon known as the lek paradox (Kirkpatrick and Ryan 1991). Hamilton and Zuk (1982) provided one possible solution to this problem by suggesting that bright colors associated with male ornaments are indicators of a male's ability to resist parasitism, with hosts that possess resistance to particular parasites being healthier and better able to produce high-quality ornaments. In this case, females benefit indirectly from a preference for males with high-quality ornaments because their offspring will inherit the resistance to parasites that such ornaments indicate. Negative associations between ornamentation and measures of parasite infection or positive associations between ornamentation and immunity are interpreted as supporting Hamilton and Zuk's hypothesis (reviewed in Møller et al. 1999). Others have questioned the casual nature of these associations and suggested more information is needed regarding the genetic mechanisms of immunity (Reid et al. 2005).

Carotenoids are responsible for the bright colors seen in many sexual ornaments across a wide range of taxa. Carotenoids are pigments that reflect light in the yellow, orange or red domain of the electromagnetic spectrum (Fox 1979). Animals do not possess the biochemical ability to manufacture carotenoids and instead must obtain them through dietary consumption (Fox 1979). In addition to their role as pigments, carotenoids serve a variety of important physiological functions (Olson and Owens 1998, Hill 1999). Carotenoids are known to have multiple stimulating effects on the immune system (reviewed in Bendich 1989). They serve as free radical scavengers, which can reduce the chance of tumor development (Burton 1989), and possess other cancer-preventative properties (Krinsky 1989, Ziegler 1989). In birds, carotenoid-based coloration of plumage is well documented (Hill and Brawner 1998; Badyaev and Hill 2000; McGraw 2005; Shawkey and Hill 2005). Carotenoids are also responsible for colorful skin parts in some birds (Bortolotti et al. 1996; Buchholz 1997; Mougeot et al. 2007). Juola et al. (2008) have shown that the gular pouches of great frigatebirds contain high concentrations of carotenoids. Spectral data have shown that some carotenoid-based coloration in birds exhibit a secondary reflectance peak in the ultra-violet (UV) wavelengths, including plumage (Burkhardt 1989, Bleiweiss 2005), beaks (Bennett et al. 1996, Peters et al. 2004) and skin parts (Mougeot et al. 2005, Mougeot et al. 2007). Many birds possess visual sensitivity to UV wavelengths (Cuthill et al. 2000), and UV signaling is thought to play a role in mate choice in some species (Bennett et al. 1996, Johnsen et al. 1998).

Empirical evidence suggests that carotenoid-based color displays play an important role in female mate choice decisions in both birds and fish (Kodric-Brown 1985, Houde 1987, Milinksi and Bakker 1990, Hill 1990, 1991, 2002; McGraw 2005). Carotenoid displays have been suggested to have several costs that might cause them to be honest signals of quality, including foraging costs (or constraint if carotenoids are rare; Grether et al. 1999), metabolic processing and transporting costs (Hill 2002), and increased predation costs associated with more conspicuous colors (Godin and McDonough 2003). Several of these costs might make carotenoid colors reliable indicators of male nutritional state. Dietary supplementation of carotenoids has increased coloration in fish and birds (Grether 2000, Hill 1994). In addition to indicating nutritional state, carotenoid coloration may also signal parasite resistance and/or immune system strength (Lozano 1994). Experimental increases in parasite loads have been shown to decrease red coloration in fish (Milinski and Bakker 1990, Houde and Torio 1992). Likewise, experimental exposure to parasite infections decreased red plumage in house finches (Brawner et al. 2000). These studies and others suggest a negative relationship between carotenoid pigmentation and parasite loads, offering support for the idea that carotenoid-based signals honestly reflect an individual's health status (Møller et al. 2000). The significance of ornamental coloration in birds has been well studied in species from the order Passeriformes (reviewed in Hill and McGraw 2006).More recently, renewed interest has been given to ornamental coloration in non-passerine birds (Massaro et al. 2003; Mougeot et al. 2007; Nolan et al. 2010).

The five species of the frigatebird family (Fregatidae) of the order Pelecaniformes are unique among seabirds in exhibiting marked sexual dichromatism. Males possess striking sexual ornaments, including iridescent nape feathers and a red gular pouch that is inflated during courtship displays (Nelson 1975). This ornamentation is often cited as a classic example of sexual selection driven by female choice (Attenborough 1998, Ligon 1999, Knight 2002). These courtship displays are often conducted in aggregations where females can assess many potential mates at the same time (Nelson 1975).Elaborate male characters are often observed in species with polygynous mating systems, but may also evolve in monogamous species in situations where the sex ratio is male biased, creating competition that results in some males failing to attract mates (Darwin 1871, Fisher 1958). Our study population is serially monogamous; with extra-pair fertilizations being extremely rare (Dearborn et al. 2001 and Chpt. 4) but with pair-bonds not persisting beyond one mating attempt (unpubl. data). The operational sex ratio in this population is strongly male biased, resulting in substantially fewer than half of all males successfully obtaining a mate in a given breeding season (Dearborn 2001; Dearborn and Anders 2006).

The appearance of male gular pouches in our study population changes drastically depending on the breeding state. At the onset of the breeding season, pouches of males attempting to breed become dark red and extend down several inches from the throat (F. Juola pers. obs). This red coloration remains intense while males actively participate in courtship displays over the course of several weeks to several months. However, once a male secures a mate, the gular

pouch retracts into the throat and the color fades rapidly from dark red to bright orange (Juola et al. 2008).This color change is noticeable within days and is complete within several weeks (F. Juola pers. obs.). Throughout the non-breeding season, male gular pouches remain retracted and bright orange in color. The rapid shift back to orange after securing a mate suggests that development and/or maintenance of this dark red color is costly. Production of this coloration is likely due to several factors. Testosterone is known to influence the development of many secondary sexual characters (reviewed in Folstad and Karter 1992). In magnificent frigatebirds, testosterone levels were highest in displaying males, and much lower in males participating in incubation and chick rearing (Chastel et al. 2005). In addition, Madsen et al. (2007b) found that testosterone was positively correlated with red coloration in the gular pouches of some displaying male magnificent frigatebirds. Previous work on our study population demonstrated that gular pouch coloration was produced by at least two factors. First, astaxanthin, a red carotenoid pigment, was present in displaying male gular pouch tissues in extremely high concentrations (>1000 µg/g; Juola et al. 2008). In addition, the spectral reflectance curves of this highly vascularized tissue revealed two absorption valleys at approximately 542 nm and 577 nm (along with a plateau at approximately 630 nm), strongly suggesting that hemoglobin also contributes to the red coloration observed (Juola et al 2008). Previously, the only investigation into the relationship between gular pouch coloration and breeding success in great frigatebirds utilized a subjective measure of color assessment (Wright and Dearborn 2009). Differences in avian

visual sensitivity compared to human visual sensitivity calls for more objective techniques of color assessment (Bennett et al. 1994, Bennett and Thery 2007). In this study, we use spectrophotometry to assess gular pouch coloration.

Here, we assess the role of the gular pouch of male great frigatebirds in signaling male quality by assessing associations between pouch color and pouch size on the one hand and body size, body condition, and circulating levels of astaxanthin on the other. In addition, we investigate the association between male pairing success and pouch size and color. Finally, we examine other male traits that might affect pairing success, such as body size and body condition.

Methods

This study was conducted on Tern Island, French Frigate Shoals, in the Northwestern Hawaiian Islands. Each year on Tern Island, there are approximately 2,000 nesting attempts by great frigatebirds (Dearborn and Anders 2006). Over the course of the 2007 breeding season (January – April), one hundred and sixty displaying adult male great frigatebirds were captured, either by hand or with a hand net. Prior to capture, each individual was photographed from both a front and side angle from a distance of approximately 2 meters. Once captured, individuals were transported to a dark room where the spectral data on pouch coloration were taken, morphological measurements were made, and a blood sample was collected. Prior to release, each individual was outfitted with an alpha-numeric wing tag so that it could be monitored for the remained of the season. Breeding status was determined by behavior and plumage.

Individuals were considered to have successfully paired if they were observed with a female and had commenced nest building.

Carotenoid Analyses

Approximately 1 ml of blood was collected from the brachial vein. Blood samples were centrifuged at 10,000 rpm for 5 minutes in order to separate plasma. All centrifugation was completed within 2 hours of blood collection, and all plasma samples were stored at -20 C for approximately 2 months, until carotenoid analyses could be completed. High-performance liquid chromatography (HPLC) was used to determine the types and amounts of carotenoids present in all plasma samples. Plasma analysis followed previously published methods (McGraw et al. 2002; 2006, Juola et al. 2008), with the exception of using 50 µl of plasma from each sample due to low pigment concentrations.In brief, reverse-phase chromatography was used with a Waters Alliance 2695 autosampler instrument (Waters Corporation, Milford, MA) equipped with a Waters YMC Carotenoid C-30 5 um column (4.6 x 250 mm), heated to 30 degrees C with a built-in column heater. An isocratic solvent system (42:42:16, methanol:acetonitrile:dichloromethane, v/v/v), was then used at a flow rate of 1.2 ml/min for 12 min to analyze polar carotenoids, after initial tests indicated the absence of any non-polar carotenoids (i.e. cryptoxanthins or carotenes). The detection limits of this system were 0.01 ug/ml (Juola et al. 2008).

Spectral Analyses

Reflectance curves were obtained from all captured males using a UV-Vis spectrophotometer (USB4000, configured range 250-850 nm, Ocean Optics Inc.) with a pulsed xenon lamp (PX-2) as a light source. Using a black rubber sheath to excluded ambient light, we held a bifurcated fiber-optic probe 3 mm from and perpendicular to the pouch tissue. All measurements were conducted indoors, in a dark room. Each curve was generated relative to a white standard (WS-1, Ocean Optics). Using SpectraSuite software (Ocean Optics), we averaged 20 sequential spectra for each of five locations selected arbitrarily along the flat portion of exposed red gular tissue below the culmen. The USB4000 spectrophotometer provides percent reflectance for every 0.21 nm. To summarize reflectance data for each displaying male, the median percent reflectance for each 5 nm increment was averaged from 5 measurements taken for each individual across the 300-700 nm range of wavelengths, corresponding to the avian visual spectrum (Endler 1990).

Color Variables

We calculated the following color variables for each male gular pouch: (1) red brightness (average reflectance at each wavelength in the interval 600-700 nm); (2) red chroma (reflectance in the interval 600-700 nm as a percent of the total brightness in the interval 300-700 nm); and since gular pouches also reflected UV wavelengths, (3) UV brightness (average reflectance at each wavelength in the interval 300-400 nm); and (4) UV chroma (reflectance in the interval 300-400

nm as a percent of the total brightness in the interval 300-700 nm). Hue was eliminated from all analyses because of the difficulty of defining a clear reflection peak (see Fig. 1) and a lack of variation, as all males exhibited a plateau in the spectral reflectance at around 630 nm.

Morphological Measurements

A single measure of structural body size was determined using a principal component analysis (PCA) based on measurements of wing length, ulna length, and culmen length for each male. We used the first principal component (PC1), which accounted for 57% of the observed variation in the three original variables, as a measure of structural body size. We calculated size-adjusted mass as the residuals of a regression of mass versus structural body size (PC1). These residuals were used as a proxy for body condition, with heavier males in better condition. Inflated gular pouch size estimates were calculated from digital photographs using the program Image J (v1.32j; NIH). Calculations were scaled on known bill lengths and bill widths for each individual. Specifically, a side view of gular pouch area was calculated first, from side photographs with bill length used as a reference. Pouch volume was then calculated by multiplying the area by the pouch width. Width was defined as the widest portion on the pouch determined from frontal photographs using bill width as a reference. A repeatability analysis (Lessels and Boag 1987) was used to test the reliability of our pouch volume calculations from digital photos. Volume was calculated 3 times each for the first 11 individuals. The inter-class correlation coefficient or repeatability (r) for pouch volume measurements of these 11 individuals was

0.988. For the remainder of individuals, volume was calculated just one time each.

Statistical Analyses

We used Pearson's product-moment correlations and multiple logistic regression analyses to test relationships between plasma astaxanthin levels, gular pouch size and coloration, body size and condition, and ultimately pairing success. A forward step-wise multiple logistic regression analysis was used to test whether pairing success was predicted by any possible combination of 8 variables: plasma astaxanthin concentration, gular pouch coloration (red chroma, red brightness, UV chroma, UV brightness), gular pouch volume, structural body size, and body condition (size-adjusted mass).

Results

Forty-two of the 160 males monitored over the course of the 2007 breeding season successfully paired (26.25%). The remaining 118 males failed to secure mates on Tern Island at any point during the breeding season.

A summary of all color variables and additional variables measured for all displaying males is presented in Table 3.1. Astaxanthin was detected in the plasma of all 160 displaying males (Table 3.1). Mean spectral reflectance curves for mated and unmated male gular pouches were nearly identical (Fig. 3.1). All displaying male gular pouches revealed high reflectance peaks and a subsequent plateau within the red color spectrum (600-700 nm), as well as a small reflectance peak within the UV range (300-400 nm; Fig. 3.1). Correlations

between the various color and morphological variables are given in Table 3.2. Plasma astaxanthin was significantly positively correlated with red chroma of gular pouch tissue (Table 3.2, Fig. 3.2), and body condition (Table 3.2, Fig 3.3), and significantly negatively correlated with red brightness, UV brightness, and UV chroma of gular pouch tissue, as well as gular pouch volume (Table 3.2). Gular pouch volume was also significantly negatively correlated with red chroma (Table 3.2) and significantly positively correlated with UV Chroma and UV brightness. Gular pouch volume was not significantly correlated with body size (Table 3.2). Likewise, body condition was not significantly correlated with gular pouch volume (Table 3.2). UV brightness was significantly positively correlated with red brightness (Table 3.2) and UV chroma (Table 3.2), and significantly negatively correlated with red chroma (Table 3.2). Finally, UV chroma was significantly positively correlated with red chroma (Table 3.2).

In a series of t-tests of all 8 measured variables, only body condition differed significantly between paired and unpaired males ($t= -2.017$, $P=0.045$, $df=158$; Fig. 3.4). Likewise, in a forward selection logistic regression analysis of pairing success, the model that best predicted pairing success included just one variable, body condition (Wald $X^2 = 3.91$, $df=1$, $P=0.0479$, $r^2=0.022$). Analyses using a backward selection and a mixed stepwise approach yielded the same result, the best model including only body condition as a significant but weak predictor of pairing success. Gular pouch size and color did not differ between mated and unmated males and did not predict mating success.

Discussion

Plasma astaxanthin concentration was significantly correlated with red chroma and red brightness in gular pouch tissue. Males with higher astaxanthin concentrations had greater red chroma scores and lower red brightness scores. This suggests that astaxanthin plays a role in the production of red color in gular pouch tissue, and that those males with higher plasma astaxanthin concentrations might partition more astaxanthin into gular pouch tissue, thus contributing to darker, redder ornaments. It has been suggested that individuals face a tradeoff between allocation of carotenoids to immune system activities versus deposition of carotenoids into colorful ornaments (Lozano 1994). If carotenoids are limiting, and males are faced with this tradeoff, a negative correlation between plasma carotenoid concentrations and ornamental coloration could be predicted. The fact that we see a positive correlation between plasma astaxanthin concentration and red chroma in gular pouches suggests that carotenoids are not limiting in this population, and that individual males are not facing an allocation tradeoff. This is in accordance with the argument put forth by Hill (1999a) that circulating levels of carotenoids are so high in species with carotenoid-based displays that carotenoids cannot be limiting for immunological function.

In red tissues in other birds, a layer of carotenoid pigment has been found to overlie a UV reflecting layer (Mougeot et al. 2007). There is evidence of such a pattern in great frigatebirds also (McGraw pers comm.). If carotenoids cover over a UV reflecting layer, this would explain the negative associations we found

between plasma astaxanthin concentration and UV brightness, and between red chroma and UV brightness. We found no association between UV reflectance and mating success in great frigatebirds, but this may not be surprising. Although UV cues have been shown to affect mate choice in some birds (Bennett et al. 1996, Johnson et al. 1998), recent molecular evidence suggests that great frigatebirds are among those species that do not have visual sensitivity to UV wavelengths (Wright and Dearborn 2009).

Body condition was higher in males that successfully paired compared with males that did not, though this difference would not be statistically significant if a simultaneous Bonferroni correction was made for multiple comparisons. All other traits measured showed no significant difference between paired and unpaired males. Logistic regression analyses using multiple predictor variables yield the same result. The model best predicting pairing success contained just one variable, body condition. Although nominally significant, this association again was weak, so that only a small amount of the variation in pairing success was explained by this variable.

The size of the gular pouch was not associated with mating success in this population of great frigatebirds. In addition, structural body size was not correlated with gular pouch volume, so females searching for potential mates cannot use pouch volume as a proxy for male body size, a potentially important trait in mate choice. Similarly, body condition was not correlated with gular pouch size. The inflation and maintenance of a gular pouch might require a certain amount of muscular coordination and/or physiological endurance. If so, males in

better physical condition may be better able to produce and maintain larger gular pouches. We found no evidence that this was the case, though our measure of condition (size-controlled mass) was limited in scope.

We found no evidence for female preferences based on gular pouch coloration. None of the color variables (red chroma, red brightness, UV chroma and UV brightness) significantly predicted pairing success. Our results differ from previous studies demonstrating female preferences for soft part coloration in other bird species (Zuk et al. 1990a, 1990b, Holder and Montgomerie 1993, Buckholtz 1995, Rintamäki et al. 2000). However, our results agree with two previous studies that investigated gular pouch color and pairing success in frigatebirds. Madsen et al. (2007a) found no association between gular pouch color and pairing success in the magnificent frigatebird based on colorimeter data. Wright and Dearborn (2009) also found no association between gular pouch color and pairing success in great frigatebirds using color analyses based on Munsell colour chips. Despite our large sample sizes and use of a more objective measure of color, we also failed to find any association between pairing success and gular pouch coloration.

In summary, our results are negative for any association between mating success and gular pouch size and color in great frigatebirds. Results of other studies on mating success and gular pouch color have been similarly negative in great frigatebirds (Wright and Dearborn 2009) and in magnificent frigatebirds (Madsen et al 2007a). Madsen et al. (2004) have suggested that the gular pouch is important in generating male courtship sounds in magnificent frigatebirds, and

properties of these sounds are related to mating success in that species (Madsen et al 2007a). In great frigatebirds, however, the same acoustic traits appear not to be associated with male mating success (Chpt. 4).

If visual display traits such as gular pouch size or color are not correlated with pairing success, how is it that that such a conspicuous ornament has been maintained in frigatebirds? Presentation of the gular pouch is one part of a very complex courtship display in great frigatebirds. The red coloration of gular pouches likely provides a sharp color contrast to the typical displaying substrate. This color contrast would seem to aid in the conspicuousness of displaying males. This visual cue may enhance a male's ability to attract females to his general area, and likewise reduce a female's search time by allowing for a quick determination of which males are attempting to breed. Once near a male, females might assess less obvious cues, such as those indicating MHC genotype. Evidence in our study population of great frigatebirds supports female choice for males whose MHC genotypes differ from their own (Chpt. 5). Therefore, display traits may function to attract females and allow them to quickly assess which males are attempting to breed, while the ultimate decision of which partner to select is made on more subtle cues such as genetic compatibility.

Chapter 3 Tables and Figures

Table 3.1 All variables measured for each of 160 displaying male great frigatebirds. Astaxanthin is reported as plasma concentration in µg/ml, red chroma is percent of the total reflectance (300-700 nm) that occurs in the red spectrum (600-700 nm), red brightness is the average reflectance within the red spectrum (600-700 nm), UV chroma is percent of the total reflectance (300-700 nm) that occurs within the UV spectrum (300-400 nm), UV brightness is the average reflectance within the UV spectrum (300-400 nm), Pouch volume is the inflated gular pouch calculated in cm^3, body size is the first principal component of PCA analysis of wing, ulna and culmen lengths, and body condition is the residuals from a size-adjusted mass regression.

Variable	Mean	Range	±SE
Astaxanthin (µg/ml)	0.63	0.16 – 1.85	0.02
Red Chroma (%)	50.93	41.01 – 60.23	0.26
Red Brightness (%)	14.54	10.53 – 19.07	0.12
UV Chroma (%)	16.92	13.04 – 21.06	0.13
UV Brightness (%)	4.89	2.93 – 7.49	0.08
Pouch Volume (cm^3)	3692.57	1524.30 – 7331.60	93.11
Body Size (PC1)	0.00	-4.34 – 2.39	0.08
Body Condition (residuals)	0.00	-220.10 – 200.65	6.80

Table 3.2 Pearson's r values for correlations of all measured display and fitness variables in male great frigatebirds (N=160 for all variables).

Variable	Body Cond.	Body Size	Pouch Volume	UV Bright.	UV Chroma	Red Bright.	Red Chroma
Astaxanthin	**0.219****	-0.089	**-0.289****	**-0.329****	**-0.327****	**-0.191***	**0.245****
Red Chroma	-0.061	-0.031	**-0.235****	**-0.792****	**-0.837****	-0.106	
Red Bright.	-0.113	-0.016	-0.011	**0.632****	0.148		
UV Chroma	-0.022	0.025	**0.207****	**0.830****			
UV Bright.	-0.060	0.011	**0.164***				
Pouch Vol.	0.067	0.081					
Body Size	0.000						

*Correlation is significant at the 0.05 level (2-tailed)
**Correlation is significant at the the 0.01 level (2-tailed)

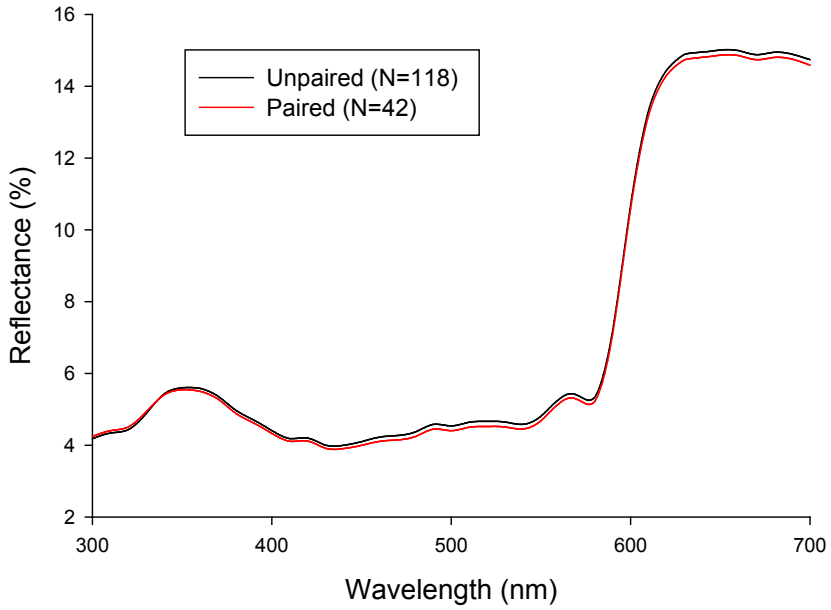

Figure 3.1 Mean spectral reflectance curve of the gular pouch of displaying male great frigatebirds (n=160). Measurements were made by averaging 20 sequential spectra for each of five locations selected arbitrarily along the flat portion of exposed gular tissue below the culmen. Black line represents the mean reflectance curve for unmated males. Red line represents the mean reflectance curve for mated males.

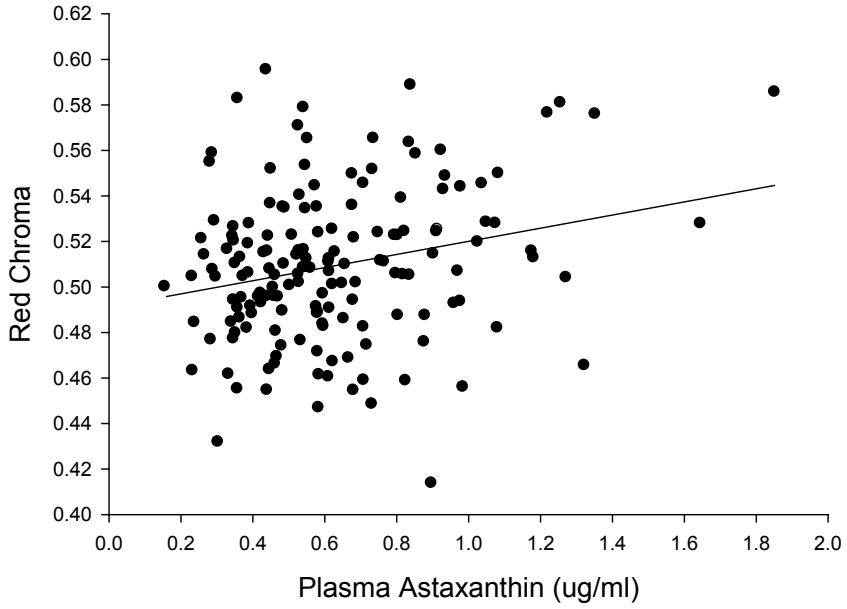

Figure 3.2 Correlation of plasma astaxanthin concentration (ug/ml) and red chroma in gular pouch tissue of displaying male great frigatebirds (r=0.245, N=160).

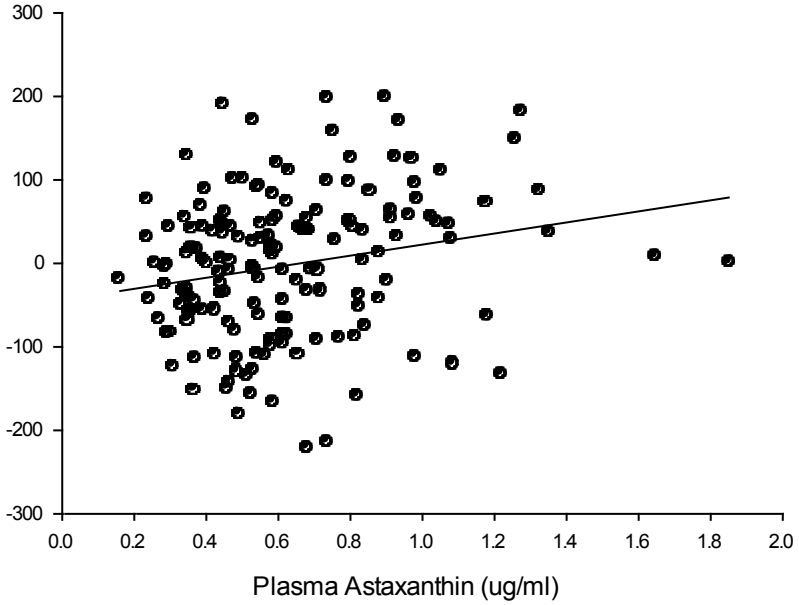

Figure 3.3 Correlation of plasma astaxanthin concentration (ug/ml) and body condition (size-controlled mass) in displaying male great frigatebirds (r=0.219, N=160).

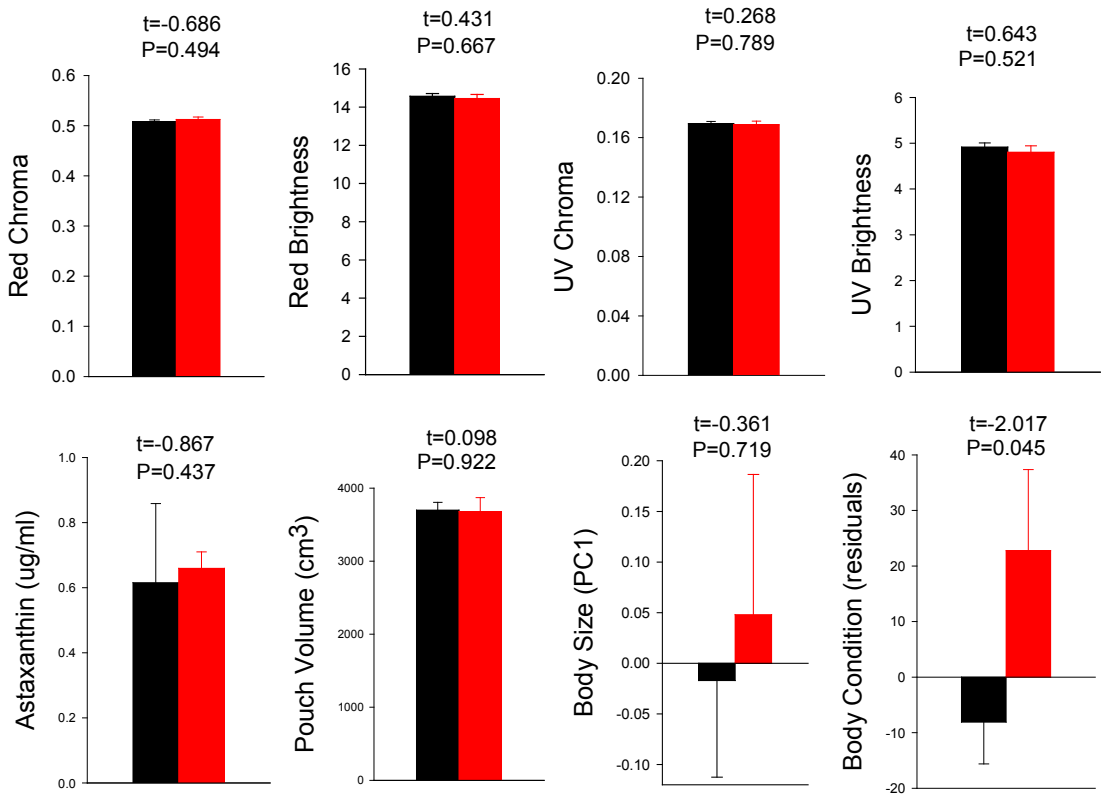

Figure 3.4 T-tests for all measured traits in male great frigatebirds. Results are given as unpaired (black bars; N=118) vs. paired (red bars; N=42). Error bars denote standard error (SE).

Chapter 4

Vocalizations reveal body condition and are associated with visual display traits in great frigatebirds (*Fregata minor*)

Summary

Acoustic displays are known to advertise aspects of male quality and to affect female choice of mates in a variety of birds, including not only songbirds but some seabirds as well. Male great frigatebirds (*Fregata minor*) produce a rapid warble vocalization that forms a prominent part of their courtship display. I investigated the relationships between aspects of this vocalization and male quality and pairing success in 103 great frigatebirds from a population in the northwestern Hawaiian Islands. I found that across males frequency bandwidth of the display was negatively related to repetition rate, enabling us to use deviation from the upper bound regression of bandwidth on rate ('vocal deviation') as one measure of vocal performance. I found that peak frequency was not associated with body size but was positively associated with size of the gular pouch, the most prominent visual ornament. Vocal deviation was significantly associated with body condition but not with pouch size or body size. Neither peak frequency nor vocal deviation significantly predicted pairing success in our study population. These results suggest that vocalizations provide honest information about male body condition and gular pouch size in great frigatebirds, but do not independently influence female choice of mates.

Background

Features of male acoustic display affect female choice of mates in a variety of animals, from acoustic insects to frogs, toads, and songbirds (Searcy and Anderson 1986, Gerhardt and Huber 2002, Catchpole and Slater 2008). Some of these animals mate at night and therefore mostly lack visual ornaments. Others mate during daylight, and some of these possess obvious visual ornaments as well as acoustic displays. The five species of frigatebirds (family Fregatidae) provide a good case in point. Males of all the frigatebird species have a large gular pouch, which assumes a brilliant red color during the mating season. The pouch is inflated to become maximally prominent when a male is actually displaying to a female (Nelson 1975, Dearborn and Ryan 2002, Juola et al. 2008). The red gular pouch is one of the most striking visual displays known for any animal, and has been widely used to illustrate sexual ornaments. Frigatebirds also produce an acoustic display as part of courtship (Nelson 1975), but it is natural to suppose that the visual ornament has the more important effects on mate choice. Surprisingly, existing evidence counters this expectation. In the great frigatebird (*Fregata minor*), Wright and Dearborn (2009) found no relationship between gular pouch hue, value, or chroma and male mating success. Juola (Chpt. 3) also found that mating success was not related to pouch color in this species, nor to pouch size. Similarly, Madsen et al. (2007a) found no relationship between pouch color and mating success in the magnificent frigatebird (*Fregata magnificens*). Body size and other morphological measurements were also poor predictors of mating success in

both of these species (Madsen et al. 2007, Wright and Dearborn 2009). Instead, Madsen et al. (2007a) found that in the magnificent frigatebird only various properties of the male acoustic display differed systematically between successful and unsuccessful males, suggesting that the acoustic display is more important to mate choice than is any visual trait in this species. Here we test the importance of acoustic traits to mate choice in the second frigatebird species in which visual traits have thus far proven unimportant, the great frigatebird.

One acoustic feature whose effect on mate choice is often analyzed is frequency, either fundamental frequency – the frequency of the lowest harmonic in a harmonic series – or peak frequency – the frequency of maximum amplitude. In some animals, females prefer acoustic displays of lower frequency, for example in some frogs (Ryan 1980, Tarano and Harrera 2003, Poole et al. 2007) and insects (Brown et al. 1996). Such preferences are sometimes attributed to an underlying preference for larger males, since frequency is often negatively related to size in these taxa (e.g. Oldham and Gerhardt 1975, Ryan 1980, Brown et al. 1996, Bee et al. 1999). In birds, however, size and frequency are often only weakly correlated within species (Logue et al. 2007, Patel et al. 2010) or not correlated at all (Cardoso et al. 2008). Frequency is one of the characters that Madsen et al. (2007a) found to differ between mated and unmated males in the magnificant frigatebird. The acoustic display of magnificant frigatebirds is termed 'drumming,' and consists of a series of repetitions of a harmonic stack, which Madsen et al. (2007a) suggest is produced by a bill clack. These authors measured fundamental frequency as the peak frequency of the lowest harmonic

in the stack, and found that mated males had significantly lower fundamentals than unmated males. Madsen et al. (2004) found that the fundamental frequency of drumming was negatively correlated with size of the gular pouch but positively correlated with body size; note that the latter correlation is opposite to the usual result.

The acoustic display used by great frigatebirds during mate attraction is very different from that of magnificent frigatebirds: it appears to be produced vocally rather than by a bill clack and does not possess multiple harmonics, instead consisting of a single frequency band that fluctuates sinusoidally (Figure 1). The vocalization has been described as a “warble” (Nelson 1975) and rendered phonetically as a repeated “whoo-hoo-ooo” (Metz and Schreiber 2002). Because this warble lacks harmonics, we measured its frequency as peak frequency rather than fundamental frequency.

Madsen et al. (2007a) measured in magnificent frigatebirds two additional attributes of drumming that were independent of fundamental frequency: the interval between harmonic bands and the coefficient of variation of the time interval between successive syllables. Both these traits also differed significantly between mated and unmated males. No analog of band interval is present in great frigatebird warbles, but we were able to measure an analog of the second measure: the coefficient of variation of the time interval between successive peaks in the warble. These variation measures can be considered to assess the consistency of performance of the display, with low variation being higher performance. Madsen et al. (2007a) indeed found that mated males had lower

variance. Consistency in vocal song performance has also proven to correlate with female preferences in one songbird species (Byers 2007) and with age and dominance in others (Botero et al. 2009, Rivera-Gutierrez et al. 2010).

We also employed another measure of vocal performance in great frigatebirds: vocal deviation (Podos 1997, 2001). Vocal deviation measures the deviation of a vocalization from an upper performance limit set by a tradeoff between frequency bandwidth and repetition rate. To produce a sound of high frequency a bird opens its bill widely, thus shortening its vocal tract and raising its resonant frequency; conversely, to produce a sound of low frequency a bird narrows its bill gape, thus lengthening the vocal tract and lowering its resonant frequency (Nowicki 1987, Westneat et al. 1993, Podos et al. 2004). Producing a warble or trill with a wide frequency bandwidth requires opening and closing the bill over a wide angle. Because birds are limited in how quickly they can move their bills, a tradeoff is thus produced between warble or trill speed and bandwidth (Podos 1997). The performance limit can be estimated using an upper bound regression, and the performance level of a given vocalization can be estimated by the deviation from this limit (Podos 2001, Ballentine et al. 2004). The predicted tradeoff has been demonstrated across species of New World sparrows (Podos 1997) and within a number of species of songbirds (Ballentine et al. 2004, Cramer and Price 2007, Illes et al. 2006). The tradeoff has also been found in the vocalizations of another seabird, the brown skua (*Catharacta antarctica*); in this species deviation from the performance limit predicted male reproductive success (Janicke et al. 2008).

Here we test for a tradeoff between frequency bandwidth and repetition rate in the warble vocalization of great frigatebirds. We then assess three attributes of the warble in male great frigatebirds: dominant frequency, consistency of performance, and vocal deviation. We relate these vocal attributes to two measures of male quality: body size and condition. Finally, we compare vocal attributes in males that succeeded in pairing and males that failed.

Materials and Methods

The study was carried out on Tern Island, French Frigate Shoals, in the northwestern Hawaiian Islands during the period February-June, 2007. Approximately 2000 breeding attempts by great frigatebirds are made on the island per year. The operational sex ratio is heavily skewed towards males, with approximately five times as many males present as females (Dearborn et al. 2001), so that only about one in five males succeeds in pairing with a female and initiating a clutch (Dearborn and Anders 2006). The breeding skew may be due in part to females skipping breeding in the year after a breeding success, while males attempt to breed every year. During the mating period, males display from the tops of shrubs to females flying overheard. Displaying males give their warble vocalization while exhibiting their inflated gular pouch and extending and trembling their wings (Nelson 1975). An interested female lands on the shrub for closer inspection of the male, and then either leaves or, eventually, nests there.

The mating system is both socially and genetically monogamous: only about 1-8% of fertilizations are extra-pair (Dearborn et al. 2001, Chpt. 5).

We recorded the warbles of displaying male frigatebirds using a Marantz recorder and a directional AudioTechnica microphone mounted on a tripod and set at a distance of 2-4 meters directly in front of the displaying male. A total of 821 vocalizations were recorded from 103 males. After recording a male, we photographed him from both the front and the side at a distance of approximately 2 meters. Once photographed, each male was captured and measured for body mass, bill width, and bill, ulna, culmen and wing length. All measurements were made by the same researcher (F. Juola). All individuals were then affixed with leg bands and wing tags so that individual behavior could be monitored for the remainder of the breeding season.

Vocal recordings were later digitized and analyzed using the sound analysis programs Syrinx (John Burt, www.syrinxpc.com), Raven (v1.2.1) and Signal (v4.0). Minimum and maximum frequencies were measured by fitting an onscreen cursor to the peaks and troughs of the spectrogram trace (Figure 4.1). Warble rate was estimated by measuring the time between the first and last trough and dividing by the number of peaks in between (Figure 4.1). Mean frequency bandwidth and mean warble rate were calculated for each individual, and from these means the upper bound regression of frequency bandwidth on warble rate was calculated as described by Podos (1997), using bins of 0.5 warbles/sec (Figure 4.2). Vocal deviation was measured as the orthogonal distance of each individual's mean vocal output from the upper-bound regression

(Podos 2001, Ballentine et al. 2004). Peak frequency, defined as the frequency within a call at which the most power was produced, was determined from analysis of power spectra. Using on-screen cursors, the time intervals between each successive peak in a warble were measured, and from these intervals a coefficient of variation was calculated for that vocalization. A mean coefficient of variation was then calculated for each male by averaging the coefficients of variation for each of that male's recorded vocalizations. We term this measure of the variation of timing "vocal consistency."

Structural body size was determined using a principal components analysis based on measurements of the wing, ulna, and culmen for each displaying male. Inflated gular pouch size estimates were calculated from digital photographs using the program Image J (v1.32j). Calculations were based on known bill lengths and bill widths for each bird. Specifically, gular pouch area was calculated first, from side photographs with bill length used as a reference. Pouch volume was then calculated by multiplying pouch area by the pouch width. Width was the widest portion on the pouch determined from frontal photographs using bill width as a reference. Body condition was determined using the residuals from a regression of body mass and structural body size. Finally, a male was considered to have successfully paired once a pair bond had formed and the pair had commenced constructing a nest.

Linear regression was used to investigate how well male quality (body size, gular pouch size and body condition) predicted the quality of vocal output (peak frequency and vocal performance). To determine the predictive value of

peak frequency, vocal consistency, and vocal deviation for pairing success, we used logistic regression analyses with vocal output measures as the independent (predictive) variables vs. successful pairing or non-successful pairing as the binary dependent variables.

Results

Of the 103 individual displaying males whose vocalizations were recorded, 27 later successfully paired, while 76 were unsuccessful in their attempts to secure a mate. This success rate is typical for the population (Dearborn and Anders 2006), and thus does not represent a negative effect of our measurements and observations on pairing success.

Frequency bandwidth of the warble was significantly, negatively associated with warble rate (Figure 4.2). The upper bound regression of warble rate on frequency bandwidth was also significant ($y = -17.1$, $x = 728.4$, $r^2 = 0.344$, $p = 0.045$, $n = 12$; Figure 4.2), enabling us to measure vocal deviation as the orthogonal deviation of a male's warbles from this regression line.

When we correlated measures of male quality with vocal traits, two significant correlations were found (Table 4.1). Peak frequency was significantly negatively associated with gular pouch size (Figure 4.3), meaning that peak frequency was lower in males with larger gular pouches. Peak frequency was not associated with body size or body condition. Vocal deviation was significantly negatively correlated with body condition (Figure 4.4), meaning that males with low vocal deviation and thus better vocal performance were in better condition.

Vocal deviation was not associated with body size or gular pouch size. Coefficient of variation in the consistency between successive warble peaks was not associated with body size, body condition, or gular pouch size.

Peak frequency was not a significant predictor of pairing success in this population (logistic regression: N = 103, Wald χ^2 = 0.165, df = 1, P = 0.684). Vocal deviation was also not a predictor of pairing success (Wald χ^2= 2.945, df = 1, P = 0.086). Finally, coefficient of variation was also not a predictor of pairing success (Wald χ^2= 0.233, df = 1, P = 0.629).

Discussion

We found an association between peak frequency and size of the gular pouch in great frigatebirds; the association was in the expected direction in that larger pouches were associated with lower frequencies. The association was a weak one, however, with only 6-7% of the variation in pouch size explained by variation in peak frequency. Madsen et al. (2004) found a much stronger negative relationship between peak frequency and pouch size in magnificent frigatebirds, and suggested that the relationship occurs because the pouch acts as a resonance chamber for the drumming display. Longer wavelengths would resonate in larger pouches, producing a negative relationship between frequency of the acoustic display and pouch size. The vocal display of great frigatebirds is much different from that of magnificent frigatebirds, in that it is produced vocally rather than by a bill clack. Thus it is not surprising that the relationship between pouch size and vocal attributes is somewhat different in the two species.

Madsen et al. (2007a) found that mated males had lower peak frequencies than unmated males in magnificent frigatebirds. In other animals, an association between frequency and mating success is often explained as due to a female preference for larger males, but in magnificent frigatebirds peak frequency is not related to body size. An alternative explanation for the relationship between peak frequency and mating success in magnificent frigatebirds is that females prefer males with large gular pouches, perhaps choosing males directly on pouch size and thus indirectly creating the correlation of mating success and frequency. In great frigatebirds, however, there is no association of gular pouch size with mating success (Chpt. 3), so this indirect mechanism would not act to produce an association of frequency with mating success, and we in fact found no such association.

Variation in time between successive warble peaks was not a good predictor of pairing success in our population. This finding differs from a similar assessment of acoustic consistency in magnificent frigatebirds. Madsen et al. (2007a) found that coefficient of variation of the time interval between successive syllables differed significantly between mated and unmated males, with mated males having lower variance. These measures of variation are considered to be assessments of the consistency of performance of the acoustic display, with low variation being higher performance. Although this has been demonstrated in magnificent frigatebirds, and previously in a songbird (Byers 2007) we did not find this to be the case in our population of great frigatebirds.

Great frigatebirds showed the negative relationship between frequency bandwidth and warble repetition rate that Podos (1997, 2001) predicted for songs consisting of trilled syllables in songbirds. The great frigatebird's vocalization is not in fact a trill, in that it does not consist of repetitions of a syllable with silent gaps between syllables. The great frigatebirds's warble does, however, contain a series of repeated frequency upsweeps and downsweeps. If a frigatebird has to open its bill during every upsweep and close its bill during every downsweep, then the same biomechanical limitations that produce a tradeoff between bandwidth and repetition rate in the trilled songs of sparrows should act on the frigatebird's warble also. Evidence for this tradeoff has previously been found in the acoustic display of another seabird, the brown skua (Janicke et al. 2008), even though the syllables in this call are not frequency-modulated (Cardoso 2008). Thus the biomechanical basis for the tradeoff is easier to understand in great frigatebirds than in brown skuas.

We found that vocal deviation was significantly associated with body condition, in the direction that would be expected: lower vocal deviation, which equates with higher vocal performance, was associated with better body condition. Again, however, the association was relatively weak, so that a female would not actually learn that much about a male's body condition by judging his vocal deviation. Vocal deviation has been found to be associated with pairing success in one songbird, swamp sparrows (*Melospiza georgiana*) (Ballentine et al. 2004). We, however, found no association of vocal deviation with pairing success in great frigatebirds.

Male frigatebirds in general have highly developed display characteristics, including both visual and vocal displays. Because operational sex ratios are highly male biased in frigatebird populations (Madsen et al. 2007a, Dearborn et al. 2001), we would expect strong sexual selection acting on males, and strong sexual selection can explain the evolution of the various male display traits. This hypothesis predicts differences in display traits between males that are successful and those that are unsuccessful in mating. Tests of this prediction with the magnificent frigatebirds have been negative with respect to the main visual display, the gular pouch, but positive with respect to the main acoustic display, drumming (Madsen et al. 2007a). Tests of this prediction with the great frigatebird, however, have been negative with respect to the gular pouch (Wright and Dearborn 2009, Chpt. 3), and now are negative with respect to acoustic traits also (this Chpt.). A third aspect of display is still available as a possible explanation for differences in male pairing success: performance of the wing extension and wing tremble that are performed along with gular pouch display and warbling by courting male great frigatebirds. Performance traits of this type have recently been argued to have a large effect on female choice of mates in a variety of animal groups (Byers et al. 2010).

Chapter 4 Tables and Figures

Table 4.1 Relationships between individual traits (body size, gular pouch size, and body condition) and peak frequency (the mean peak frequency of each individual's calls), vocal deviation (the mean orthogonal distance from the upper-bound regression line of warble rate vs. bandwidth plots), and coefficient of variation (the mean coefficient of variation in time between successive warbles within all calls). N = 103 males in each case, *denotes significance (less than 0.05).

Trait		Peak Frequency	Vocal Deviation	Coef. Of Variation
Body Size	R^2	0.021	0.016	0.015
	F	2.20	1.656	1.562
	P	0.141	0.201	0.214
Pouch Size	R^2	0.066	0.004	<0.001
	F	7.039	0.359	0.021
	P	**0.009***	0.550	0.885
Body Condition	R^2	<0.001	0.040	0.037
	F	0.014	4.197	3.889
	P	0.907	**0.043***	0.051

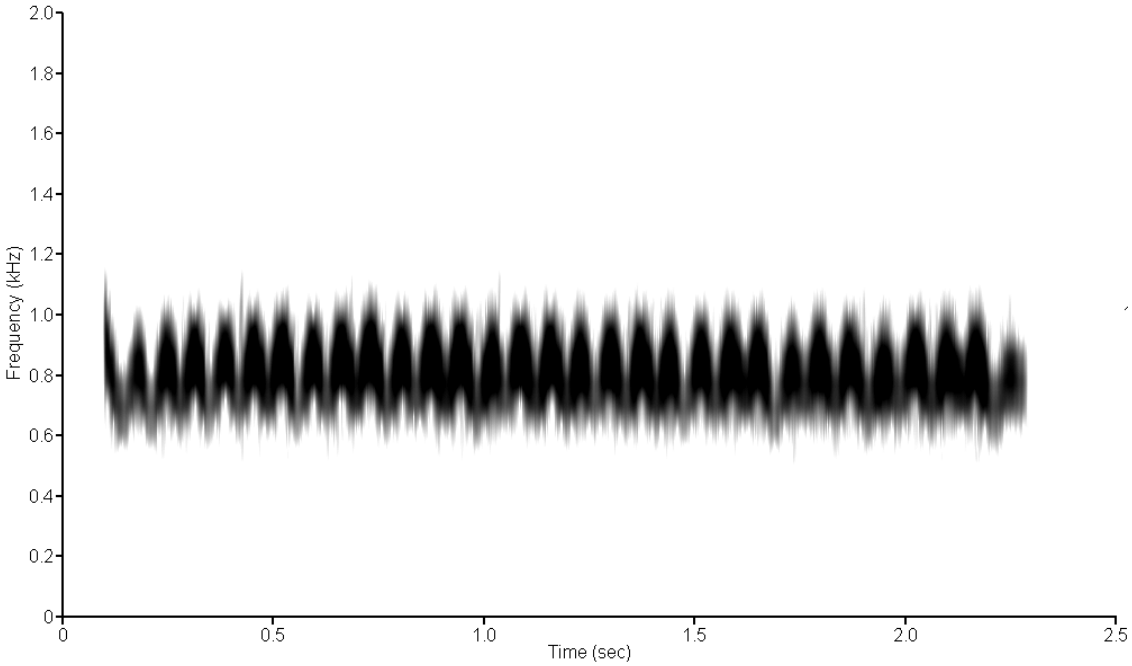

Figure 4.1 Visual representation of male display vocalization. Spectrogram with time (x-axis) and KHz (y-axis) used to calculate warble speed and frequency bandwidth.

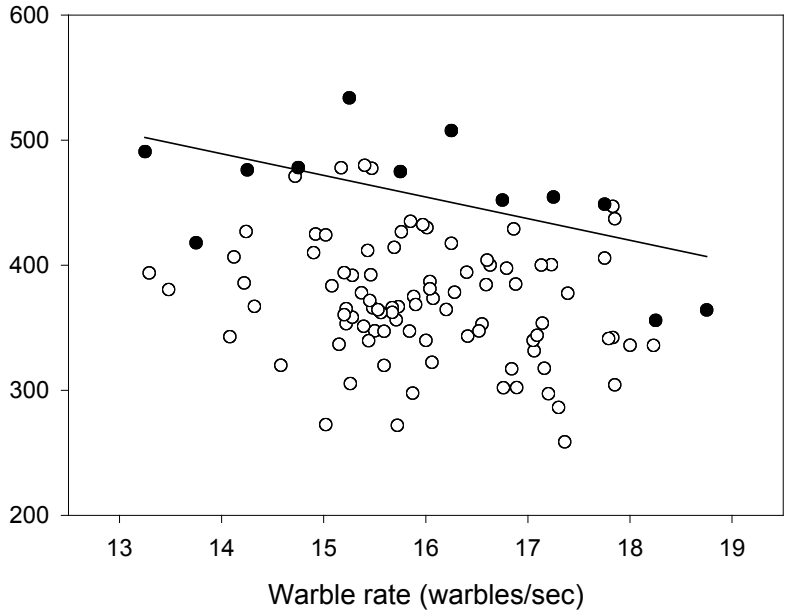

Figure 4.2 Mean warble rate by frequency bandwidth plot and upper-bound regression line for 103 individuals (means based on 821 individual calls). Dark circles represent the points used to calculate the upper-bound regression.

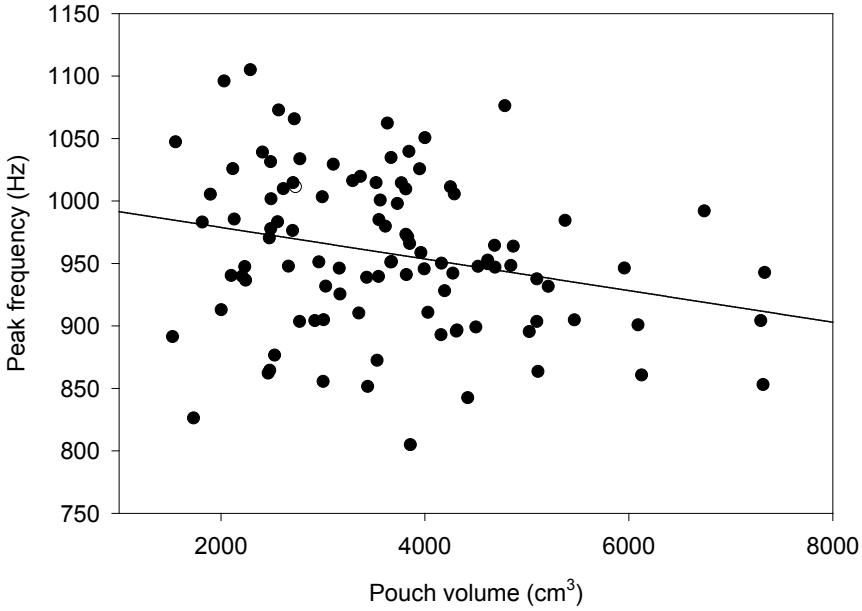

Figure 4.3 Pouch volume was a significant predictor of peak frequency. Males with larger pouches produced vocalizations with lower peak frequencies.

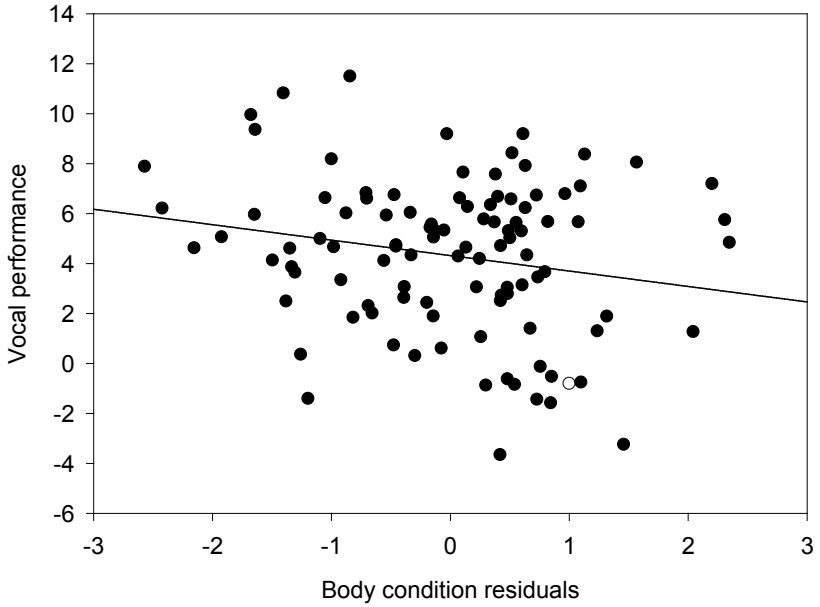

Figure 4.4 Body condition was a significant predictor of vocal deviation. Males in better condition produced higher quality vocalizations.

Chapter 5

Sequence-based evidence for MHC-disassortative mating in a colonial seabird

Summary

The major histocompatibility complex (MHC) is a highly polymorphic multi-gene family associated with immune defense and has been proposed to play a role in mate choice. Under the genetic compatibility hypothesis, females choose mates that differ genetically from their own MHC genotypes, avoiding inbreeding and/or enhancing immunocompetence of their offspring. We tested the prediction of disassortative mating based on MHC genotypes in a population of great frigatebirds (*Fregata minor*), by sequencing the second exon of the MHC class II ß gene. We compared MHC amino acid differences of 46 mated pairs to that of 10,000 simulations of 46 random pairings using the same individuals. In addition, we repeated this analysis using a similarity index from a set of neutral markers (12 microsatellite loci) to distinguish between MHC-based mate choice and mate choice based on overall genetic similarity. Analysis showed significant disassortative mating amongst mated pairs compared to random pairings based on MHC genotypes, but not for neutral microsatellite markers. This result suggests a role for MHC genes in mate choice in this population of great frigatebirds, and in particular supports the hypothesis that females choose mates that differ genetically from themselves at MHC loci.

Background

A fundamental goal in evolutionary biology is to gain a better understanding of forces that create and maintain genetic variation in natural populations. When natural selection is consistently directional, it can eliminate genetic variation over time. However, heterozygote advantage is one situation in which genetic variation can be maintained, rather than eliminated, by natural selection (Takahata and Nei 1990, MacDougall-Shackleton et al. 2005). Mounting evidence suggests that evolution at the major histocompatibility complex (MHC) might emerge as a taxonomically broad example of selection acting to maintain genetic diversity, through heterozygote advantage and other processes (Hedrick 2002), though empirical data are primarily from mammals and fishes (Bodmer 1972, Potts and Wakeland 1990, Hughes and Hughes 1995, Lie et al. 2009). The major histocompatibility complex is a highly polymorphic gene assemblage that plays a critical role in the development and activation of both the T-cell mediated and humoral arms of immune defense (Klein 1986, Penn and Potts 1999). Heterozygous individuals possess a wider array of MHC alleles and thus may be able to mount more efficient defenses against a greater diversity of pathogens compared with more homozygous individuals.

Because of the fitness variation among MHC genotypes (Paterson et al. 1998, Carrington et al. 1999, Senseney et al. 2000, Kurtz et al. 2004, Madsen and Ujvari 2006, Fernandez-de-Mera et al. 2009), and because MHC genotypes might be detectable by olfaction (Boehm and Zufall 2006), MHC could be an important target of mate choice (Yamazaki et al. 1976, Zelano and Edwards

2002). One potentially important model suggests that individuals will choose mates whose genotypes are different from their own (Trivers 1972, Zeh and Zeh 1996, Tregenza and Wedell 2000), resulting in offspring that are more likely to be heterozygous at targeted loci such as MHC. Studies from mammals and fishes provide evidence for such MHC-disassortative mating (Yamazaki et al. 1976, Potts et al. 1991, Ober et al. 1997, Penn and Potts 1999, Landry et al. 2001, Forsberg et al. 2007, Consuegra and de Leaniz 2008, Setchell et al. 2010, but see Hedrick and Black 1997, Paterson and Pemberton 1997), and we would expect to see similar patterns in other taxa such as birds. To date, however, tests of this idea in birds have been limited in scope (Freeman-Gallant et al. 2003, Ekblom et al. 2004, Westerdahl 2004, Richardson et al. 2005, Bonneaud et al. 2006), with no clear evidence of disassortative mating based on MHC. Part of the difficulty has been methological: taxonomic diversity in MHC research is constrained by a lack of locus-specific primers for non-mammalian MHC genes. This is problematic because MHC gene complexes exhibit a high degree of gene duplication and concerted evolution (Edwards and Hedrick 1998). Consequently, previous studies of MHC-based mate choice in birds have used indirect measures such as restriction fragment length polymorphisms or denaturing gradient gel electrophoresis to quantify genotypes and estimate polymorphism (Freeman-Gallant et al. 2003, Ekblom et al. 2004, Westerdahl 2004, Richardson et al. 2005, Bonneaud et al. 2006).

In this study, we developed primers and sequenced the second exon of the MHC class II ß gene, which codes for the most polymorphic segment of the

peptide-binding region (Alcaide et al. 2007). Using this approach, we tested whether great frigatebirds (*Fregata minor*) choose mates with complementary MHC genotypes. Great frigatebirds are colonial-breeding seabirds. In our study population and probably elsewhere, there is serial monogamy and a male-biased operational sex ratio, such that females have many options as they choose mates for each breeding attempt (Nelson 1975, Dearborn et al. 2001, Dearborn and Anders 2006). The frequency of extra-pair paternity is low, such that choice of social mate and genetic mate are the same for most pairs (Dearborn et al. 2001).

Importantly, dissimilar MHC genotypes in mates could arise from general inbreeding avoidance, rather than from mate choice based on MHC complementarity alone. Few studies have been able to make this distinction (Landry et al. 2001, Neff et al. 2008, Miller et al. 2009, Setchell et al. 2010). In the case of general inbreeding avoidance, mates should show dissimilar genotypes not only at MHC but also at a variety of loci across the genome. Alternatively, if MHC is an important target of mate choice per se, we would expect mates to have dissimilar genotypes at MHC but not at neutral genomic markers. We combined MHC sequencing with microsatellite genotyping of mated pairs to test whether great frigatebirds choose mates with complementary MHC genotypes. Disassortative mating at both MHC and microsatellite loci would suggest a genome-wide pattern of outbreeding. Disassortative mating at MHC but random mating at microsatellite loci would suggest, instead, that MHC dissimilarity is the actual target of mate choice.

Materials and Methods

Study System

We studied great frigatebirds on Tern Island, in the Northwestern Hawaiian Islands (23°45'N, 166°17'W). Tern Island encompasses 14 ha and is one of 10 small islands in the French Frigate Shoals atoll. Approximately 4,000 great frigatebirds nest on Tern Island each year (Dearborn and Anders 2006). Egg laying typically commences in February and lasts through May. In 2007, we monitored the breeding activities of 46 pairs. All 92 of these individuals were captured by hand while at their nests, and approximately 50ul of blood was collected from their brachial vein. Blood samples were also collected from the single offspring of each of these 46 breeding pairs. Blood samples were stored in 1 ml of Longmire's lysis buffer at room temperature for the remainder of the field season.

Genetic Analyses

We digested all blood samples using proteinase K. Genomic DNA was extracted using a standard alcohol precipitation protocol. In order to develop primers that would amplify our locus of interest, the second exon of MHC class II ß gene in great frigatebirds, we initially used a primer pair designed to amplify a large portion of this gene region in birds. Using PCR, we successfully amplified an approximately 1,500 bp sequence of the MHC class II ß gene encompassing part of exon 1, all of exon II and part of exon III. Using this sequence, we designed a new primer pair flanking exon II (481F-CACACTGCCAGTCCTACCG and 1038R-

AGGGACTCGTGTCCTCATGG). Sequences amplified with this new primer pair were 557 bp in length including the primer sequences. For all 92 adult birds, each individual DNA sample was PCR amplified 3 separate times (10 ul reactions each) to limit PCR artifacts, using the proof-reading enzyme Pfx (Platinum Pfx, Invitrogen). After PCR, all three amplification products from an individual were combined and gel purified using a 2.5% agarose gel and gel purification kit (Wizard SV, Promega Corp.). After gel purification, DNA was transformed and cloned using a haploid cloning vector and high efficiency cloning cells (Clone Jet, Fermentas; JM109 competent cells, Promega Corp.). This separate cloning of PCR products allowed for precise determination of sequences from all heterozygous individuals. Cloning products were then grown up for 24 hours on standard LB/amp plates. Colonies produced from these plates were then used as template DNA for PCR screening. All clones were PCR and gel screened (primers 481F/1038R, 1.5% agarose gels) to insure the presence of the proper insert (exon II). After gel screening, PCR products were cleaned and sequenced using the BigDye Terminator Cycle sequencing kit (v. 3.1) and a Genetic Analyzer (Applied Biosystems, Inc., 3130x). For every individual, 24 – 28 positive clones were selected at random for sequencing.

Sequence Screening

All sequences were aligned, edited and analyzed using BioEdit v 7.0.0 (Ibis Therapeutics) and MEGA4 (Tamura et al. 2007). MHC sequences occurring just once within an individual and differing by less than 3 bp from any other sequence

found from that same individual were considered artifacts of PCR error and were removed from further analyses (Edwards 1995, Alcaide et al. 2007). In addition, since the recombination of cloned PCR products can result in chimeras (Bradley and Hillis 1996) all alleles were compared with direct sequences of uncloned PCR products to check for similarity of polymorphic sites (Alcaide et al. 2007). Sequences that did not match polymorphic sites of their respective uncloned sequences were also removed from further analyses.

Microsatellites

We used microsatellite genotypes to obtain an overall index of genetic similarity between mates (calculated as Wang's r; Wang 2002). Because choice of genetic mate and social mate are decoupled in some species, we genotyped nestlings at these same microsatellite loci and conducted a paternity exclusion analysis with Cervus 3.0 (Kalinowski et al. 2007).We genotyped all 92 adults and 46 offspring at 12 di- or tetra-nucleotide microsatellite loci previously developed for this population (Dearborn et al. 2008). We used three 10-µl multiplex PCR reactions of four loci each, with slightly different recipes for the three reactions. For loci Fmin04, Fmin06, Fmin13, and Fmin15, we used 1x GeneAmp Gold buffer (Applied Biosystems, Inc.), 0.2mM each dNTP, 1.5 mM MgCl2, 0.5 µM of forward and reverse primers for Fmin13, 0.3 µM of forward and reverse primers for each of the other three loci (Fmin04, Fmin06, and Fmin15), 0.4 U AmpliTaq Gold polymerase (Applied Biosystems, Inc.), and 10 ng DNA. Cycle parameters were

95o for 7 min; 35 cycles of 95o for 30 sec, 53o for 3 min, 70o for 30 sec; and 70o for 15 min.

For loci Fmin01, Fmin03, Fmin11, and Fmin18, we used 1x GeneAmp Gold buffer (Applied Biosystems, Inc.), 0.2mM each dNTP, 2.0 mM MgCl2, 0.2 μM of forward and reverse primers for Fmin11, 0.4 μM of forward and reverse primers for each of the other three loci (Fmin01, Fmin03, and Fmin18), 7.5 μM bovine serum albumin, 0.5 U AmpliTaq Gold polymerase (Applied Biosystems, Inc.), and 10 ng DNA. Cycle parameters were 95o for 7 min; 35 cycles of 95o for 30 sec, 58o for 30 sec, 70o for 3 min; and 70o for 15 min. For loci Fmin02, Fmin10, Fmin14, and Fmin17, we used 1x GeneAmp Gold buffer (Applied Biosystems, Inc.), 0.2mM each dNTP, 2.0 mM MgCl2, 0.3 μM of forward and reverse primers for each locus, 7.5 μM bovine serum albumin, 0.5 U AmpliTaq Gold polymerase (Applied Biosystems, Inc.), and 10 ng DNA. Cycle parameters were 95o for 7 min; 35 cycles of 95o for 30 sec, 56o for 30 sec, 70o for 3 min; and 70o for 15 min. Dye-labeled products were run on an ABI PRISM 3730XL, with manual verification of allele calls in GeneMapper 4.0 (Applied Biosystems, Inc.). To estimate the per-allele error rates of microsatellite genotyping, we blindly repeat-genotyped a large sample of individuals (Hoffman and Amos 2005).

Data Analyses

MHC sequences were connected in a parsimony-based haplotype network using TCS (Clement et al. 2000). For all analyses of selection and mate choice, MHC

sequences were converted from nucleotide to amino acid sequences prior to provide for a more meaningful comparison of functional differences between individuals. The modified Nei-Gojobori test was used to determine whether all unique exon II ß gene amino acid sequences were under positive selection. We investigated whether mated pairs were less similar at the MHC exon II ß gene than would be expected with random mating in several ways, all based on amino acid sequences. First, we calculated the allele sharing percentages of known pairs using a standard formula, where the proportion of alleles shared within a pair is twice the number of alleles shared by the pair members divided by the sum of the total number of alleles for each individual [$D=2F_{ab}/(F_a+F_b)$] (Wetton et al. 1987, Bonneaud et al. 2006). We then compared the mean allele sharing value of these 46 known pairs to the mean allele sharing values generated from 10,000 simulations of 46 random male-female pairings selected from the same 92 individuals. Because this first analysis of allele sharing does not consider the degree to which allele sequences differ from one other, we also compared the sum of all pairwise amino acid differences between known pairs. The mean of the sum of all pairwise amino acid difference of these 46 known pairs was then compared to the mean of the sum of all pairwise amino acid difference generated from 10 000 simulations of 46 random pairings selected from the same 92 individuals, just as with the allele sharing analysis (Landry 2001). Lastly, to test for disassortative mating at non-MHC loci, we used the average relatedness for mated pairs (Wang's r) based on the 12 microsatellite loci. The mean Wang's r was calculated for all 46 known pairs and was then compared to the mean

Wang's r generated from 10,000 simulations of 46 random pairings selected from the same 92 individuals. For all simulations of randomly assigned pairs, one-tailed tests were used to determine significance between known pairs and random pairs because our predicted outcomes were unidirectional.

Results

MHC

Genotypes based on sequenced clones of the second exon of the class II ß gene were determined for all 92 adults (46 pairs). Multiple clones were sequenced for every individual: after removal of erroneous sequences (PCR error and chimeras), a total of 2,025 sequenced clones remained (range 17-26 sequenced clones per individual, mean = 22.01 ± 0.20 SE). Amongst the 92 individuals, 44 unique exon II ß gene nucleotide sequences were isolated. Nucleotide sequences differed by 1-36 bp, with a large number of moderate-frequency haplotypes (Fig. 5.1). All remaining results are based on conversion to amino acid sequences in order to make more meaningful comparisons using functional units. There were a total of 39 unique exon II ß gene amino acid sequences amongst the 92 individuals. Sequences differed by 1-23 amino acids. Each individual possessed between 2-4 unique alleles (2 alleles in 15 individuals (16%), 3 alleles in 36 individuals (39%), 4 alleles in 41 individuals (45%)). This pattern indicated that 2 loci were being amplified. There were no signs of pseudogenes amongst the unique sequences (no stop codons or frame shift

mutations) and the sequences were consistent with expressed loci subject to positive selection (modified Nei-Gojobori Z = 2.33, P = 0.011).

Microsatellites

We successfully genotyped 89% of individuals at all 12 loci; the remaining individuals were genotyped at 11 loci. Based on blind repeat PCR and repeat sizing of 1,236 single-locus genotypes, the genotyping error rate was 0.00202 per allele. Wang's r for genetic similarity between mated pairs ranged from -0.398 to 0.519 (n = 46 pairs). Extra-pair fertilization is a rare phenomenon in this population (Dearborn et al. 2001), but paternity exclusion analysis in this sample of 46 single-chick families identified four likely cases of extra-pair paternity, with the four offspring mismatching their social fathers at 3, 3, 4, and 6 loci, respectively. Assuming that the female is the genetic parent of the offspring (0 mismatches; and see Dearborn et al. 2001), the LOD score for a parent-offspring relationship between the social father and the offspring was < -5.5 for these four pairs, versus LOD scores ranging from +2.6 to 17.1 among the remaining 42 pairs.

Disassortative mating

To determine whether pairs mated disassortatively with respect to their MHC exon II sequences, we compared MHC amino acid sequences of mated pairs based on allele identity and based on allele similarity. First, we compared sharing of identical alleles between mated pairs (n=53) with 10,000 simulations of

randomly assigned pairs. There was no difference in allele sharing between mated pairs and randomly assigned pairs (allele sharing$_{mated}$=0.364 ± 0.03 SE, allele sharing$_{random}$=0.360 ± 0.00 SE, p=0.546; Fig. 5.2). We then compared the sum of all pairwise amino acid sequence differences between mated pairs to that of 10,000 simulations of randomly assigned pairs. Mated pairs had significantly greater pairwise amino acid differences compared to the randomly assigned pairs (AAdistance$_{mated}$=130.85 ± 8.02 SE, AAdistance$_{random}$=126.31 ± 0.02 SE, p=0.018; Fig. 5.3). To test whether MHC itself is a target of mate choice, we also compared mates' genotypes at 12 microsatellite loci. There was no difference in microsatellite distance between mated pairs and 10,000 randomly assigned pairs (Wang's r_{mated}=-0.021 ± 0.029 SE, Wang's r_{random}=-0.008 ± 0.0002 SE, p=0.276; Fig. 5.4).

Discussion

Our data on MHC amino acid sequences and microsatellite genotypes suggest that mate choice in this system favors disassortative amino acid sequences at MHC loci, in a manner that is not consistent with simple inbreeding avoidance. This pattern is unusual in general, and in particular has not previously been shown in birds.

Specifically, amino acid sequences from two loci associated with the 2nd exon of the class II ß gene indicated that dissimilarity influenced female mate choice in this population of great frigatebirds. Disassortative mating was not evident when we considered only the proportion of amino acid sequences shared

between pair members, without consideration of how different these sequences were from one another. Once the magnitude of the sequence differences was considered, by comparing the sum of all pairwise amino acid differences, actual pairs were significantly more disassortative in their mating compared with the average similarity based on 10,000 simulations of randomly assigned pairs. In other words, mates did not differ from random in the number of alleles that they shared; but among the alleles that mates did not share, the dissimilarity between those alleles was greater than would be expected by chance. This result highlights the importance of considering the actual amino acid sequences, and critically, how different these are from one another, rather than simply making a comparison based only on the proportion of shared nucleotide haplotypes.

Our data from microsatellite loci allowed us to consider whether disassortative mating based on functional MHC genes was more likely to be driven by generalized inbreeding avoidance (Jordan and Bruford 1998) or by selection for increased MHC diversity in offspring (Zelano and Edwards 2002). If MHC-disassortative mating were tied to inbreeding avoidance, mates should also show disassortative mating across other parts of the genome, including our microsatellite loci. To the contrary, our analyses revealed that microsatellite genotypes did not differ significantly between mated pairs and randomly assigned pairs. This result suggests that MHC complementarity is an actual target of mate choice in this system. Similar evidence for disassortative mating based on MHC genotypes but not on microsatellites has been found recently in tuataras (Miller et al. 2009); however this is the first demonstration of such

patterns in a bird. It has been theorized that females might best complement their own MHC genotypes by selecting mates with an intermediate level of MHC dissimilarity (Bateson 1983). Evidence for intermediate levels of MHC dissimilarity has been found in some previous studies (Forsberg et al. 2007, Eizaguirre et al. 2009, Lenz et al. 2009), though we did not find any evidence for selection of intermediate levels of dissimilarity in this study.

Choosing mates to yield MHC-heterozygous offspring could be advantageous in populations such as this one. In general, oceanic islands characterized by extreme densities of nesting birds are likely to foster high transmission rates of pathogens and parasites (Muzaffar and Jones 2004). Great frigatebirds, like many marine birds, are colonial nesters. The breeding colony on Tern Island encompasses approximately 4,000 great frigatebirds that are included amongst approximately 200,000 marine birds representing 15 species. The available nesting habitat totals approximately 10 ha; therefore, birds congregating to breed on this island do so in extraordinarily high densities. Furthermore, Tern Island includes hematophagous flies (Hippoboscidae) that can serve as disease vectors of frigatebirds and other seabirds (Work and Rameyer 1996).

Although the pattern of MHC-disassortative mate choice in this study is compelling, several caveats are important to consider. First, conclusions about genetic patterns of mate choice can be misleading if extra-pair paternity is frequent and undetected. Previous work has shown that extra-pair fertilizations are rare in this population (1.1%; Dearborn et al. 2001). In our current study,

microsatellite data revealed that 4 of 46 offspring were likely the product of extra-pair fertilizations, with the offspring genetically related to the social mother, but not to the social father. In these cases, if the females did participate in extra-pair copulations, selective fertilization and/or selective abortion could allow a female to control the level of genetic compatibility she shares with her genetic mate (Schwensow et al. 2008), regardless of her social mate. A female that shares more MHC amino acids with her social mate may have more incentive to seek extra-pair fertilizations from males that are more different at MHC loci. One prediction, therefore, would be that the sum of the pairwise amino acid differences between the social parents of these 4 extra-pair offspring would be lower (i.e., their genotypes would be more similar) than for all other pairs. Interestingly, we did find a trend towards smaller pairwise amino acid differences in the 4 social pairs with extra-pair offspring (116.50 ± 24.30 SE) compared to the remaining 42 social pairs (132.21 ± 55.23 SE), though with such a small sample of extra-pair offspring, this trend was not significant ($t_{44} = 0.610$, $P = 0.586$).

Second, this study is unique in its sequencing of avian MHC loci, but there are also several limits to what can be learned from this methodology. Our approach of haploid cloning and repeated sequencing allowed us to be confident we had uncovered all unique alleles within each individual. However, because of the similar flanking sequence of these two MHC loci, we were unable to assign alleles to a particular locus; therefore we could not conduct independent analyses of mate choice based on each locus separately. Our study also did not include analyses of gene expression; therefore we cannot say definitively that all

MHC sequences presented here produced functional MHC molecules involved in the immune repertoire (Knapp 2007). Nonetheless, several pieces of indirect evidence suggest that both loci are functional: analyses of all unique sequences revealed no stop codons or frameshift mutations, and significant positive selection was detected on the entire dataset. Furthermore, inclusion of a locus that is not a functional gene in our analysis would serve only to weaken our ability to detect a pattern of disassortative mating. Our findings of a significant result, therefore, suggest that either both loci were functional genes, or that the strength of mating preferences on one of the loci was sufficient to allow for detection of a disassortative pattern.

Third, the mechanism for disassortative mating by MHC genotype is unknown, though it most likely depends on olfactory discrimination. In mammals and fish it has been demonstrated that peptides derived from MHC proteins contribute to individual olfactory profiles that subsequently play a role in mate choice decisions (Leinders-Zufall et al. 2004, Milinski et al. 2005, Spehr et al. 2006). Furthermore, it has been shown that MHC molecules and their associated peptide ligands are detected by specific subsets of sensory neurons in the mammalian nose (Boehm and Zufall 2006). Far less is known about birds' abilities to detect MHC-dependent odors. Traditionally birds have been thought of as being largely visually oriented, with olfaction playing little or no role in their sensory repertoires. However, in recent years, more evidence of the olfactory abilities of birds has been accumulating, including examples across a wide range of avian orders (Balthazart and Taziaux 2009). Furthermore, a functional

olfactory system, similar in structure to that found in other vertebrates, has been found in every bird studied (Bang and Wenzel 1985, Roper 1999). Experimental evidence now links avian olfactory abilities to such behaviors as orientation (Gagliardo et al. 2008, Holland et al. 2009), locating food (Nevitt et al. 1995, Nevitt and Bonadonna 2005, Nevitt et al. 2008), recognition of familiar odors (Bonadonna et al. 2003, Bonadonna and Nevitt 2004), and locating nest burrows (Grubb 1974, Bonadonna et al. 2003). Despite the accumulating evidence, investigations into the social utility of species- or individual-specific odors in birds have been largely neglected. However, several studies have presented evidence suggesting olfaction could play a role in mate choice (Hagelin et al. 2003, Bonadonna and Nevitt 2004, Soini et al. 2007, Balthazart and Taziaux 2009). Given the evidence for olfactory-mediated behaviors in many avian species, including individual recognition, coupled with what we know about how MHC molecules mediate odor profiles, the idea that female great frigatebirds would use olfactory cues when assessing potential mates seems an intriguing possibility.

Male great frigatebirds are known for their secondary sexual characters and elaborate courtship displays. These types of traits are thought to signal "good genes" to potential suitors (Zahavi 1975, Hamilton and Zuk 1982), though to date there is mixed evidence for a role of these traits in mate choice in this species (Wright and Dearborn 2009). Even if males with good ornaments possess good genes, they may not possess the "most suitable" genes for females attempting to maximize their genetic compatibility (Foerster et al. 2003,

Stapleton et al. 2007). Social, ecological and genetic factors all potentially play a role in which mate choice strategy a female may choose to employ (Mays and Hill 2004). These two seemingly contradictory strategies for selecting a mate are not necessarily mutually exclusive. Females could use nested rule based preferences (Candolin 2003, Mays and Hill 2004), where they initially select a subset of males with attractive ornaments (using visual or auditory cues), then from this subset, choose the most genetically compatible male (perhaps based on olfactory cues). Females in our study population assess potential mates aerially, before landing next to a potential suitor. Once in close proximity the potential pairs engage in mutual preening, but this behavior does not always lead to pair formation (Dearborn and Juola pers. obs.). If mate choice is a multi-stage process, it could be possible to maintain directional selection for elaborate ornaments while still observing mate selection for genetically dissimilar partners (Oh and Badyaev 2006). Thus, female preference for MHC complementarity can exert selective pressures that coexist with female preference for exaggerated ornaments. If our new MHC primers can be applied to other bird species, there may soon be an even broader taxonomic view of the evolutionary role of MHC complementarity in mate choice.

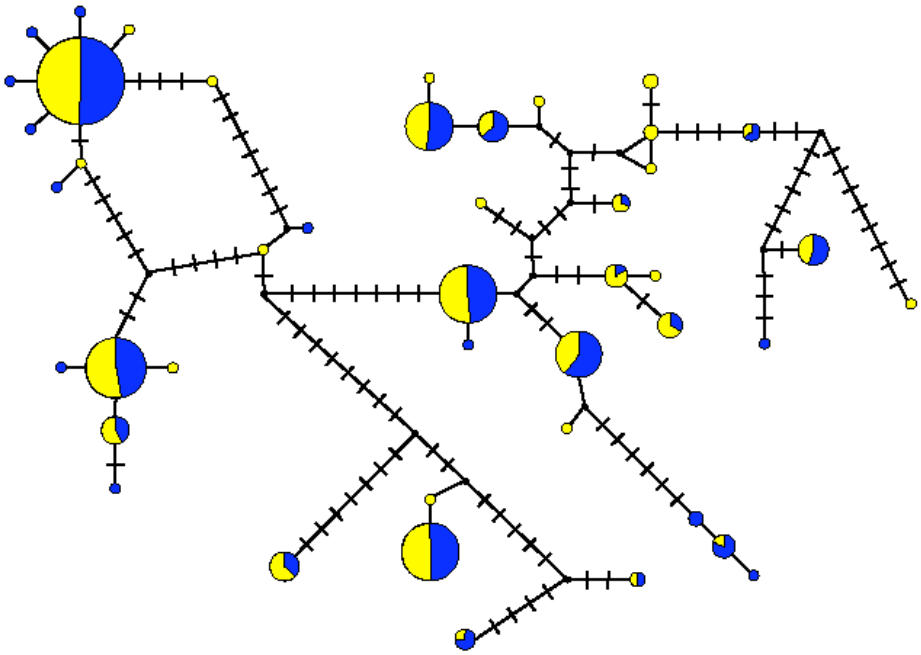

Figure 5.1 Network of nucleic acid haplotypes, based on statistical parsimony. Area of each circle is proportional to number of copies of that haplotype in our sample, with males shaded blue and females shaded yellow. Each connecting line segment represents a change of one base pair.

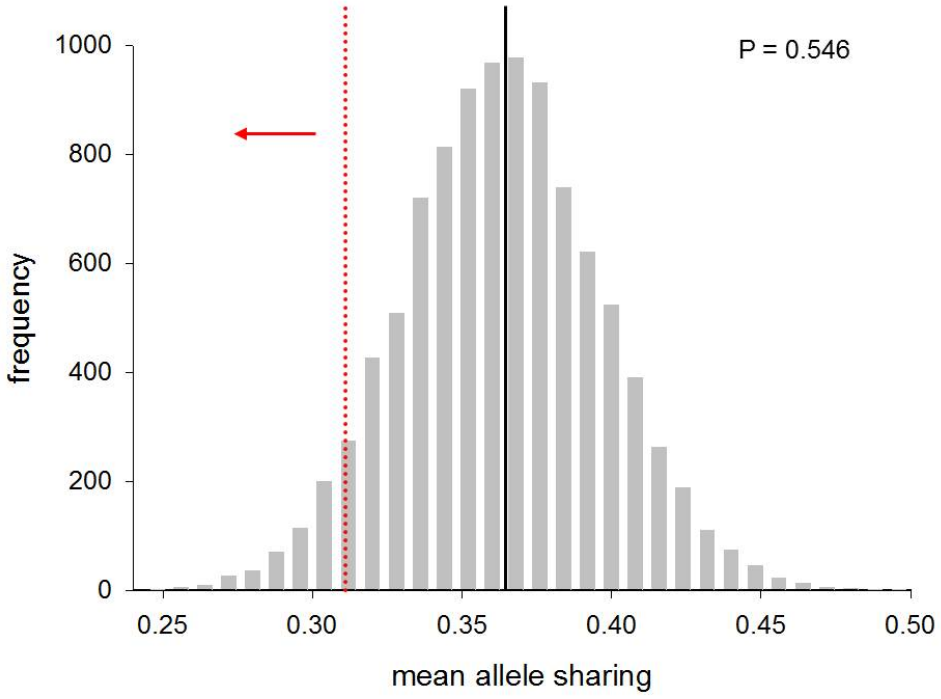

Figure 5.2 Mean allele sharing value of 46 known pairs compared to the distribution of mean allele sharing values generated from 10,000 simulations of 46 random male-female pairings selected from the same 92 individuals. Analysis is based on the amino acid sequences for the entire exon II of the MHC class II ß gene.

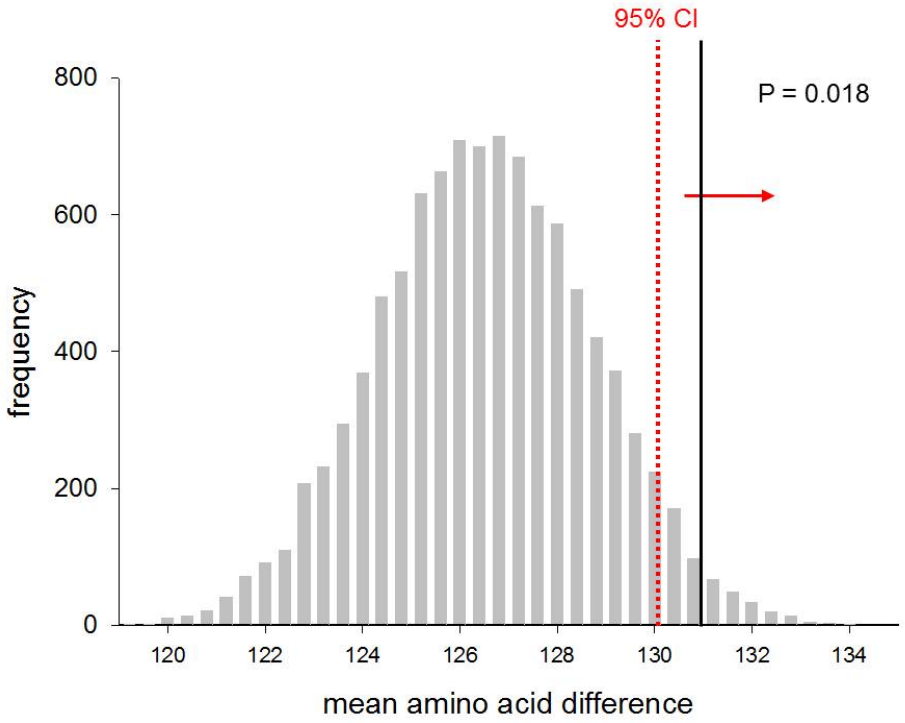

Figure 5.3 Mean sum of all pairwise amino acid differences for 46 pairs compared to the distribution of the mean sum of all pairwise amino acid differences generated from 10,000 simulations of 46 random pairings selected from the same 92 individuals. Analysis is based on the amino acid sequences for the entire second exon of the MHC class II ß gene.

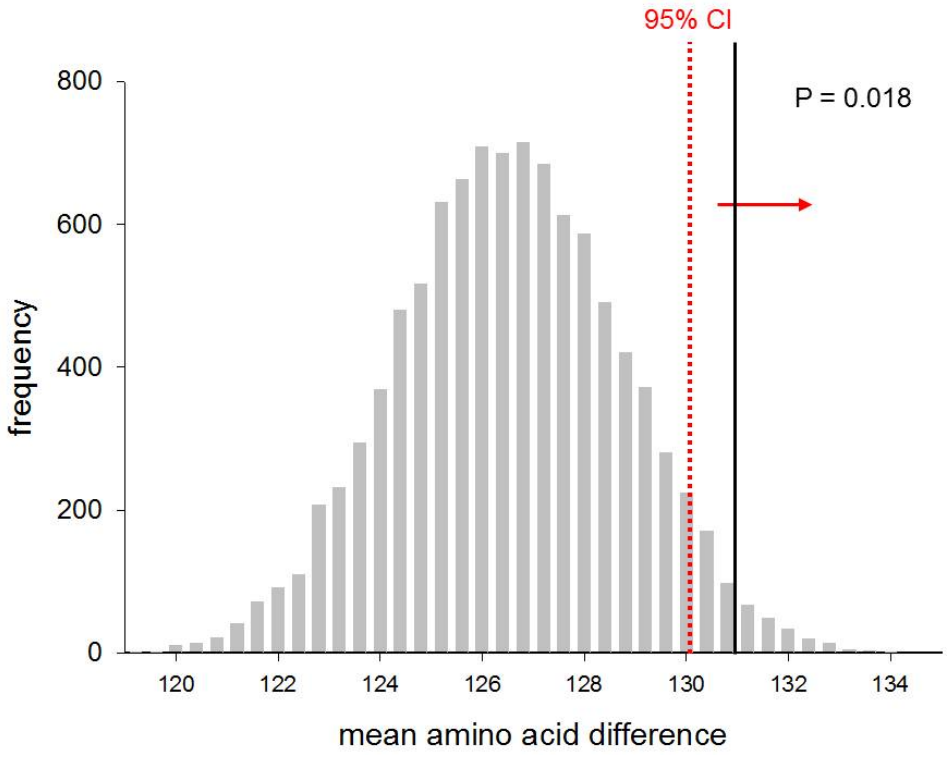

Figure 5.4 Mean average relatedness for 46 pairs compared to the distribution of the mean average relatedness generated from 10,000 simulations of 46 random pairings selected from the same 92 individuals. Analysis is based on Wang's r statistic of relatedness from 12 microsatellite loci.

Chapter 6

Conclusion

In this dissertation, I systematically investigated mate choice in great frigatebirds. I first examined the possibility that carotenoids play a part in coloration of male gular pouches. I discovered that astaxanthin, a red carotenoid pigment, is present in the gular pouch tissue of displaying males, and that this pigmentation isat least partially responsible for the red coloration exhibited in gular pouches during the breeding season when males are displaying.

This was the first study in great frigatebirds and the second study in any frigatebird species to investigate the role of gular pouch size in mate choice. Similarly, this was the first study to investigate gular pouch color and mate choice using objective spectrophotometric techniques. Similar to previous findings in the magnificent frigatebird, neither the size nor the coloration of male gular pouches was a good predictor of female mate choice preferences.

This was also the first study to provide empirical data on great frigatebird vocalizations. Quantitative descriptions of vocal display traits in male great frigatebirds should be of general interest, as these traits have been relatively little studied in non-passerine birds. The courtship vocalizations of male great frigatebirds showed the tradeoff between frequency bandwidth and repetition rate that has been found in the trilled songs of a number of passerine species; consequently the deviation of a male's vocalizations from the upper limit defined by this tradeoff could be used as a measure of vocal performance. This and other

aspects of male vocal display were associated with aspect of male quality. Nevertheless, vocal performance and other vocal display traits were not good predictors of mate choice in my study population.

Finally, the investigation into MHC-influenced mate choice is an area of research currently receiving much attention. Since it has been determined that MHC genes can influence mate choice decisions in other mammals and fish, it has become particularly interesting to determine if such effects are possible in other taxa, including birds. Previously work in other avian species has yielded somewhat equivocal results. My finding of a significant pattern of disassortative mating based on the amino acid sequence of an MHC loci is the most convincing evidence to date of an effect of MHC genotype on mate choice in birds, utilizing gene sequence and amino acid sequence data in the analyses.

In conclusion, my dissertation has taken a wide-ranging look at display traits and mate choice in great frigatebirds. This was a thorough investigation that utilized modern techniques, such as spectrophotometry and gene sequencing. My finding of non-significant results for nearly all visual and acoustic display traits I measured is intriguing. Previous studies on frigatebirds are scarce, though my results to corroborate the two previous studies, one of magnificent frigatebirds and one of great frigatebirds, where most all display traits measured were also found to not be related to mating success. Several possibilities exist that could explain why this is the case. First of all, additional display traits not investigated in this study could play a role in mate choice. The lifting and shaking of wings by males during their active display is something that

was not addressed in this study. In addition, the finding of a disassortative mating pattern based on amino acid sequences of an MHC locus suggests that genetic compatibility may be an important factor in mate choice. Individual females may therefore have different preferences in mates, based on their own genetic makeup. If this is the case, then mate selection could be a two-part process. Male visual and acoustic display traits may serve to attract attention and to allow females to quickly locate those males attempting to breed. Females then inspect individual males more closely, and select their mating partner on more subtle clues, such as MHC genotype.

References

Alcaide M, Edwards SV, Negro JJ (2007) Characterization, polymorphism, and evolution of MHC class II B genes in birds of prey. Journal of Molecular Evolution 65:541-554.

Alonso-Alvarez C, Bertrand S, Devevey G, Gaillard M, Prost J, Faivre B, Sorci G (2004) An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. American Naturalist 164:651-659.

Alonso JC, Magaña M, Martĩn CA, Palacĩn C (2010) Sexual traits as quality indicators in lekking male great bustards. Ethology 116:1084-1098.

Andersson M (1994) Sexual Selection. Princeton University Press, Princeton, New Jersey.

Andersson S, Prager M (2006) Quantifying colors. In: Hill GE, McGraw KJ (eds) Bird coloration. Mechanisms and measurements, vol. 1. Harvard University Press, Cambridge, MA.

Attenborough D (1998) The life of birds. Princeton, NJ: Princeton University Press.

Badyaev AV, Hill GE (2000) Evolution of sexual dichromatism: contribution of carotenoid- versus melanin based coloration. Biological Journal of the Linnean Society 69:153–172.

Ballentine B, Hyman J, Nowicki S (2004) Vocal performance influences female response to male bird song: an experimental test. Behavioral Ecology 15:163-168.

Balthazart J, Taziaux M (2009) The underestimated role of olfaction in avian reproduction? Behavioral Brain Research 200:248-259.

Bang BG, Wenzel BM (1985) Nasal cavity and olfactory system. In: King AS, McClelland J (eds) Form and function in birds, vol 3. Academic Press, London, pp 195-225.

Bateson PPG (1983) Optimal Outbreeding. In: Bateson PPG (ed) Mate choice. Cambridge University Press, Cambridge, pp 257-277.

Bee MA, Perrill SA, Owen PC (1999) Size assessment in simulated territorial encounters between male green frogs (*Rana clamitans*). Behavioral Ecology and Sociobiology 45:177-184.

Bendich A (1989) Carotenoids and the immune response. Journal of Nutrition 119, 112-115.

Bennett ATD, Cuthill IC, Norris KJ (1994) Sexual selection and the mismeasure of color. American Naturalist 144:848-860.

Bennett ATD, Cuthill IC, Partridge JC, Maeir ES (1996) Ultraviolet vision and mate choice in zebra finches. Nature 380:433-435.

Bennett ATD, Thery M (2007) Avian color vision and coloration: multidisciplinary evolutionary biology. American Naturalist 169:S1-S6.

Blas J, Pérez-Rodríguez L, Bortolotti GR, Viñuela J, Marchant TA (2006) Testosterone increases bioavailability of carotenoids: insights into the honesty of sexual signaling. Proceedings of the National Academy of Sciences USA 103:18633-18637.

Bleiweiss R (2005) Variation in ultraviolet reflectance by carotenoid bearing feathers of tanagers (Thraupini: Emberizinae: Passeriformes). Biological Journal of the Linnean Society 84:243–257.

Blount JD, Houston DC, Moller AP (2000) Why egg yolk is yellow. Trends in Ecology and Evolution 15:47-49.

Blount JD, Metcalfe NB, Birkhead TR, Surai PF (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. Science 300:125-127.

Bodmer WF (1972) Evolution significance of the HLA system. Nature 237:139-145.

Boehm T, Zufall F (2006) MHC peptides and the sensory evaluation of genotype. Trends in Neuroscience 29:100-107.

Bonadonna F, Cunnignham GB, Jouventin P, Hesters F, Nevitt GA (2003) Evidence for nest-odour recognition in two species of diving petrel. Journal of Experimental Biology 206:3719-3722.

Bonadonna F, Nevitt GA (2004) Partner-specific odor recognition in an Antarctic seabird. Science 306:835.

Bonneaud C, Chastel O, Federici P, Westerdahl H, Sorci G (2006) Complex mhc-based mate choice in a wild passerine. Proceedings of the Royal Society B 273:1111-1116.

Bortolotti G, Negro JJ, Tella JL, Marchant TA, Bird DM (1996) Sexual dichromatism in birds independent of diet, parasites and androgens. Proceedings of the Royal Society B 263:1171-1176.

Bortolotti G, Blas J, Negro JJ, Tella JL (2006) A complex plumage pattern as an honest social signal. Animal Behaviour 72:423-430.

Botero CA, Rossman RJ, Caro LM, Stenzler LM, Lovette IJ, de Kort SR, Vehrencamp SL (2009) Syllable type consistency is related to age, social status and reproductive success in the tropical mockingbird. Animal Behaviour 77:701-706.

Bradley RD, Hillis DM (1996) Recombinant DNA sequences generated by PCR amplification. Molecular Biology and Evolution 14:592-593.

Brawner WR, Hill GE, Sunderman CA (2000) Effects of coccidial and mycoplasmal infections on carotenoid-based plumage pigmentation in male house finches. Auk 117:952-963.

Brown WD, Wideman J, Andrade MCB, Mason AC, Gwynne DT (1996) Female choice for an indicator of male size in the song of the black-horned tree cricket *Oecanthus nigricornis* (Orthoptera: Gryllidae: Oecanthinae). Evolution 50:2400-2411.

Buchholtz R (1995) Female choice, parasite load and male ornamentation in wild turkeys. Animal Behaviour 50:929-943.

Buchholz R (1997) Male dominance and variation in fleshy head ornamentation in wild turkeys. Journal of Avian Biology 28:223–230.

Burkhardt D (1989) UV vision: a bird's eye view of feathers. Journal of Comparative Physiology A 164:787–796

Burton GW (1989) Antioxidant action of carotenoids. Journal of Nutrition 119:109-111.

Byers BE (2007) Extrapair paternity in chestnut-sided warblers is correlated with consistent vocal performance. Behavioral Ecology 18:130-136.

Byers J, Hebets E, Podos J (2010) Female mate choice based upon male motor performance. Animal Behaviour 79:771-778.

Candolin U (2003) The use of multiple cues in mate choice. Biological Reviews 78:575–595.

Cardoso GC (2008) On the performance of brown skua, *Catharacta antarctica*, vocalizations. Animal Behaviour 76:E1-E2.

Cardoso GC, Mamede AT, Atwell JW, Mota PG, Ketterson ED, Price TD (2008) Song frequency does not reflect differences in body size among males in two oscine species. Ethology 114:1084-1093.

Carrington M, Nelson GW, Martin MP, Kissner T, Vlahov D, Goedert JJ, Kaslow R, Buchbinder S, Hoots K, O'Brien SJ (1999) HLA and HIV-1: Heterozygote advantage and B*35-Cw*04 disadvantage. Science 283:1748-1752.

Casagrande S, Csermely D, Pini E, Bertacche V, Tagliavini J (2006) Skin carotenoid concentration correlates with male hunting skill and territory quality in the kestrel *Falco tinnunculus*. Journal of Avian Biology 37:190-196.

Catchpole CK, Slater PJB (2008) Bird song: biological themes and variations. 2nd edition. Cambridge University Press, Cambridge, United Kingdom.

Chastel O, Barbraud C, Weimerskirch H, Lormee H, Lacroix A, Tostain O (2005) High levels of LH and testosterone in a tropical seabird with an elaborate courtship display General and Comparative Endocrinology 140:33-40.

Clement M, Posada D, Crandall K (2000) TCS: a computer program to estimate gene genealogies. Molecular Ecology 9:1657-1660.

Coleman SW, Patricelli GL, Borgia G (2004) Variable female preferences drive complex male display. Nature 428:742-745.

Consuegra S, de Leaniz CG (2008) MHC-mediated mate choice increases parasite resistance in salmon. Proceedings of the Royal Society B 275:1397-1403.

Cotton S, Fowler K, Pomiankowski A (2004) Do sexual ornaments demonstrate heightened condition dependent expression as predicted by handicap hypothesis? Proceedings of the Royal Society B 271: 771-783.

Cramer ERA, Price JJ (2007) Red-winged blackbirds *Ageliaus phoeniceus* respond differently to song types with different performance levels. Journal of Avian Biology 38:122-127.

Cuthill IC, Partridge JC, Bennett ATD, Church SC, Hart NS, Hunt S (2000) Ultraviolet vision in birds. Advances in the Study of Behavior 29:159–214.

Darwin C (1859) On the origin of species by means of natural selection. Murray, London.

Darwin C (1871) The descent of man, and selection in relation to sex. Murray, London.

Dearborn DC (2001) Body condition and retaliation in parental effort decisions of incubating great frigatebirds (*Fregata minor*). Behavioral Ecology 12:200-206.

Dearborn DC, Anders AD, Parker PG (2001) Sexual dimorphism, extrapair fertilizations, and operational sex ratio in great frigatebirds (*Fregata minor*). Behavioral Ecology 12:746-752.

Dearborn DC, Ryan MJ (2002) A test of the Darwin–Fisher theory for the evolution of male secondary sexual traits in monogamous birds. Journal of Evolutionary Biology 15:307-313.

Dearborn DC, Anders AD, Schreiber EA, Adams RMM, Mueller UG (2003) Inter-island movements and population differentiation in a pelagic seabird. Molecular Ecology 12:2835-2843.

Dearborn DC, Anders AD (2006) Demography and reproductive ecology of great frigatebirds. Atoll Research Bulletin 543:159-171.

Dearborn DC, Hailer F, Fleischer RC (2008) Microsatellite primers for relatedness and population structure in great frigatebirds (Pelecaniformes: Fregatidae). Molecular Ecology Research 8:1399-1401.

Edwards SV (1995) Dynamics of mhc evolution in birds and crocodilians: amplification of class II genes with degenerate primers. Molecular Evolution 4:719-729.

Edwards SV, Hedrick PW (1998) Evolution and ecology of MHC molecules: from genomics to sexual selection. Trends in Ecology and Evolution 13:305-311.

Eizaguirre C, Yeates SE, Lenz TL, Kalbe M, Milinski M (2009) MHC-based mate choice combines good genes and maintenance of MHC polymorphism. Molecular Ecology 18:3316-3329.

Ekblom R, Saether SA, Grahn M, Fiske P, Kålås JA, Höglund (2004) Major histocompatibility complex variation and mate choice in a lekking bird, the great snipe (*Gallinago media*). Molecular Ecology 13:3821-3828.

Endler JA (1980) Natural selection on color patterns in *Poecilia reticulate*. Evolution 34:76-91.

Endler JA (1990) On the measurement and classification of colour in studies of animal colour patterns. Biological Journal of the Linnean Society 41:315-352.

Fernandez-de-Mera IG, Vicente J, Naranjo V, Fierro Y, Garde JJ, de la Fuente J, Gortazar C (2009) Impact of major histocompatibility complex class II polymorphisms on Iberian red deer parasitism and life history traits. Infection, Genetics and Evolution 9:SI1232-1239.

Figuerola J, Gutierrez R (1998) Sexual differences in levels of blood carotenoids in cirl buntings *Emberiza cirlus*. Ardea 86:245–248.

Fisher RA (1930) The genetical theory of natural selection. Oxford: Clarendon Press.

Fisher RA (1958) The Genetical Theory of Natural Selection. 2d ed. Dover, New York.

Fletcher DL (1992) Methodology for achieving pigment specifications. Poultry Science 71:733-743.

Foerster K, Delhey K, Johnsen A, Lifjeld JT, Kempenaers B (2003) Females increase offspring heterozygosity and fitness through extra-pair matings. Nature 425:714–717.

Folstad I, Karter AJ (1992). Parasites, bright males, and the immunocompetence handicap. American Naturalist 139:603–622.

Forsberg LA, Dannewitz J, Petersson E, Grahn M (2007) Influence of genetic dissimilarity in the reproductive success and mate choice of grown trout – females fishing for optimal MHC dissimilarity. Journal of Evolutionary Biology 20:1859-1869.

Freeman-Gallant CR, Meguerdichian M, Wheelwright NT, Sollecito SV (2003) Social pairing and female mating fidelity predicted by restriction fragment length polymorphism similarity at the major histocompatibility complex in a songbird. Molecular Ecology 12:3077-3083.

Fox DL (1979) Biochromy: Natural Coloration of Living Things. University of California Press, Berkeley.

Gagliardo A, Ioale P, Savini M, Wild M (2008) Navigational abilities of homing pigeons deprived of olfactory or trigeminally mediated magnetic information when young. Journal of Experimental Biology 211:2046-2051.

Gerhardt HC, Huber F (2002) Acoustic communication in insects and anurans: common problems and diverse solutions. University Chicago Press, Chicago, IL.

Goden JG, McDonough HE (2003) Predator preference for brightly colored males in the guppy: a viability cost for a sexually selected trait. Behavioral Ecology 14:194-200.

Goodwin TW (1984) The biochemistry of the carotenoids. Animals, vol 2. Chapman and Hall, New York, NY.

Grafen A (1990) Biological signals as handicaps. Journal of Theoretical Biology 144:517-546.

Grether GF, Hudon J, Millie DF (1999) Carotenoid limitation of sexual coloration along an environmental gradient in guppies. Proceedings of the Royal Society B 266:1317-1322.

Grether GF (2000) Carotenoid limitation and mate preference evolution: a test of the indicator hypothesis in guppies (*Poecilia reticulate*). Evolution 54:1712-1724.

Grubb TC (1974) Olfactory navigation to nesting burrow in leach's petrel (*Oceanodroma leucorrhoa*). Animal Behaviour 22:192-202.

Hagelin JC, Jones IL, Rasmussen LEL (2003) A tangerine-scented social odour in a monogamous seabird. Proceedings of the Royal Society B 270:1323-1329.

Hamilton WD, and Zuk M (1982) Heritable true fitness and bright birds: a role for parasites? Science 218:384-387.

Harrison P (1985) Seabirds: an identification guide. Houghton Mifflin Company, Boston, MA.

Hedrick PW, Black FL (1997) HLA and mate selection: no evidence in South Amerindians. American Journal of Human Genetics 61:505-511.

Hedrick PW (2002) Pathogen resistance and genetic variation at the MHC loci. Evolution 56:1902-1908.

Hill GE (1990) Female house finches prefer colourful males: sexual selection for a condition-dependent trait. Animal Behaviour 40:563-572.

Hill GE (1991) Plumage coloration is a sexually selected indicator of male quality. Nature 350:337-339.

Hill GE (1994) House finches are what they eat: a reply to Hudon. Auk 111:221-225.

Hill GE (1995) Seasonal variation in circulating carotenoid pigments in the house finch. Auk 112:1057-1061.

Hill GE, Brawner WR (1998) Melanin-based plumage coloration in the house finch is unaffected by coccidial infection. Proceedings of the Royal Society B 265:1105–1109.

Hill GE (1999a) Is there an immunological cost to carotenoid-based ornamental coloration? American Naturalist 154:589-595.

Hill GE (1999b) Mate choice, male quality, and carotenoid-based plumage coloration. Proceedings of the International Ornithological Congress 22:1654-1668.

Hill GE (2002) A red bird in a brown bag. Oxford University Press, Oxford.

Hill GE, Inouye CY, Montgomerie R (2002) Dietary carotenoids predict plumage coloration in wild house finches. Proceedings of the Royal Society B 269:1119-1124.

Hill GE, McGraw KJ (2006) Bird coloration. Function and Evolution, vol. II. Harvard University Press, Cambridge, MA.

Hoffman JI, Amos W (2005) Microsatellite genotyping errors: detection approaches, common sources and consequences for paternal exclusion. Molecular Ecology 14:599-612.

Holder K, Montgomerie R (1993) Context and consequences of comb displays by male rock ptarmigan. Animal Behaviour 45:457-470.

Holland RA, Thorup K, Gagliardo A, Bisson IA, Knecht E, Mizrahi D, Wikelski M (2009) Testing the role of sensory systems in the migratory heading of a songbird. Journal of Experimental Biology 212:4065-4071.

Houde AE (1987) Mate choice based upon naturally occurring color-pattern variation in a guppy population. Evolution 41:1-10.

Houde AM, Torio AJ (1992) Effect of parasitic infection on male color pattern and female choice in guppies. Behavioral Ecology 3:346-351.

Hughes AL, Hughes MK (1995) Natural selection on the peptide-binding regions of major histocompatibility complex molecules. Immunogenetics 42:233-243.

Hunt S, Cuthill IC, Bennett ATD, Griffiths R (1999) Preferences for ultraviolet partners in the blue tit. Animal Behaviour 58:809-815.

Hunt S, Kilner RM, Langmore NE, Bennett ATD (2003) Conspicuous, ultraviolet-rich mouth colours in begging chicks. Proceedings of the Royal Society B (suppl.) 270:S25-S28.

Hussein G, Sankawa U, Goto H, Matsumoto K, Watanabe H (2006) Astaxanthin, a carotenoid with potential in human health and nutrition. Journal of Natural Products 69:443-449.

Illes AE, Hall ML, Vehrencamp SL (2006) Vocal performance influences male receiver response in the banded wren. Proceedings of the Royal Society B 273:1907-1912.

Janicke T, Hahn S, Ritz MS, Peter HU (2008) Vocal performance reflects individual quality in a nonpasserine. Animal Behaviour 75:91-98.

Johnsen A, Andersson S, Ornborg J, Lifjeld JT (1998) Ultraviolet plumage ornamentation affects social mate choice and sperm competition in bluethroats (Aves: Luscinia s. svecica): a field experiment. Proceedings of the Royal Society B 265:1313–1318.

Jordan WC, Bruford MW (1998) New perspectives on mate choice and the MHC. Heredity 81:239-245.

Jourdie V, Moureau B, Bennett ATD, Heeb P (2004) Ultraviolet reflectance by the skin of nestlings. Nature 431:262.

Juola FA, Haussmann MF, Dearborn DC, Vleck CM (2006) Telomere shortening in a long-lived marine bird: Cross-sectional analysis and test of an aging tool. Auk 123:775-783.

Juola FA, Dearborn DC (2007) Does the differential cost of sons and daughters lead to sex ratio adjustment in great frigatebirds *Fregata minor*? Journal of Avian Biology 38:94-104.

Juola FA, McGraw K, Dearborn DC (2008) Carotenoids and throat pouch coloration in the great frigatebird (*Fregata minor*) Comparative Biochemistry and Physiology B 149:370-377.

Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Molecular Ecology 16:1099-1106.

Kirkpatrick M, Ryan MJ (1991) The evolution of mating preferences and the paradox of the lek. Nature 350:33-38.

Klein J (1986) Natural history of the major histocompatibility complex. John Wiley & Sons, New York.

Knapp LA (2007) A matter of form over function. Heredity 99:241-242.

Knight J (2002) Sexual stereotypes. Nature 415:254-256.

Kodric-Brown A (1985) Female preference and sexual selection for male coloration in the guppy (*Poecilia reticulate*). Behavioral Ecology and Sociobiology 17:199-205.

Kodric-Brown A (1989) Dietary carotenoids and male mating success in the guppy: an environmental component to female choice. Behavioral Ecology and Sociobiology 25:393-401.

Krinsky NI (1989) Carotenoids and cancer in animal models. Journal of Nutrition. 119:123-126.

Kurtz J, Kalbe M, Aeschlimann PB, Häberli MA, Wegner KM, Reusch TBH, Milinski M (2004) Major histocompatibility complex diversity influences parasite resistance and innate immunity in sticklebacks. Proceedings of the Royal Society B 271:197-204.

Landry C, Garant D, Duchesne P, Bernatchez L (2001) 'Good genes as heterozygosity': the major histocompatibility complex and mate choice in Atlantic salmon (*Salmo salar*). Proceedings of the Royal Society B 268:1279-1285.

Leinders-Zufall T, Brennan P,Widmayer P, S Chandramani P, Maul-Pavicic A, Jager M, Xiao-Hong Li X, Breer H, Zufall F, Boehm T (2004) MHC class I peptides as chemosensory signals in the vomeronasal organ. Science 306:1033-1037.

Lie HC, Simmons LW, Rhodes G (2009) Does genetic diversity predict health in humans? PLOS One 4:e6391.

Lenz TL, Eizaguirre C, Scharsack JP, Kalbe M, Milinski M (2009) Disentangling the role of MHC-dependent 'good genes' and 'compatible genes' in mate-choice decisions of three-spined sticklebacks *Gasterosteus aculeatus* under semi-natural conditions. Journal of Fish Biology 75:2122-2142.

Lessells CM, Boag PT (1987) Unrepeatable repeatabilities: a common mistake. Auk 104:116-21.

Ligon JD (1999) The evolution of avian breeding systems. Oxford: Oxford University Press.

Logue DM, Droessler EE, Roscoe DW, Vokey JR, Rendall D, Kunimoto RM (2007) Sexually antithetical song structure in a duet singing wren. Behaviour 144:331-350.

Lozano A (1994) Carotenoids, parasites, and sexual selection. Oikos 70:309-311.

MacDougall-Shackleton EA, Derryberry EP, Foufopoulos J, Dobson AP, Hahn TP (2005) Parasite-mediated heterozygote advantage in an outbred songbird population. Biology Letters 1:105-107.

Madsen T, Ujvari B (2006) MHC class I variation associates with parasite resistance and longevity in tropical pythons. Journal of Evolutionary Biology 19:1973-1978.

Madsen V, Balsby TJS, Dabelsteen T, Osorno JL (2004) Bimodel signaling of a sexually selected trait: gular pouch drumming in the magnificent frigatebird. Condor 106:156-160.

Madsen V, Dabelsteen T, Osorio D, Osorno JL (2007a) Morphology and ornamentation in male magnificent frigatebirds: variation with age class and mating success. American Naturalist 169:S93-S111.

Madsen V, Valkiūnas G, Iezhova TA, Mercade C, Sanchez M, Osorno JL (2007b) Testosterone levels and gular pouch coloration in courting magnificent frigatebird (*Fregata magnificens*): Variation with age-class, visited status and blood parasite infection. Hormones and Behavior 51:156-163.

Massaro M, Davis LS, Darby JT (2003) Carotenoid-derived ornaments reflect parental quality in male and female yellow-eyed penguins (*Megadyptes antipodes*). Behavioral Ecology and Sociobiology 55:169-175.

Mays HLJ, Hill GE (2004) Choosing mates: good genes versus genes that are a good fit. Trends in Ecology and Evolution 19:554-559.

McGraw KJ, Hill GE, Stradi R, Parker RS (2001) The influence of carotenoid acquisition and utilization on the maintenance of species-typical plumage pigmentation in male American Goldfinches (*Carduelis tristis*) and Northern Cardinals (*Cardinalis cardinalis*). Physiological and Biochemical Zoology 74:843-852.

McGraw KJ, Adkins-Regan E, Parker RS (2002) Anhydrolutein in the zebra finch: a new, metabolically derived carotenoid in birds. Comparative Biochemistry and Physiology B 132:811-818.

McGraw KJ, Ardia DR (2003) Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. American Naturalist 162:704-712.

McGraw KJ, Gregory AJ, Parker RS, Adkins-Regan E (2003) Diet, plasma carotenoids, and sexual coloration in the zebra finch (*Taeniopygia guttata*). Auk 120:400-410.

McGraw KJ, Gregory AJ (2004) Carotenoid pigments in male American goldfinches: what is the optimal biochemical strategy for becoming colourful? Biological Journal of the Linnean Society 83:273-280.

McGraw KJ, Hill GE, Navara KJ, Parker RS (2004) Differential accumulation and pigmenting ability of dietary carotenoids in colorful finches. Physiological and Biochemical Zoology 77:484-491.

McGraw KJ (2005a) Interspecific variation in dietary carotenoid assimilation in birds: links to phylogeny and color ornamentation. Comparative Biochemistry and Physiology B 142:245-250.

McGraw KJ**,** Ardia DR (2005) Sex differences in carotenoid status and immune performance in zebra finches. Evolutionary Ecology Research 7:251-262.

McGraw KJ (2006) Mechanics of carotenoid-based coloration. In: Hill GE, McGraw KJ (eds) Bird coloration. Mechanisms and measurements, vol 1. Harvard University Press, Cambridge, MA.

McGraw KJ, Hardy LS (2006) Astaxanthin is responsible for the pink plumage flush in Franklin's and Ring-billed gulls. Journal of Field Ornithology 77:29-33.

McGraw KJ, Nolan PM, Crino OL (2006) Carotenoid accumulation strategies for becoming a colourful House Finch: analyses of plasma and liver pigments in wild moulting birds. Functional Ecology 20:678-688.

McGraw KJ, Toomey MB, Nolan PM, Morehouse NI, Massaro M, Jouventin P (2007) A description of unique fluorescent yellow pigments in penguin feathers. Pigment Cell Research 20:301-304.

Metz VG, Schreiber EA (2002) Great frigatebird (*Fregata minor*). In: Poole A, Gill F (eds) The birds of North America, No. 681. The Academy of Natural Sciences, Philadelphia, and The American Ornithologists' Union, Washington, DC.

Milinski M, Bakker TCM (1990) Female sticklebacks use male coloration in mate choice and hence avoid parasitized males. Nature 344:330-333.

Milinski M, Griffiths S, Wegner KM, Reusch TBH, Haas-Assenbaum A, Boehm T (2005) Mate choice decisions of stickleback females predictably modified by MHC peptide ligands. Proceedings of the National Academy of Sciences USA 102:4414-4418.

Miller C, Moore JA, Nelson NJ, Daugherty CH (2009) Influence of major histocompatibility complex genotype on mating success in a free-ranging reptile population. Proceedings of the Royal Society B 276:1695-1704.

Møller AP, Christe P, Lux E (1999) Parasitism, host immune function, and sexual selection. Quarterly Review of Biology 74:3-20.

Møller AP, Biard C, Blount JD, Jouston DC, Ninni P, Saino N, Surai PF (2000) Carotenoid-dependent signals: indicators of foraging efficiency, immunocompetence or detoxification ability? Avian Poultry Science Review 11:137-159.

Mougeot F, Redpath SM (2004) Sexual ornamentation relates to immune function in male red grouse *Lagopus lagopus scoticus*. Journal of Avian Biology 35:425-433.

Mougeot F, Redpath SM, Leckie F (2005) Ultra-violet reflectance of male and female red grouse, *Lagopus lagopus scoticus*: sexual ornaments reflect nematode parasite intensity. Journal of Avian Biology 36:203-209.

Mougeot F, Martínez-Padilla J, Pérez-Rodríguez L, Bortolotti GR (2007) Carotenoid-based colouration and ultraviolet reflectance of the sexual ornaments of grouse. Behavioral Ecology and Sociobiology 61:741-751.

Muzaffar SB, Jones IL (2004) Parasites and diseases of the auks (Alcidae) of the world and their ecology - a review. Marine Ornithology 32:121-146.

Neff BD, Garner SR, Heath JW, Heath DD (2008) The MHC and non-random mating in a captive population of Chinook salmon. Heredity 101:175-185.

Negro JJ, Botolotti GR, Tella JL, Fernie KJ, Bird DM (1998) Regulation of integumentary colour and plasma carotenoids in American kestrels consistent with sexual selection theory. Functional Ecology 12:307-312.

Negro JJ, Garrido-Fernandez J (2000) Astaxanthin is the major carotenoid in tissues of White storks (*Ciconia ciconia*) feeding on introduced crayfish (*Procambarus clarkii*). Comparative Biochemistry and Physiology B 126:347-352.

Negro JJ, Grande JM, Tella JL, Garrido J, Hornero D, Donázar JA, Sanchez-Zapata JA, BenÍtez JR, Barcell M (2002) Coprophagy: an unusual source of essential carotenoids. Nature 416:807-808.

Negro JJ, Sarasola JH, Fariñas F, Zorrilla I (2006) Function and occurrence of facial flushing in birds. Comparative Biochemistry and Physiology A 143:78-84.

Nelson JB (1975) The breeding biology of frigatebirds: a comparative review. Living Bird 14:113-155.

Nevitt GA, Veit RR, Kareiva P (1995) Dimethyl sulphide as a foraging cue for Antarctic procellariiform seabirds. Nature 376:680-682.

Nevitt GA, Bonadonna F (2005) Seeing the world through the nose of a bird: New developments in the sensory ecology of procellariiform seabirds. Marine Ecology Progress Series 287:292-295.

Nevitt GA, Losekoot M, Weimerskirch H (2008) Evidence for olfactory search in wandering albatross, *Diomedea exulans*. Proceedings of the National Academy of Sciences USA 105:4576-4581.

Nolan PM, Dobson FS, Nicolaus M, Karels TJ, McGraw KJ, Jouventin P (2010) Mutual mate choice for colorful traits in king penguins. Ethology 116:635-644.

Nowicki, S. (1987) Vocal tract resonances in oscine bird sound production: evidence from birdsongs in a helium atmosphere. Nature 325:53-55.

Oldham RS, Gerhardt HC (1975) Behavioral isolation of the treefrogs *Hyla cinerea* and *Hyla gratiosa*. Copeia 1975:223–231.

Ober C, Weitkamp LR, Cox N, Dytch H, Kostyu D, Elias S (1997) HLA and mate choice in humans. American Journal of Human Genetics 61:497-504.

Oh KP, Badyaev AV (2006) Adaptive genetic complementarity in mate choice coexists with selection for elaborate sexual traits. Proceedings of the Royal Society B 273:1913–1919.

Olson VA, Owens IPF (1998) Costly sexual signals: are carotenoids rare, risky or required? Trends in Ecology and Evolution 13:510-514.

Parker RS (1996) Absorption, metabolism, and transport of carotenoids. FASEB Journal 10:542-551.

Patel R, Mulder RA, Cardoso GC (2010) What makes vocalisation frequency an unreliable signal of body size in birds? A study on black swans. Ethology 116:554-563.

Paterson S, Pemberton JM (1997) No evidence for major histocompatibility complex-dependent mating patterns in a free-living ruminant population. Proceedings of the Royal Society B 264:1813-1819.

Paterson S, Wilson K, Pemberton JM (1998) Major histocompatibility complex variation associated with juvenile survival and parasite resistance in a large unmanaged ungulate population. Proceedings of the National Academy of Sciences USA 95:3714-3719.

Penn DJ, Potts WK (1999) The evolution of mating preferences and major histocompatibility complex genes. American Naturalist 153:145-164.

Peters RH (1983) The ecological implications of body size. Cambridge University Press, Cambridge, U.K.

Peters A, Denk AG, Delhey AG, Kempenaers B (2004) Carotenoid based bill colour as an indicator of immunocompetence and sperm performance in male mallards. Journal of Evolutionary Biology 17:1111–1120.

Podos J (1997) A performance constraint on the evolution of trilled vocalizations in a songbird family (Passeriformes: Emberizidae). Evolution 51:537–551.

Podos J (2001) Correlated evolution of morphology and vocal signal structure in Darwin's finches. Nature 409:185–188.

Podos J, Southall JA, Rossi-Santos M (2004) Vocal mechanics in Darwin's finches: correlation of beak gape and song frequency. Journal of Experimental Biology 207:607–619.

Poole KG, Murphy CG (2007) Preferences of female barking treefrogs, *Hyla gratiosa*, for larger males: univariate and composite tests. Animal Behaviour 73:513-524.

Potts WK, Wakeland EK (1990) Evolution of diversity at the major histocompatibility complex. Trends in Ecology and Evolution 5:181-187.

Potts WK, Manning CJ, Wakeland EK (1991) Mating patterns in semi-natural populations of mice influenced by MHC genotype. Nature 352:619-621.

Richardson DS, Komdeur J, Burke T, von Schantz T (2005) MHC-based patterns of social and extra-pair mate choice in the Seychelles warbler. Proceedings of the Royal Society B 272:759-767.

Reid JM, Arcese P, Cassidy A, Marr AB, Smith J, Keller LF (2005) Hamilton and Zuk meet heterozygosity? Song repertoire size indicates inbreeding and immunity in song sparrows (*Melospiza melodia*). Proceedings of the Royal Society B 272:481–487.

Rintamäki PT, Höglund J, Karvonen I, Alatalo RV, Björklund N, Lundberg A, Rätti O, Vouti J (2000) Combs and sexual selection in black grouse (*Tetrao tetrix*). Behavioral Ecology 11:465-471.

Rivera-Gutierrez HF, Pinxten R, Eens M (2010) Multiple signals for multiple messages: great tit, *Parus major*, song signals age and survival. Animal Behaviour 80:451-459.

Roper TJ (1999) Advances in the Study of Behavior, vol. *28* (eds) Slater PJB, Rosenblatt JS, Snowdon CT, Roper TJ. Academic Press, Boston, Massachusetts, pp 247-332.

Royle NJ, Surai PF, McCartney RJ, Speake BJ (1999) Parental investment and egg yolk lipid composition in gulls. Functional Ecology 13:298-306.

Ryan MJ (1980) Female mate choice in a neotropical frog. Science 209:523-525.

Saks L, McGraw KJ, Horak P (2003) How feather colour reflects its carotenoid content. Functional Ecology 17:555-561.

Schwensow N, Eberle M, Sommer S (2008) Compatibility counts: MHC-associated mate choice in a wild promiscuous primate. Proceedings of the Royal Society B 275:555-564.

Searcy WA, Anderson M (1986) Sexual selection and the evolution of song. Annual Review of Ecology and Systematics 17:507–533.

Senseney HL, Briles WE, Abplanalp H, Taylor RL (2000) Allelic complementation between MHC haplotypes B-Q and B-17 increases regression of Rous sarcomas. Poultry Science 79:1736-1740.

Setchell JM, Charpentier MJE, Abbott KM, Wickings EJ, Knapp LA (2010) Opposites attract: MHC-associated mate choice in a polygynous primate. Journal of Evolutionary Biology 23:136-148.

Shawkey MD, Hill GE (2005) Carotenoids need structural colours to shine. Biology Letters 1:121-124.

Slifka KA, Bowen PE, Stacewicz-Sapuntzakis M, Crissey SD (1999) A survey of serum and dietary carotenoids in captive wild animals. Journal of Nutrition 129:380-390.

Soini HA, Schrock SE, Bruce KE, Wiesler D, Ketterson ED, Novotny MV (2007) Seasonal variation in volatile compound profiles of preen gland secretions of the dark-eyed junco (*Junco hyemalis*). Journal of Chemical Ecology 33:183-198.

Spehr M, Kelliher KR, Li X-H, Boehm T, Leinders-Zufall T, Zufall F (2006) Essential role of the main olfactory system in social recognition of major histocompatibility complex peptide ligands. Journal of Neuroscience 26:1961-1970.

Stapleton MK, Kleven O, Lifjeld JT, Robertson RJ (2007) Female tree swallows (*Tachycineta bicolor*) increase offspring heterozygosity through extrapair mating. Behavioral Ecology and Sociobiology 61:1725-1733.

Stradi R (1999) Pigmenti e sistematica degli uccelli. In: Brambilla L, Canali G, Mannucci E, Massa R, Saino N, Stradi R, Zerbi G (eds) Colori in volo: il piumaggio degli uccelli. Universita degli Studi di Milano, Milan, Italy.

Takahata N, Nei M (1990) Allelic genealogy under overdominant and frequency dependent selection and polymorphism of major histocompatibility complex loci. Genetics 124:967-978.

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24:1596-1599.

Tarano Z, Herrera EA (2003) Female preferences for call traits and male mating success in the Neotropical frog *Physalaemus enesefae*. Ethology 109:121–134.

Tella JL, Negro JJ, Rodriguez-Estrella R, Blanco G, Forero MG, Blazquez MC, Hiraldo F (1998) A comparison of spectrophotometry and color charts for evaluating total plasma carotenoids in wild birds. Physiological Zoology 71:708-711.

Tella JL, Figuerola J, Negro JJ, Blanco G, Rodriguez-Estrella R, Forero MG, Blazquez MC, Green AJ, Hiraldo F (2004) Ecological, morphological, and phylogenetic correlates of interspecific variation in plasma carotenoid concentrations in birds. Journal of Evolutionary Biology 17:156-164.

Tregenza T, Wedell N (2000) Genetic compatibility, mate choice and patterns of parentage: invited review. Molecular Ecology 9:1013-1027.

Trivers RL (1972) Parental investment and sexual selection. In Campbell B (ed) Sexual selection and the descent of man. Chicago, IL: Aldine-Atherton, pp136-179.

Velando A, Beamonte R, Torres R (2006) Pigment-based skin colour in the blue-footed booby: an honest signal of current condition used by females to adjust reproductive investment. Oecologia 149:543-552.

Wang JL (2002) An estimator for pairwise relatedness using molecular markers. Genetics 160:1203-1215.

Westerdahl H (2004) No evidence of an MHC-based female mating preference in great reed warblers. Molecular Ecology 13:2465-2470.

Westneat MW, Long JH, Hoese W, Nowicki S (1993) Kinematics of birdsong: functional correlation of cranial movements and acoustic features in sparrows. Journal of Experimental Biology 182:147–171.

Wetton JH, Carter RE, Parkin DT, Walter D (1987) Demographic study of a wild house sparrow population by DNA fingerprinting. Nature 327:147-149.

Work TM, Rameyer RA (1996) *Haemoproteusiwa* n. sp. in great frigatebirds (*Fregata minor* [Gmelin]) from Hawaii: parasite morphology and prevalence. Journal of Parasitology 82:489-491.

Wright SG, Dearborn DC (2009) Male ornament variation in a sexually dimorphic seabird with variable male mating success. Evolutionary Ecology Reseach 11:759-770.

Yamazaki K, Boyse EA, Miké V, Thaler HT, Mathieson BJ, Abbott J, Boyse J, Zayas ZA, Thomas L (1976) Control of mating preferences in mice by genes in major histocompatibility complex. Journal of Experimental Medicine 144:1324-1335.

Zahavi A (1975) Mate selection – a selection for a handicap. Journal of Theortical Biology 53:205-214.

Zeh JA, Zeh DW (1996) The evolution of polyandry I: intragenomic conflict and genetic incompatability. Proceedings of the Royal Society B 263:1711-1717.

Zelano B, Edwards SV (2002) An mhc component to kin recognition and mate choice in birds: predictions, progress, and prospects. American Naturalist 160:S225-S237.

Ziegler RG (1989) A review of epidemiologic evidence that carotenoids reduce the risk of cancer. Journal of Nutrition 119:116-122.

Zonios G, Bykowski J, Kollias N (2001) Skin melanin, hemoglobin, and light scattering properties can be quantitatively assessed *in vivo* using diffuse reflectance spectroscopy. Journal of Investigative Dermatology 117:1452-1457.

Zuk M, Thornhill R, Ligon JD (1990a) Parasites and mate choice in red jungle fowl. American Zoologist 30:235-244.

Zuk M, Thornhill R, Ligon JD, Johnson K, Austad S, Ligon S, Thornhill NW, Costin C (1990b) The role of male ornaments and courtship behavior in female mate choice of red jungle fowl. American Naturalist 136:458-473.