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# Parasites, Immune Response, and Sexual Selection in Western Bluebirds

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of the requirements for the degree of

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Evolution, Ecology, and Organismal Biology

by

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Chapter 1: **Sexual Selection and Parasites: Do Mechanisms Matter?**

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Chapter 4: **What determines the rates of double brooding in Western Bluebirds?**

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## Dedication

To my mother, Catherine Jacobs, for getting me interested in this crazy field in the first place. I'll be lucky if I end up half the woman you are.

ABSTRACT OF THE DISSERTATION

Parasites, Immune Response, and Sexual Selection in Western Bluebirds

by

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The Hamilton-Zuk hypothesis proposes that females choose elaborately ornamented males as mates because such ornaments indicate parasite resistance. Females benefit from selecting resistant males by passing resistance genes on to the offspring. Many studies have tested this hypothesis using immunity as a proxy for parasite resistance; however, the relationship between the strength of the immune response and the quality of the sexual signal remains controversial, and such studies rarely consider whether immunity provides a good measure of parasite resistance. Here I examined the relationship between immune response, parasite load, and sexual signaling, as well as what factors affect mating and reproductive success in western bluebirds (*Sialia mexicana*). Coloration appeared to act as a signal in social mate choice, and social pairs mate assortatively by UV-blue coloration. Males with brighter UV-blue coloration also sired more of the offspring within their own nests. However, coloration played no role in extra-pair mate choice. Likewise, immune response did not predict coloration (sexual signal quality) or male reproductive success. Immune response did relate to parasite loads, however, and

individuals that had a stronger immune response tended to have lower levels of infection with avian malaria. Parasite infection and age both predicted male success at siring extra-pair offspring; extra-pair males tended to be older and uninfected. This suggests that females select extra-pair males based on their ability to resist parasites, consistent with the Hamilton-Zuk hypothesis. However, uninfected males did not have higher reproductive success than infected males, despite the advantage of siring extra-pair offspring. Double brooding, another method by which birds can increase their reproductive success in a season, occurred rarely in our population, with only 5% of pairs producing second broods. Pairs that produced second broods tended to breed earlier and the females in these pairs tended to be heavier. Environment also played a role in double brooding, with more pairs producing second broods during years in which the breeding season was long, warm, and dry. This highlights the importance of parasites and environmental factors in determining mate choice and reproductive success.

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## Dissertation Introduction

Many animals possess elaborate traits, such as the long tail of a peacock or the large antlers on a male elk, that seem detrimental to the fitness of their bearers. Darwin (1871) was the first to propose an explanation for how these traits could evolve. Rather than enhancing survival, such traits enhanced fitness by allowing their bearers, usually males, better access to mates. Darwin described two classes of such traits: weapons, which are used in male-male competition for access to females, and ornaments, which evolve through female choice and are used by males to attract more mates.

The function of weapons is fairly straightforward and clear; however, ornaments provide more of a puzzle. Why would certain traits, like a long tail on a bird or the song of a male cricket, make females more likely to mate with the males bearing them? Several hypotheses have been proposed for why females prefer ornamented males (Andersson 1994). Fisher (1958) proposed that such traits are largely arbitrary, and that such ornaments evolve via runaway selection. Under this system, arbitrary female preference leads choosy females to mate with elaborately ornamented males. These males therefore enjoy a higher fitness as a result of this preference, and any offspring produced will inherit the gene(s) for the preference as well as the gene(s) for the ornament, leading to a simultaneous increase of the preference genes and the ornamentation genes in the population. Over time, this runaway process is expected to result in the evolution of increasingly elaborate ornaments, until the costs of natural selection acting against ornament bearers matches the benefit gained by having an ornament (Fisher 1958).

The Fisherian runaway selection model assumes that any female preference for male ornaments is purely arbitrary and that ornaments themselves do not indicate anything about male other than his attractiveness to females. However, others have suggested that ornaments may indicate some other aspect of male quality, and that females selecting ornamented males benefit because they gain superior viability genes for their offspring (Pomiankowski 1987, Andersson 1994). The offspring of these preferred males survive better, thus conferring indirect fitness benefits on the female (Petrie 1994).

Precisely which genes qualify as "genes for fitness"? There are many possible candidates, but special attention is given to genes for parasite resistance. Parasites are ubiquitous, and infection can severely reduce host fitness. The idea that sexual signals evolved, at least in part, to signal parasite infection status came from Hamilton and Zuk (1982). They proposed that females would benefit from paying attention to signals that revealed parasite infection because, by avoiding highly parasitized mates, they could select individuals with genes for resistance and ensure that their offspring had such genes as well. Moreover, because hosts and parasites constantly coevolve, genes for resistance would change over time and never go to fixation in the population. The Hamilton-Zuk hypothesis has generated a number of studies which pose the question of whether sexual signals provide information about parasite infection and whether females pay attention to these signals. Many have turned to immune response as a proxy for parasite resistance to test this hypothesis (Møller et al. 1999), assuming that a stronger immune response results in greater resistance and lower parasite burdens.

While the use of immunity in place of parasites seems reasonable, several critical issues have arisen. First, while many assume that a strong immune response results in lower parasite burdens, few studies actually attempt to validate this assumption. In some cases, certain measures of immune response may have no effect on an animal's ability to resist parasites (Adamo 2004), and in other cases, the individuals that mount the strongest immune response may actually be the most susceptible (Auld et al. 2010). Certain genes, such as MHC alleles, may confer resistance or susceptibility to infection (Dunn et al. 2012, Westerdahl et al. 2012), but may not be associated with a change in immune response (Bonneaud et al. 2009). Thus, rather than relying exclusively on immune response as a measure of an animal's ability to resist parasites, it is also critical to include measurements of individual parasite burden when considering the relationship between immunity and sexual signaling (Graham et al. 2011).

In addition to considering the immune system's relationship to parasite burden, one must also consider the costs of immunity. Researchers agree that immunity is costly (Sheldon and Verhulst 1996), and mounting an immune response can take resources away from other processes, resulting in trade-offs between immune response and survival (Hanssen et al. 2004), reproductive investment (Råberg et al. 2000, Hanssen et al. 2005, McKean et al. 2008), and sexual signal quality (Verhulst et al. 1999, Jacot et al. 2004). These costs are thought to help maintain the honesty of sexual signaling, such that only a high-quality individual can afford to produce a good signal without succumbing to parasites (Folstad and Karter 1992, Sheldon and Verhuslt 1996). However, different researchers have interpreted this different ways, with some predicting that, due to the

costs of immunity, individuals with high-quality sexual signals should have reduced immune response (Skarstein and Folstad 1996), while others predict the opposite (López and Martín 2005). In some cases, positive and negative correlations between immune response and signal quality have been reported within the same species, depending upon the measure of immunity used (Møller and Petrie 2002, Garvin et al. 2008). Thus the relationship between immunity and sexual signaling remains controversial.

Much of the work testing for a relationship between sexual signals and parasite and/or immune response has been done in birds (Møller 1990, Zuk et al. 1990, Lindström and Lundström 2000, Griggio et al. 2010). Traditionally, many species of birds were thought to be strictly monogamous (Lack 1968). However, the use of molecular methods has revealed that, in many cases, passerines are not truly monogamous if one considers their genetic mating system. Rather, they are socially monogamous; one male will pair up with one female to raise the offspring in a given nest, but one or both members of the pair will mate with other individuals outside the pair bond (Griffith et al. 2002). These extra-pair matings can allow males to greatly increase their reproductive success and can increase the strength of sexual selection (Albrecht et al. 2009, Balenger et al. 2009a).

Socially monogamous birds provide an excellent system in which to examine mate choice because they allow us to tease apart the influence of direct and indirect benefits on mate choice. As female choice can be costly, one expects that females gain some benefit from selecting certain males (Pomiankowski 1987). These can come in the form of direct benefits (such as food, superior nest sites, etc.) and/or indirect (genetic) benefits, which increase the fitness of a female's offspring. Social mate choice, or female

choice for a social partner, may be largely determined by direct benefits. During the breeding season, females will often share a territory with the male, and males can provide a substantial amount of care to the offspring (Lack 1968, Mock 1991). Thus females should choose social mates with good territories (Alatalo 1986) and/or males that can provide parental care (Hoelzer 1989). In contrast, extra-pair males rarely provide females with direct benefits; females may choose such males to gain genetic benefits for their offspring (Griffith et al. 2002).

While extra-pair copulations can greatly increase a male's reproductive success (O'Brien and Dawson 2011), many other factors also contribute to the successful production of surviving offspring and therefore individual fitness. For example, in birds, predation may account for a large number of nest failures (Ricklefs 1969) and thus dramatically affect reproductive success independent of mate choice. Aside from production of extra-pair offspring, individuals can increase the number of offspring they have by increasing brood size or by increasing the number of broods they produce in a given season. This latter method, double brooding, is common in many species of birds (Geupel and DeSante 1990, Moore and Morris 2005, Monroe et al. 2008) and can greatly increase an individual's reproductive success in a given season (Morrison and Bolger 2002, Monroe et al. 2008). The ability of a pair to produce a second brood may depend, at least in part, on some of the same factors that determine an individual's ability to get a mate, such as a high-quality territory (Nagy and Holmes 2005).

This dissertation focuses on sexual selection and reproductive success in wild birds using the western bluebird (*Sialia mexicana*) as the primary study species. I

examine how parasites and sexual signals relate to one another in the wild and also the role these factors play in sexual signal quality and mate choice in this species. In particular, I test whether a male's parasite load and ability to mount an immune response predict the quality of his sexual signal (plumage coloration) and his reproductive success, including both within-pair and extra-pair young. I also ask what factors affect the likelihood that pairs will increase their reproductive success by producing a second brood.

Western bluebirds are socially monogamous passerines, and up to 45% of nests can have extra-pair offspring (Dickinson 2003). They nest in pre-existing cavities in trees, although they will readily use human-made nestboxes (Guinan et al. 2008), allowing for ease of nest-finding and access to the nestlings. Bluebirds display brightly-colored plumage, with UV-blue coloration covering most of the body and a large chestnut patch on the breast (Siefferman and Hill 2003, McGraw et al. 2004). This species is sexually dichromatic, with males displaying brighter plumage than females, implying that plumage may be a sexually selected signal (Darwin 1871, Møller and Birkhead 1994). Studies on other species of bluebird have suggested that coloration may play a role in female choice (Balenger et al. 2009b), making it a probable target for sexual selection.

In the first chapter, I review past studies looking for a relationship between sexual signaling and immune response. Many such studies have found conflicting results, with some reporting that a stronger immune response correlates with better sexual signal quality, and others reporting the opposite result. I discuss possible reasons for the discrepancies, as well as future avenues for research in this area.

In chapter two, I examine whether plumage coloration functions as a sexual signal in western bluebirds. Plumage coloration acts as an important sexual signal in many birds (Hill 2006), and I test whether male coloration predicts success at siring both within-pair and extra-pair offspring. Both male and female bluebirds display colorful plumage, and I examine whether mutual mate choice can account for this pattern by determining whether social pairs mate assortatively by color. I also looked at whether females and their extra-pair mates mated assortatively by color.

Chapter three goes on to determine how parasites and immune response may influence sexual signal quality and mate choice. I measure infection with avian malaria, predicting that individuals with the strongest immune response will have the lowest parasite burdens. Uninfected individuals should also have higher mating success and sire more extra-pair offspring than their infected counterparts. I also hypothesize that, due to the costs of immunity, too strong of an immune response may result in lower fitness and such males will not be preferred by females. Rather males that have an immune response of intermediate strength will have the highest mating success.

In the fourth chapter, I examine double brooding in this population, which has the potential to greatly increase an individual's reproductive success. I determine whether individual factors, such as mass and breeding date, make a pair more likely to produce a second brood in a season, as well as the environmental factors that play a role in determining population rates of double brooding.

**References**


Adamo S.A. (2004) Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. *Journal of Insect Physiology* 50:209-216.

Alatalo R.V., Lundberg A., and Glynn C. (1986) Female pied flycatchers chose territory quality and not male characteristics. *Nature* 323:152-153.

Albrecht T., Vinkler M., Schnitzer J., Poláková R., Munclinger P., and Bryja J. (2009) Extra-pair fertilizations contribute to selection on secondary male ornamentation in a socially monogamous passerine. *Journal of Evolutionary Biology* 22:2020-2030.

Andersson M. (1994) *Sexual Selection*. Princeton: Princeton University Press.

Auld S.K.J.R., Scholefield J.A., and Little T.J. (2010) Genetic variation in the cellular response of *Daphnia magna* (Crustacea, Cladocera) to its bacterial parasite. *The Proceedings of the Royal Society B* 277:3291-3297.

Balenger S.L., Johnson L.S., Mays H.L., and Masters B.S. (2009a) Extra-pair paternity in the socially monogamous mountain bluebird *Sialia currucoides* and its effect on the potential for sexual selection. *Journal of Avian Biology* 40: 173-180.

Balenger S.L., Johnson L.S., and Masters B.S. (2009b) Sexual selection in a socially monogamous bird: male color predicts paternity success in the mountain bluebird. *Behavioral Ecology and Sociobiology* 63: 403-411.

Bonneaud C., Sinsheimer J.S., Richard M., Chastel O., and sorci G. (2009) Mhc polymorphisms fail to explain the heritability of phytohaemagglutinin-induced skin swelling in a wild passerine. *Biology Letters* 5:784-787.

Darwin C. (1871) *The Descent of Man, and Selection in Relation to Sex*. (Reprinted 2006) New York: W.W. Norton and Company, Inc.

Dickinson J.L. (2003) Male share of provisioning is not influenced by actual or apparent loss of paternity in western bluebirds. *Behavioral Ecology* 14: 360-366.

Dunn P.O., Bollmer J.L., Freeman-Gallent C.R., and Whittingham L.A. (2012) MHC variation is related to a sexually selected ornament, survival, and parasite resistance in common yellowthroats. *Evolution* 67:679-687.

Fisher R.A. (1958) *The Genetical Theory of Natural Selection* (2nd Ed.). New York: Dover.

Folstad I. and Karter A.J. (1992) Parasites, bright males, and the immunocompetence handicap hypothesis. *American Naturalist* 139:603-622.

Garvin J.C., Dunn P.O., Whittingham L.A., Steeber D.A., and Hasselquist D. (2008) Do male ornaments signal immunity in the common yellowthroat? *Behavioral Ecology* 19:54-60.

Geupel G.R., and DeSante D.F. (1990) Incidence and determinants of double brooding in Wrentits. *Condor* 92:67-75.

Graham A.L., Shuker D.M., Pollitt L.C., Auld S.K.J.R., Wilson A.J., and Little T.J. (2011) Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology. *Functional Ecology* 25:5-17.

Griffith S.C., Owens I.P.F., and Thuman K.A. (2002) Extra-pair paternity in birds: a review of interspecific variation and adaptive function. *Molecular Ecology* 11: 2195-2212.

Griggio M., Zanollo V., and Hoi H. (2010) UV plumage color is an honest signal of quality in male budgerigars. *Ecological Research* 25:77-82.

Guinan J.A., Gowaty P.A., and Eltzroth E.K. (2008) Western Bluebird (Sialia mexicana),. In A. Poole, Ed. *The Birds of North America Online* Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online: http://bna.birds.cornell.edu/bna/species/510doi:10.2173/bna.510

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

Hanssen S.A., Hasselquist D., Folstad I., and Erikstad K.E. (2004) Costs of immunity: immune responsiveness reduces survival in a vertebrate. *Proceedings of the Royal Society: B* 271:925-930.

Hanssen S.A., Hasselquist D., Folstad I., and Erikstad K.E. (2005) Cost of reproduction in a long-lived birds: incubation effort reduces immune function and future reproduction. *Proceedings of the Royal Society: B* 272:1039-1046.

Hill G.E. (2006) Female mate choice for ornamental coloration. In: Hill G.E. and McGraw K.J. ed. *Bird Coloration: Function and Evolution* (Vol. 2). Cambridge: Harvard University Press.

Hoelzer G.A. (1989) The good parent process of sexual selection. *Animal Behaviour* 38:1067-1078.

Jacot A., Scheuber H., and Brinkhof M.W.G. (2004) Costs of an induced immune response on sexual display and longevity in field crickets. *Evolution* 58:2280-2286.

Lack D. (1968) *Ecological Adaptations for Breeding in Birds*. London: Methuen & Co. Ltd.

Lindström K. and Lundström J. (2000) Male greenfinches (*Carduelis chloris*) with brighter ornaments have higher virus infection clearance rate. *Behavioral Ecology and Sociobiology* 48:44-51.

López P. and Martín J. (2005) Female Iberian wall lizards prefer male scents that signal a better cell-mediated immune response. *Biology Letters* 1:404-406.

McGraw K.J., Wakamatsu K., Ito S., Nolan P.M., Jouventin P., Dobson F.S., Austic R.E., Safran R.J., Siefferman L.M., Hill G.E., and Parker R.S. (2004) You can't judge a pigment by its color: carotenoid and melanin content of yellow and brown feathers in swallows, bluebirds, penguins, and domestic chickens. *Condor* 106:390-395.

McKean K.A., Yourth C.P., Lazzaro B.P., and Clark A.G. (2008) The evolutionary costs of immunological maintenance and deployment. *BMC Evolutionary Biology* 8:76.

Mock P.J. (1991) Daily allocation of time and energy of western bluebirds feeding nestlings. *Condor* 93:598-611.

Møller A.P. (1990) Effects of a haematophagous mite on the barn swallow (*Hurundo rustica*). *Evolution* 44:771-784.

Møller A.P. and Birkhead T.R. (1994) The evolution of plumage brightness in birds is related to extrapair paternity. *Evolution* 48:1089-1100.

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

Møller A.P. and Petrie M. (2002) Condition dependence, multiple sexual signals, and immunocompetence in peacocks. *Behavioral Ecology* 13:248-253.

Monroe A.P., Hallinger K.K., Brasso R.L., and Cristol D.A. (2008) Occurrence and implications of double brooding in a southern population of Tree Swallows. *Condor* 110:382-386.

Moore D.J. and Morris R.D. (2005) The production of second clutches in the Common Tern: proximate effects of timing and food supply. *Waterbirds* 28:458-467.

Morrison S.A., and Bolger D.T. (2002) Variation in a sparrow's reproductive success with rainfall: food and predator-mediated processes. *Oecologia* 133:315-324.

Nagy L.R. and Holmes R.T. (2005) To double-brood or not? Individual variation in the reproductive effort in Black-throated Blue Warblers (*Dendroica caerulescens*). *Auk* 122:902-914.

O'Brien E.L. and Dawson R.D. (2011) Plumage color and food availability affect male reproductive success in a socially monogamous bird. *Behavioral Ecology* 22:66-72.

Petrie M. (1994) Improved growth and survival of offspring of peacocks with more elaborate trains. *Nature* 371:598-599.

Pomiankowski A. (1987) The costs of choice in sexual selection. *Journal of Theoretical Biology* 128:195-218.

Råberg L., Nilsson J., Ilmonen P., Stjernman P., and Hasselquist D. (2000) The cost of an immune response: vaccination reduces parental effort. *Ecology Letters* 3:382-386.

Ricklefs R.E. (1969) An analysis of nesting mortality in birds. *Smithsonian Contributions to Zoology* 9:1-48.

Sheldon B.C. and Verhulst S. (1996) Ecological immunity: costly parasite defenses and trade-offs in evolutionary ecology. *Trends in Ecology and Evolution* 11:317-321.

Siefferman L. and Hill G.E. (2003) Structural and melanin coloration indicate parental effort and reproductive success in male eastern bluebirds. *Behavioral Ecology* 14:855-861.

Skarstein F. and Folstad I. (1996) Sexual dichromatism and the immunocompetence handicap: an observational approach using arctic charr. *Oikos* 76:359-367.

Verhulst S., Dieleman S.J., and Parmentier H.K. (1999) A trade-off between immunocompetence and sexual ornamentation in domestic fowl. *Proceedings of the National Academy of Sciences USA* 96:4478-4481.

Westerdahl H., Asghar M., Hasselquist D., and Bensch S. (2012) Quantitative disease resistance: to better understand parasite-mediated selection on major histocompatibility complex. *Proceedings of the Royal Society: B* 279:577-584.

Zuk M., Thornhill R., Ligon J.D., and Johnson K. (1990) Parasites and mate choice in red jungle fowl. *American Zoologist* 30:235-244.

# Chapter 1

## Sexual Selection and Parasites: Do Mechanisms Matter?

## Introduction

Parasites have long been appreciated as a challenge that most, if not all, organisms must face, one that may impact many aspects of host biology and fitness. In particular, the effects of parasites on host mating decisions and mating behavior have become an area of active study in recent years. The potential importance of parasites in mate choice was first pointed out by Hamilton and Zuk (1982). The Hamilton-Zuk hypothesis postulates that exaggerated ornaments and sexual signals arose in part because they can signal parasite resistance, and that females should prefer flashier mates because the genes for resistance would be passed on to their offspring (Hamilton and Zuk 1982).

The Hamilton-Zuk hypothesis is not alone in suggesting that females may benefit by choosing parasite-free mates. Other ideas, such as the transmission avoidance hypothesis and the resource provisioning model (also sometimes called the good parent hypothesis) also make this prediction (Clayton 1991, Møller 1994). The transmission avoidance hypothesis states that females should avoid parasitized mates to avoid becoming infected themselves, while the resource provisioning model assumes that parasite-free mates should be better at providing resources to females and offspring (Clayton 1991). However, one key difference between these models and the Hamilton-Zuk hypothesis is that the Hamilton-Zuk hypothesis specifically states that parasite resistance is heritable and preference for healthy individuals is favored because females obtain genes for resistance for their offspring. This idea that females select for resistance genes is an appealing one, as co-evolution between hosts and pathogens may explain why genes for fitness do not go to fixation, even when subjected to strong selection pressure

from female preference. The Hamilton-Zuk hypothesis has generated both intra- and inter-specific predictions about the connection between parasite levels and sexual signals. Within a species, one expects preferred mates to have flashier sexual signals and fewer parasites. When comparing multiple species, one would expect selection pressure on sexual ornaments to be strongest in species infected with more parasites, as females would benefit more from choosing resistant mates in such species. Thus one would predict that the species with the flashiest sexual signals would be infected by the greatest number of parasites.

After the publication of the Hamilton-Zuk hypothesis, many scientists set out to test the relationship between parasites and sexual signals (reviewed in Clayton 1991, Zuk 1992, Møller et al. 1999). Most of the studies focused on the intra-specific effects of parasites on mate choice. Researchers often picked a single parasite species to work with, studying its effects on host sexual signaling and mating preference. For instance, Møller (1990) found negative effects of an ectoparasitic mite on tail length in barn swallows, a species in which the length of the tail is an important sexual signal. Zuk et al. (1990) found that nematode infection decreased sexual signal quality while having few or no effects on non-sexually selected traits in jungle fowl, and that females selecting mates preferred unparasitized males with better sexual signals.

However, while the Hamilton-Zuk hypothesis set very clear and testable predictions about the relationship between parasites and sexual signals, the authors also cautioned that not every parasite species could be expected to influence the evolution of sexual signals in the same way. Parasites that killed hosts too quickly would leave only

resistant individuals in the population, thus ensuring that every individual encountered would be resistant and removing the need for choosiness. On the other hand, benign parasites would not cause enough harm for choosy females to benefit by selecting mates with genes for parasite resistance. Thus, the hypothesis suggested that the parasites likely to be most important to the evolution of sexual signals were those that caused chronic, debilitating diseases (Hamilton and Zuk 1982). This creates a potential problem for researchers testing the Hamilton-Zuk hypothesis, as picking the "wrong" parasite to work with might result in there being no relationship observed between parasites and sexual signaling.

While many studies have examined the effects of a parasite that causes a chronic infection on sexual signals, one may question how broadly the results of such studies can be interpreted. Examining the effects of a single parasite on sexual signals can provide useful information, but it may not tell us how an animal may respond to other parasites and pathogens. As animals are exposed to many species of parasites over the course of their lifetimes, it is difficult to evaluate the importance of any one in particular. Are females interested in males capable of resisting that one species, or do they prefer males that can respond to parasites in general and avoid or resist infection by a large number of pathogens? Answering the latter question when examining parasites alone is difficult, as it is often unfeasible to measure the total parasite burden of an animal. Thus, many scientists have instead chosen to look at immune response. The immune system has evolved to deal with most, if not all, of the parasites an animal may encounter, and thus should provide an acceptable idea of an animal's parasite resistance (Møller et al. 1999).

This new direction brought scientists interested in sexual selection into the field of ecological immunology (Sheldon and Verhuslt 1996, Møller et al. 1999, Lawniczak et al. 2007).

Ecological immunology is based on the principle that immune response is a necessary but costly trait in terms of energy and resources, and that these costs make immune defense a candidate for trade-offs with other life-history traits, such as reproductive effort (Zuk 1990, Sheldon and Verhulst 1996, Zuk and Stoehr 2002, French et al. 2009). The issue of costs of immunity is an important one from the perspective of sexual signaling because it helps explain how signal honesty is maintained (Kotiaho 2001). In order for signals to remain honest indicators of quality, some cost must be associated with both signals and immune response, creating a negative relationship between immunity and signal quality within an individual. These costs impose a handicap that can maintain signal reliability, as low-quality males will not be able to pay the costs associated with high signal quality (Zahavi 1975, Nur and Hasson 1984, Getty 2002).

While the idea that immune response may be linked to sexual signals started as a development of the Hamilton-Zuk hypothesis, the Hamilton-Zuk hypothesis is not quite the same as many of the ideas put forward in ecological immunology. Both seek to put immunology in a more evolutionary context by pointing out that parasites and pathogens represent an important selection pressure to their hosts and that defenses to fight off these pathogens should be under strong selection (Zuk 1994, Sheldon and Verhulst 1996, Zuk and Stoehr 2002). However, the Hamilton-Zuk hypothesis emphasizes genetic-based

parasite resistance as the factor a female should care most about in a mate. Only a resistant male would have low enough parasite burdens to produce a high-quality sexual signal. Also, the Hamilton-Zuk hypothesis relies on co-evolutionary cycles between hosts and their pathogens to generate a constant source of genetic variability in quality (Hamilton and Zuk 1982). In contrast, ecological immunologists tend to focus more on the ways in which energy allocation to immunity versus signaling can influence sexual signal quality. This explanation does not include parasites directly, although parasites are certainly important in that they impose a cost on individuals who allocate too many resources away from the immune system. Instead of focusing on parasites and how they interact with their hosts directly, ecological immunology relies more on the costs of immunity as the all-important explanation behind signal honesty. This causes ecological immunologists to emphasize the importance of an animal's general immune response, rather than resistance to a given pathogen (Westneat and Birkhead 1998).

While fundamental differences exist between the Hamilton-Zuk hypothesis and ecological immunology's emphasis on energetic trade-offs, both predict that sexual signaling and immunity should be tied together. Much of the work examining sexual selection and immunology has attempted to link variation in immune response with variation in sexual signals and/or mating success. To do this, many studies have used various measures of immune response and looked to see whether these measures are correlated with the quality of sexual signals. Table 1.1 shows a summary of such correlational studies and their results. Other studies have experimentally manipulated ornaments or challenged the immune system with an antigen. For instance, Saino and

Møller (1996) manipulated the tail length of male barn swallows (a sexual signal in this species) and found that immune response decreased in males with lengthened tails, but that males that had had longer tails to begin with were better able to cope with the manipulation, implying that individual quality and immune response are linked. López et al. (2009) found that challenging the immune systems of male lizards caused them to develop duller ventral coloration.

While much research suggests that immunity and sexual signaling are linked, several questions about the nature of that relationship remain. First, we lack clearly defined predictions about the expected relationship between immune response and sexual signal quality. Studies that find opposite results may both be interpreted as supporting the same hypothesis, leading to a great deal of confusion. The actual, detailed mechanisms that link immunity and signaling remain controversial. Many different hypotheses have been proposed (Fig. 1.1), and a considerable body of work has been devoted to trying to test these hypotheses. However, we propose that most of the hypotheses generated specifically to explain how sexual signaling relates to immunity are insufficient and do not properly address or explain the conflicting results obtained in the field. We use this chapter to address the following questions: 1) What is the predicted relationship between sexual signal quality and immune response? 2) What results have been obtained by studies looking for a relationship between sexual signals and immunity? 3) In cases where different studies have obtained conflicting results, how can we explain these discrepancies? We begin with a brief overview of the prominent mechanisms proposed to explain how immunity relates to sexual signal quality (Fig. 1.1). We then

explain why we think that these hypotheses by themselves are insufficient and we draw on work done in other areas of the field of ecological immunology in an attempt to explain some of the conflicting results obtained to date.

## What Is Immunity and How Do We Measure It?

Anyone who has studied immunity can agree at least on one point: the immune system is extremely complex. In fact, in vertebrates, immunity is often broken down into many parts, each corresponding to a certain "arm" of the immune system. When one takes into account the many parts of the immune system, defining what constitutes a strong immune system can become extremely difficult and tricky, as we discuss later. Many scientists talk about an animal's "immunocompetence", or the overall ability of that animal to use its immune system to resist parasites. In practice, of course, measuring immunocompetence is next to impossible (Zuk and Stoehr 2002). However, many studies try to be as thorough as possible by employing a variety of immunological measures to try to quantify an animal's overall immune response. The commonly used divisions of the immune system as well as methods used to quantify them are as follows.

### *Innate Immunity*

Innate immunity describes the actions of many cells or molecules that are always present and designed to fight off many different potential threats. These include natural killer cells, compliment, macrophages, etc. Some immunologists also include physical barriers to infection, such as skin, in this category (Weir and Stewart 1993), although the

ecological immunology literature does not usually consider such barriers. To quantify innate immunity, many researchers measure some part of the defenses already present, usually using a blood sample. One such popular method is to count the number and varieties of leukocytes in the blood (Skarstein and Folstad 1996, Zuk and Johnson 1998), although such measures may be difficult to interpret (Sheldon and Verhulst 1996, Norris and Evans 2000). A more recent, and more easily interpreted, method involves measuring the ability of blood to kill bacteria (Millet et al. 2007).

*Acquired Immunity*

Acquired immunity differs from innate immunity in that it is activated in response to a specific challenge. In vertebrates, it also produces immunological memory, whereby the immune system "remembers" specific pathogens that the organism has encountered in the past. This enables the mounting of a more efficient secondary response to that pathogen if it is ever encountered again. Methods to measure this type of immunity usually involve challenging the animal in some way with a novel antigen and measuring the response. Acquired immunity is further broken down into cell-mediated and humoral immunity (Weir and Stewart 1993).

Cell-mediated immunity describes the response of the T-lymphocytes. One very popular field method to measure this is the phytohaemagglutinin (PHA) test, in which a mitogen is injected into the animal's body and the resulting swelling is measured (Møller and Petrie 2002, Martín et al. 2007, Garvin et al. 2008, Griggio et al. 2010). PHA is used to stimulate T-lymphocyte proliferation in the lab, and thus this test has been thought to

indicate cell-mediated immunity (Kennedy and Nager 2006). However, while T-lymphocytes are certainly involved in producing this swelling, other cells from the innate portion of the immune system may respond as well (Martin et al. 2006b, Tella et al. 2008). Thus, ecological immunologists must be cautious in their interpretations of this test (Kennedy and Nager 2006).

Humoral immunity covers the actions of the B-lymphocytes and the antibodies they produce. Popular tests to measure this involve challenges with various antigens designed to elicit an antibody response. The antigens commonly used include sheep red blood cells (Møller and Petrie 2002, Bonneaud et al. 2005), tetanus-diphtheria vaccines (Råberg et al. 2000; Hanssen et al. 2008), and others (Aguilera and Amat 2007). For more a complete discussion of methods to measure immunity, see reviews by Norris and Evans (2000), Grasman (2002), and Salvante (2006).

## Sexual Selection and Immune Response: Popular Mechanisms

### *Immunity as a Life History Trait*

Perhaps the simplest way of explaining the link between immune response and sexual signals is the one first described by Sheldon and Verhulst (1996). Under this scenario, immunity is viewed as a life history trait, the costs of which must be balanced with competing costs in a way that maximizes reproductive success. If an animal allocates more energy to immunity, less energy will be available for breeding or other functions. The males best able to balance these costs or those which have more energy available to allocate will be the most successful. This implies that such energetic trade-

offs are what determine the quality and honesty of sexual signals. Genetic resistance to parasites and the co-evolutionary cycles that are expected to result from that do not play a role in this mechanism, setting it apart from the original Hamilton-Zuk hypothesis (Westneat and Birkhead 1998). The life history explanation of immune response and sexual signaling is very general, and some of the more detailed physiological mechanisms proposed to explain why sexual signals may indicate immunity can be seen as extensions of this idea.

As mentioned earlier, under this hypothesis immune response must be costly to keep sexual signals honest. The costs of immunity have been demonstrated numerous times (reviewed in Zuk and Stoehr 2002, Schmid-Hempel 2003). Evidence for these costs comes both from studies that show a physiological and/or behavioral cost to immune activation (e.g. Råberg et al. 2000, Whitaker and Fair 2002, Jacot et al. 2004, Marzal et al. 2007), and from selection experiments on laboratory populations. For instance, in domestic fowl, males selected for high immune response had smaller sexual ornaments (Verhulst et al. 1999), while in *Drosophila melanogaster*, males selected for increased ability to secure mates had lower immune responses (McKean and Nunney 2008). The costs associated with immune response may ensure that only high-quality individuals are able to afford such costs and still produce a good sexual signal.

*The Immunocompetence Handicap Hypothesis*

One of the first and most prominent ideas explaining the link between immunity and signaling is the immunocompetence handicap hypothesis (Folstad and Karter 1992).

This hypothesis suggests that testosterone provides the mechanistic link between signals and immune response. Because testosterone can be immunosuppressive and is necessary for the development of many sexual signals in vertebrates, it may create a system of trade-offs such that only males of high quality can afford to suppress their immune systems to the point necessary for signal development. Like the life-history explanation of immunity, the immunocompetence handicap hypothesis predicts that, within an individual, there should be a trade-off between immunity and sexual signal quality. However, immunocompetence handicap hypothesis is novel in that it relies on hormones (and testosterone in particular) to create this trade-off and maintaining signal honesty. This put testosterone's regulation of immune response and sexual signals into a larger, evolutionary picture.

The immunocompetence handicap hypothesis as it was initially proposed differed greatly from the life history explanation of immunity and signaling. Although some of their basic predictions are similar, the immunocompetence handicap hypothesis relied solely on testosterone as the factor mediating trade-offs between immunity and sexual signaling, without having to invoke any energy or resource-based explanations for why immune response should decrease as sexual signal quality increased. However, one could argue that if testosterone alone was responsible for this trade-off, there would be nothing to prevent the spread of a mutant "cheater" whose immune system did not react to testosterone. Such a mutant would benefit from having highly exaggerated ornamentation without having to pay the costs in terms of immunosuppression. To explain why such a mutant could not arise, Wedekind and Folstad (1994) proposed that

immunosuppression by testosterone was adaptive because it allowed for the reallocation of resources from the immune system to the sexual signal. Seen in this light, suppression of immunity by testosterone is merely a proximate explanation for the changes in immunity predicted by life history theory. Thus, while the immunocompetence handicap hypothesis was initially proposed as a mechanism by which the Hamilton-Zuk hypothesis could operate, it deviates from the Hamilton-Zuk hypothesis by emphasizing the importance of trade-offs between immunity and signaling. Under this scenario, genetic resistance to parasites, which is crucial to the Hamilton-Zuk hypothesis, may become less important.

The immunocompetence handicap hypothesis has been extremely popular in the field of ecological immunology and a great deal of work has focused on testing some of its predictions (Evans et al. 2000, Cox and John-Alder 2007, Mougeot et al. 2009, Ros et al. 2009). However, it also suffers from several problems. One of these concerns the validity of a key assumption underlying the hypothesis, namely that testosterone is immunosuppressive. While Folstad and Karter (1992) cited numerous instances in which testosterone appeared to suppress immunity, a more recent review by Roberts et al. (2004) found at best weak support for this assertion in most vertebrate taxa. Others have suggested that testosterone does not truly suppress immunity, but that cells of the immune system are redistributed from the blood to the tissues, making them harder to detect. This response is thought to promote wound healing and avoidance of infection after stress and competitive encounters (Braude et al. 1999). This redistribution effect was proposed to explain observed changes in only certain measures of immune response, namely

lymphocyte numbers; other branches of the immune system, such as the humoral response, should not be affected by testosterone (Braude et al. 1999). Thus, the immunoredistribution hypothesis does not help to explain why immune response as a whole should appear to be lower. Also, the redistribution of immune response is thought to occur in response to stress, and there is reason to believe that stress may simply suppress immunity rather than redistributing it (Råberg et al. 1998, Adamo and Parsons 2006).

While current evidence seems to indicate that testosterone does not always suppress immune response, this does not mean that the hypothesis as a whole is completely useless. Even if testosterone itself cannot suppress immunity, it may act indirectly through other means. Many researchers have suggested that corticosterone, a stress hormone which is often elevated in individuals with high testosterone, may provide the missing link (Evans et al. 2000, Owen-Ashley et al. 2004, Roberts et al. 2004), or that corticosterone and testosterone somehow interact to produce immunosuppression (Buchanan et al. 2003). For example, Evans et al. (2000) manipulated testosterone levels in house sparrows and looked at the effects on corticosterone levels and antibody production. They found that increasing testosterone levels simultaneously increased corticosterone and that once the effects of corticosterone were accounted for, testosterone appeared to actually improve immune response. Stress hormones such as corticosterone may also be involved in the suppression of immune response during times of stress, which is thought to be adaptive (Råberg et al. 1998). Given the stress breeding males likely experience, this mechanism seems highly plausible (Zuk 1990). However, as

increased testosterone levels may be accompanied by increasing corticosterone levels (Evans et al. 2000), disentangling the effects of these two hormones may not be simple.

Other work suggests that testosterone may act by increasing damage done by oxidative stress, and that this damage reduces the quality of sexual signals (Alonso-Alvarez et al. 2007). This idea incorporates a newer mechanism, discussed below, which suggests that oxidative stress, and not testosterone per se, plays the main role in maintaining signal honesty. It should be pointed out, however, that regardless of the minute details, all of these ideas can be considered subsets of the immunocompetence handicap hypothesis, as they all suggest that testosterone is somehow related to signal development and immunosuppression, whether directly or indirectly. Thus, all of these modifications propose the same basic outcome, namely that immune response somehow trades off with sexual signal quality.

Perhaps the greatest limitation of the immunocompetence handicap hypothesis arises from the fact that this mechanism, by its very nature, only applies to certain taxa, specifically vertebrates. Invertebrates lack testosterone, and thus the hypothesis has created a divide between work done on vertebrates and work done on invertebrates. Clearly one cannot expect to find testosterone-mediated immunosuppression or signal enhancement in invertebrates, and thus most researchers interested in the immunocompetence handicap hypothesis have focused on vertebrates and largely ignored work done on invertebrates. However, whether such a divide should really exist is questionable. Compared to vertebrates, the invertebrate immune system uses very different mechanisms to fight off invaders, yet despite these differences evidence

suggests that invertebrate sexual signals may be related to immune response as well (e.g. Siva-Jothy 2000, Ahtiainen et al. 2004, Simmons et al. 2005). Ahtiainen et al. (2004) found that male wolf spiders (*Hygrolycosa rubrofasciata)* that displayed faster drumming rates (a sexual signal) also had a stronger encapsulation response (a measure of immunity). These results are similar to results obtained in many vertebrate studies (Table 1.1), implying that sexual signals may convey information about immune response in invertebrates just as they are thought to in vertebrates (Lawniczak et al. 2007).

The similarity of the results obtained for vertebrate and invertebrate work raises the question of how signal honesty is maintained. The trade-off between immune response and sexual signaling seen in vertebrates also seems to be present in invertebrates as well (Jacot et al. 2004, McKean and Nunney 2008, Leman et al. 2009), but if testosterone does not mediate such a trade-off, what does? Some researchers have proposed that the immunocompetence handicap hypothesis may still apply to invertebrates as long as some other substance performs the same function as testosterone by increasing signal quality while decreasing immune response (Rantala et al. 2003). Several candidate substances have been proposed, including juvenile hormone, which is thought to simultaneously promote traits such as dominance and sexual signaling while depressing immunity (Rantala et al. 2003, Contreras-Garduño et al. 2009). Thus, juvenile hormone is thought to act like testosterone in insects. However, juvenile hormone differs from testosterone in that it is present in both sexes, whereas testosterone is primarily associated with males (Folstad and Karter 1992, Contreras-Garduño et al. 2009). Females not producing a sexual signal would presumably have no need to suppress

immune response to promote signal expression. This hypothesis needs to focus more on how the effects of juvenile hormone differ between the sexes, if there is such a difference.

Others have suggested melanin as a substance that creates the immune-signaling trade-off. In some insect species, such as damselflies (*Calopteryx splendens xanthostoma*), melanin is used for pigmentation of sexual signals but also plays an important role in the insect immune response, making it a potentially limiting resource (Siva-Jothy 2000, Cotter et al. 2008). This argument relies more on direct allocation of resources and not hormones, and thus does not closely resemble the immunocompetence handicap hypothesis (Lawniczak et al. 2007). Regardless of the substance proposed, all of these explanations predict a negative relationship between immunity and signal quality within an individual, just as the immunocompetence handicap hypothesis does in vertebrates. However, despite the potential similarities between patterns of immunity and sexual signaling across taxa, many of the studies focusing on vertebrates neglect the invertebrate literature. This divide is unfortunate, as invertebrate species may prove to be excellent model systems for the study of ecological immunology (Lawniczak et al. 2007). It also highlights one of the major shortcomings of the immunocompetence handicap hypothesis, namely its lack of universal applicability. One might argue that this hypothesis can still be applied to invertebrates as long as researchers find some substance that acts in the same manner as testosterone by increasing signal expression and decreasing immune response in the more highly ornamented sex. However, until researchers find more evidence that such a substance exists, it seems simpler and more

universal to invoke the life history explanation to explain trade-offs between immunity and sexual signals.

*Carotenoid-based Signals*

The immunocompetence handicap hypothesis is by no means the only hypothesis attempting to explain how sexual ornaments may reliably signal immunity. Some scientists have suggested that carotenoids may provide the link. Carotenoids are molecules that are used by animals to produce red, orange, or yellow coloration of various body parts (Olson and Owens 1998, McGraw 2006). They are also thought to play a role in immunity, possibly as immunostimulants or as anti-oxidants that mitigate damage caused by the free radicals generated during an immune response (Lozano 1994, von Schantz et al. 1999). Animals lack the means to synthesize carotenoids themselves and must obtain them from their diet (Olson and Owens 1998, McGraw 2006). Such a restriction means that these molecules may be in short supply, and only healthy individuals would be able to divert enough carotenoids from their immune systems to display the bright colors often preferred in sexual signals. This can be viewed as a special case of the resource allocation issue described by Sheldon and Verhuslt (1996), in which a limiting resource can be devoted either to the immune system or to other fitness-enhancing traits (Zuk and Stoehr 2002). It should also be noted that the idea that allocation of carotenoids is important for signal honesty and the immunocompetence handicap hypothesis are not mutually exclusive ideas. Testosterone may play a role in

determining the amount of carotenoids circulating in the blood or moved to sexual signals (e.g. Alonso-Alvarez et al. 2009, Mougeot et al. 2009).

Carotenoid-based mechanisms have received quite a bit of attention. A review of the initial Hamilton-Zuk hypothesis noted that many of the sexual signals which had been successfully linked to parasites were carotenoid-based (Zuk 1992), and many different studies have looked at the effects of carotenoid supplementation on immune response (Aguilera and Amat 2007), sexual signaling (Hill 1991), or both (Blount et al. 2003, Clotfelter et al. 2007, Biard et al. 2009). More recently, however, some have started to doubt whether carotenoids deserve this special attention. For one thing, some doubt exists about the ability of carotenoids to combat oxidative damage, which is one of the assumptions often made about why carotenoids are important to immunity (Pérez-Rodríguez 2009). Some have even suggested that high levels of carotenoids may be harmful to animals, although more evidence is needed on this subject (Olson and Owens 1998, Vinkler and Albrecht 2010). Furthermore, not all studies have found a relationship between carotenoid levels and sexual signals, even in cases where carotenoids appear to influence immune response (Biard et al. 2009).

In some ways, the hypothesis that carotenoids determine signal honesty suffers from one of the same problems as the immunocompetence handicap hypothesis, namely that it cannot be universally applied. This mechanism by its very nature deals only with a limited number of signals. Carotenoid-based signals are hardly uncommon and may be found in many taxa, although most work has looked at birds (Olson and Owens 1998, McGraw 2006) or fishes (Olson and Owens 1998, Clotfelter et al. 2007). However, many

sexual and social signals exist that do not seem to involve carotenoids in any way. For example, much work has gone into studying the signaling honesty of various badges of status, such as the throat patches in house sparrows, which are not carotenoid-based displays (Møller 1987, Johnstone and Norris 1993, Evans et al. 2000). Evidence of previous immune challenges may be reflected in bird plumage that is not colored by carotenoids (Hanssen et al. 2008). Moreover, recent work by Dunn et al. (2010) suggests that carotenoid-based and non-carotenoid based ornaments may convey the same information about immunity to prospective mates. The authors examined two populations of the common yellowthroat warbler (*Geothlypis trichas*) and found that in one population, immune response was best correlated with the size and color of carotenoid-based bib plumage, but in the other population, immune response was best predicted by the size of a melanin-based facial mask. These results call into question whether carotenoid-based signals deserve special recognition among other sexual signals.

Given the many studies which have found an effect of carotenoids on immunity, it seems likely that some connection between carotenoids and parasites or immune response exists (Blount et al. 2003, Aguilera and Amat 2007, Clotfelter et al. 2007). However, the exact mechanism by which carotenoids influence immune response and vice versa still requires verification (Pérez-Rodríguez 2009, Vinkler and Albrecht 2010). Until this relationship is resolved, researchers should be cautious about asserting that carotenoid-based ornaments are necessarily better honest indicators of immune function than other sexual signals.

*Reactive Oxygen Species*

One of the more recent mechanisms proposed to explain how signals may honesty indicate immunity suggests that oxidative stress from reactive oxygen species may create the trade-off between immunity and signal quality (von Schantz et al. 1999, Alonso-Alvarez et al. 2007, Dowling and Simmons 2009). Reactive oxygen species are generated as a byproduct of metabolism, but they are also generated by immune responses (Constantini and Møller 2009, Dowling and Simmons 2009). These reactive oxygen species damage host tissues, and this damage may result in reduced quality of sexual signals (Dowling and Simmons 2009). Thus, males that can mount a more efficient immune response may suffer less oxidative damage and thereby remain healthier and better able to produce flashy sexual signals (von Schantz et al. 1999). Originally, von Schantz et al. (1999) proposed that genes for parasite resistance played an important role in this process by increasing the efficiency of the immune response. Males lacking these genes would have to mount a costly general response to parasites and would suffer more oxidative damage. This reliance on specific genes for parasite resistance is similar to what Hamilton and Zuk (1982) proposed. However, more recent modifications of this idea have tied oxidative stress in more with the immunocompetence handicap hypothesis by suggesting that testosterone may also play an important role by increasing susceptibility to oxidative damage (Alonso-Alvarez et al. 2007). This newer approach has moved away from the focus on genetic resistance.

Given that this hypothesis is relatively recent, fewer studies test it directly. However, some support has been found linking testosterone and oxidative stress.

Mougeot et al. (2009) found that red grouse with either testosterone supplementation or parasitic infection showed increased oxidative damage. In the case of the males given testosterone, this oxidative damage occurred even though these males had higher levels of antioxidants circulating in the blood, suggesting either that something about testosterone in particular makes individuals more susceptible to oxidative stress or that oxidative stress was increased to the point that even the higher levels of antioxidants were unable to prevent damage. Other studies have also found that testosterone increases susceptibility to oxidative stress (Alonso-Alvarez et al. 2007). However, most studies to date give at best an incomplete picture of the relationship between oxidative stress, immunity, and sexual signals. Many studies do not include any measure of an individual's ability to repair the damage caused by reactive oxygen species, which could represent an important component of fitness (Monaghan et al. 2009). The original hypothesis set forth by von Schantz et al. (1999) emphasized the importance of underlying resistance genes in determining which males could mount more efficient immune response and thus suffer lower oxidative damage, yet this component of oxidative stress has been largely ignored (but see Kurtz et al. 2006). Thus, while the oxidative stress hypothesis seems plausible, more evidence is needed to determine whether or not this mechanism plays a prominent role in the honesty of sexual signaling.

**The Problem: Conflicting Predictions and Results**

While the mechanisms above suggest various ways that immunity may be linked to sexual signaling, they face a common problem when it comes to actually generating

predictions about the relationship between immune response and signal quality. As mentioned above, all the mechanisms predict that there should be a trade-off between strength of the immune system and sexual signal quality, and at the level of the individual, the predictions are clear and straightforward. All of the mechanisms described predict that a greater investment in sexual signaling should result in a decrease in immune response and vice versa, and empirical data from selection experiments and experimental immune challenges support this assertion (Verhulst et al. 1999, Jacot et al. 2004, McKean and Nunney 2008, Leman et al. 2009, López et al. 2009).

Problems arise, however, when scientists try to examine the correlation between immunity and sexual signaling across multiple individuals rather than within a single animal. If there is a negative relationship between immune response and signal quality within individuals, do we expect this same pattern to hold among different individuals as well? In other words, should males with the most preferred signals also have the weakest immune response, at least during the breeding season, as a consequence of having to pay the costs associated with signaling? If one considers only that immune response is costly, such a scenario makes sense. However, this view ignores the effects of differences in individual quality which could lead to differences among individuals in their ability to pay the costs associated with signaling. Obviously, as stated above, there should be a negative relationship within an individual between immune response and signal quality. However, if the costs of immune response act as a condition-dependent handicap, individuals in better condition should be better able to pay these costs and still produce a good sexual signal (Zahavi 1977, Nur and Hasson 1984, Maynard Smith 1991, Getty

2002). This pattern is sometimes referred to as the car/house paradox after an example from economics. Within an individual, if money is spent on a car, less money will be available for buying a house. However, if one looked at the relationship across individuals, one tends to find that people with more expensive cars also have more expensive houses because they started off with more money to spend. This reasoning can also be applied to the trade-offs faced by individuals between investing in immune response and investing in sexual signals; individuals that had more resources to begin with should be better at both (Zuk et al. 1996). Many studies provide support for this idea by showing that higher quality individuals seem to be better able to bear the costs associated with immune response and/or sexual signaling (e.g. Saino and Møller 1996, Ardia 2005, Mougeot et al. 2009).

At least some of the problems to date can be blamed on people becoming confused about the predictions of the handicap model and expecting to find a negative relationship between immunity and signaling among different individuals rather than just within a single individual (Getty 2002). This has led to some people claiming that a highly ornamented individual should have a weaker immune response while others claim that such individuals should have a stronger immune response. If researchers consider either outcome as support for the hypotheses described above, falsification of these hypotheses becomes difficult or impossible (Braude et al. 1999). Thus, it is important for researchers to take into account differences in individual quality and how these differences determine the relative costs individuals pay for signals and immunity (Nur and Hasson 1984, Getty 2002).

Some of the confusion in the field cannot be explained away so simply, however. Part of the problem is that different studies looking for a correlation between immune response and sexual signal quality have obtained opposite results. Table 1.1 shows a summary of such studies. While most of the work done suggests that more ornamented individuals have better immunity, as one would predict, many have obtained the exact opposite result (Table 1.1). For example, Skarstein and Folstad (1996) found that male arctic charr that had brighter red coloration also tended to have lower lymphocyte counts, which presumably implied lower immunity. The authors interpreted this as a result of the costs of immune response. Conversely, López and Martín (2005) examined male scents in Iberian wall lizards and found opposite results, namely that females preferred the scents of males able to mount a stronger immune response. They concluded that these scent marks were honest indicators of quality and immunity, and that females used these scents to select high-quality mates. Interestingly, both of these studies mention testosterone as a possible substance responsible for producing the observed relationship (Skarstein and Folstad 1996, López and Martín 2005). This is just one example of two studies testing the same idea, namely that sexual signal quality conveys information about immunity to prospective mates, and obtaining opposite results with regards to whether a preferred sexual signal indicates a strong or a weak immune response. Such conflicting results continue to plague the field even in recent work. In a study by Aguilera and Amat (2007), preferred breast coloration in green finches was associated with lower antibody response. In contrast, Griggio et al. (2010) found that male

budgerigars whose breast plumage had higher UV chroma also were able to mount a stronger swelling response to PHA.

These contradictory results represent an underappreciated and largely unaddressed problem to the field of sexual selection and ecological immunology. Furthermore, all of the mechanisms described above predict essentially the same relationship between immunity and sexual signals, and yet none of them can really be invoked to explain why the results of correlational studies have been so variable. In other words, they cannot explain why the relationship between immune response and sexual signal quality sometimes appears to be negative and sometimes appears to be positive. In many ways, the field has become bogged down by a focus on mechanisms that do not generate new predictions about the relationship between immune response and sexual signaling, while few efforts have been made to reconcile the different results that have been obtained. For example, several studies have attempted to determine whether testosterone suppresses immune response directly or indirectly by increasing some other substance such as corticosterone (Evans et al. 2000, Owen-Ashley et al. 2004). This distinction is important to researchers interested in the effects of different hormones on immune response; however for researchers interested in explaining relationships between immune response and sexual signals, such a distinction is less helpful. Even if testosterone does suppress immune response by increasing corticosterone rather than having a direct effect, the predicted relationship between the quality of the sexual signal and immune response remains the same. The answer to the question of whether or not testosterone is directly immunosuppressive will not help us explain why some studies find a positive relationship

between signal quality and immune response while others find a negative one. It seems pointless to argue about the minute physiological details of a mechanism when that mechanism as a whole does not accurately predict the trends actually observed in the field.

If the hypotheses previously discussed are unable to explain the different results described above, where then should we look for an answer? The first step may be to better understand the relationships between immunity and resistance as well as to understand the relationships and trade-offs that occur within the immune system itself. There has been a considerable body of work done on these subjects in the field of ecological immunology. Much of it has not focused directly on sexual selection and sexual signaling. However, we feel that many of findings of these studies can be applied to sexual selection and that this would benefit both fields. There are many reasons, reviewed below, why researchers may not be able to find a consistent relationship between immune response and signal quality. Here, we attempt to review the literature to determine what needs to be known in order to generate testable predictions about the link between parasites, immune response, and sexual signaling and discuss possible directions for future work.

## Immunity: What We Know and What We Need to Consider

### *Specific vs. General Immunity*

When trying to generate predictions about how immune response relates to signaling, it is important to keep in mind that there may be a difference between the

strength of the immune system (as measured by some challenge that stimulates an immune response) and its ability to fight off an individual pathogen of interest (Westneat and Birkhead 1998, Adamo 2004a, Viney et al. 2005). Many of the immune tests popular in the literature (e.g. PHA response, antibody response to sheep red blood cells, white cell counts, etc.) are designed to test "general immunocompetence" rather than an organism's ability to fight off any one pathogen. These methods have both their strengths and weaknesses. On one hand, the ability of an organism to mount a response to a novel pathogen should tell one something about its ability to fight off the numerous pathogens it already faces. However, a strong response to a generalized immune challenge may not always translate into the ability to resist a given pathogen (Adamo 2004a). Indeed, Westneat and Birkhead (1998) state that such immunological methods do not really test the original Hamilton-Zuk hypothesis. They argue that under the Hamilton-Zuk hypothesis, females select for genes that confer resistance to a given pathogen or pathogens of interest. This selection for genetic resistance against a single pathogen can produce an arms race between host and parasite which may be crucial for maintaining genetic variability in quality (Hamilton and Zuk 1982). However, it may not lead to greater investment in the immune system as a whole because males with superior genetic resistance do not need to maintain as strong an immune system. Thus, such selection is less likely to be captured using general tests of immunity. Possession of genetic resistance may even appear as a negative relationship between immunity and sexual signals, because males that are genetically resistant can afford to take more resources away from immunity and devote them to signaling (Zuk 1994).

Some studies that have found a negative relationship between immune response and sexual signaling have attributed their results to this phenomenon, namely that individuals with genetic resistance can afford to suppress immunity without increasing disease susceptibility (Aguilera and Amat 2007). However, few studies reporting such a negative relationship have measured resistance directly. Lindström and Lundström (2000) found that when male greenfinches (*Carduelis chloris*) were infected with a specify virus type, males with larger yellow tail patches had lower viral levels and cleared the infection faster; however, this clearance rate had no connection to antibody response, implying that such males had neither a higher nor a lower immune response than average. Whether or not genetically resistant males can suppress immunity and still have lower parasite loads and attract mates is unclear. A study by Zala et al. (2008) in mice found that females preferred males from a strain that was genetically resistant to infection by *Salmonella* even before such males were infected, implying that, at least in some circumstances, females can select directly for resistance genes. Males in the resistant strain were also able to maintain higher testosterone levels when infected, implying that such males could tolerate higher levels of immunosuppression.

If knowing the strength of an immune response is not enough to predict whether or not an animal can resist parasites, researchers must also look at the effects of pathogens. Ideally, measures of immune response will correlate with parasite resistance, but this may not always be the case (Adamo 2004a,b, Viney et al. 2005). In cases where differences in immune response measurements do not seem to translate into differences in resistance, one must question what these differences in immune response actually mean.

Is immune response simply correlated to condition and not to pathogen resistance, and if so, which is the better predictor of male ornament quality and mating success, immune response or resistance? It is also possible that different measures of immune response correlate to resistance to different pathogens. Different parts of the immune system may specialize in fighting off different types of pathogens (Weir and Stewart 1993); thus, it may be important for researchers to consider which pathogens play the most important role in the lives of their study animal, and the kinds of immunological defenses needed to combat them. Which immune defenses are optimal may differ depending on the organism under study and the infectious agents that organism is likely to face (Schmid-Hempel and Ebert 2003, Lee 2006). For example, Adamo (2004b) found that one measure of immune response, increase in lysozyme-like activity, was correlated with the ability of crickets to resist three different strains of pathogenic bacteria. However, another measure of immune response, phenoloxidase activity, did not influence resistance at all. Thus, if crickets consistently face challenges by pathogenic bacteria, one might predict that lysozyme-like activity would be important for survival and probably also mate choice.

In light of the distinction between immune response and pathogen resistance, researchers should consider the following questions when trying to predict the relationship between immune response and signal quality. 1) Are there genetic underpinnings to resistance, and how do they relate to immune response? To address this, researchers must first locate genes involved in parasite resistance, then look to see whether individuals possessing such genes have any measureable differences in the

strength of their immune response. 2) Do the tests of immune response used actually reflect the organism's ability to fight off pathogens? Theoretically, if animals have a higher level of immune response, they should be better able to fight off pathogens. Experimental infections of individuals whose immune responses have already been measured may be helpful in answering this question. 3) If a higher immune response is not related to greater resistance, which trait seems to be more important to female choice, specific resistance or overall, general immune response? Future studies should consider such questions to try to reconcile some of the differences in the field. This approach will doubtless prove challenging, as determining the many pathogens that animals may be exposed to under natural conditions is extremely difficult, and locating different genotypes that may confer resistance to these pathogens may prove more so.

While locating genes involved in resistance will doubtless be a long and difficult process, one possible starting point is to more closely examine genes in the major histocompatability complex (MHC). The MHC plays an important role in immune response by coding for cell surface antigens involved in the recognition and presentation of antigens to T-cells (Milinski 2006). Thus, it seems likely that these gene complexes are involved in pathogen resistance. Some empirical work supports this hypothesis; Westerdahl et al. (2005) found evidence suggesting that certain alleles of the major histocompatability complex (MHC) confer malaria resistance in songbirds, and certain MHC haplotypes may also play a role in resistance to ectoparasites in domestic chickens (Owen et al. 2008). The role of different MHC alleles in immune response is less certain, although some studies have a relationship between the two. Kurtz et al. (2004) found that

sticklebacks possessing lower MHC diversity displayed higher innate immunity, which the authors interpreted as compensating for a less-efficient adaptive immune system. Whether MHC alleles also influence the strength of adaptive immune response is less clear. In one study by Bonneaud et al. (2005), house sparrows possessing a particular MHC allele tended to have higher swelling responses to PHA and higher antibody responses when injected with sheep red blood cells. However, another study in nestlings of the same species failed to find any relationship between response to PHA and MHC alleles (Bonneaud et al. 2009).

While MHC alleles are not the only source of resistance to pathogens, they may be a promising starting point, and integrating studies looking at the role of MHC alleles in resistance and mate choice with those examining general immune response may prove fruitful (Zuk 1994, Drury 2010). Researchers pursuing this avenue should be cautious, however, to note that predicting that a female will choose a male because he possesses a specific MHC haplotype that confers resistance is different from predicting that a female will select a male that has a different MHC type than she does. Much of the work on the MHC and its role in mate choice has focused on this latter prediction, namely that females should select mates with dissimilar MHC alleles to increase the heterozygosity of the MHC in offspring. This increased heterozygosity is thought to increase the fitness of the offspring by allowing them to recognize a greater variety of pathogens (Milinski 2006).

*Methods for Measuring Immunity*

Given that the immune system consists of more than one branch, it is unlikely for any one test of immune response to accurately represent "immunocompetence" as a whole (Norris and Evans 2000, Zuk and Stoehr 2002, Adamo 2004a). This realization has led scientists to employ multiple tests of immune response when trying to link immunity and signal quality. However, the results of these tests suggest that different components of the immune system may not all be linked to sexual signals in the same way. For instance, Møller and Petrie (2002) found that in peacocks, male train length was related positively to PHA response, but negatively to the peacocks' ability to produce antibodies in response to stimulation by a novel antigen (in this case sheep red blood cells). Similar results, in which one measure of immune response increases with the quality of the sexual signal while another decreases, have been obtained for other species as well. Zuk and Johnson (1998) found that, during the breeding season, male jungle fowl with larger combs had fewer lymphocytes in the blood but a higher response to PHA. Faivre et al. (2003) reported that blackbirds (*Turdus merula*) with preferred bill coloration had lower secondary antibody responses, but higher swelling response to PHA. In contrast, Garvin et al. (2008) found exactly opposite results, namely that yellowthroat warblers (*Geothlypis trichas*) with larger facial masks had higher antibody levels but lower response to PHA.

Such variation obviously makes the results of these studies somewhat difficult to interpret. It also suggests the existence of more complex relationships between the different branches of the immune system. Some authors have suggested that trade-offs

between different branches of the immune system can make it impossible to simultaneously produce a strong response in innate immunity, cell-mediated immunity, and humoral immunity (Møller and Petrie 2002, Forsman et al. 2008, Garvin et al. 2008). Forsman et al. (2008) compared three different measures of immunity in house wren broods: response to PHA, ability to produce antibodies, and ability of the blood to kill bacteria. Broods of nestlings that produced a stronger antibody response tended to have a weaker PHA response and vice versa (Forsman et al. 2008). These results imply that nestlings (and presumably other types of individuals) that have invested strongly in one branch of the immune system cannot invest as much in the other branches, creating trade-offs between different aspects of immunity. Other studies employing multiple measures of immunity have found similar trade-offs between different types of immune response in the presence of stressors such as parasites (Johnsen and Zuk 1999, Arriero 2009). These trade-offs within the immune system may explain why some studies have found a positive response between signal quality and immune response while other have found a negative one. In studies that only measure one component of immunity, the component measured may or may not be the one in which the animal has invested the most. Thus, one would expect that a study measuring only one type of immune response might find different results from another study measuring a different type of immune response (Norris and Evans 2000, Martin et al. 2007).

Having one branch of the immune system exhibit a strong response while another branch has a weak response makes things difficult for researchers hoping to find a clean, easily interpretable relationship between immune response and sexual signals. However,

one should expect to find such "messy" results quite commonly due to the way animals allocate energy to the immune system. Life history theory predicts that not all animals should invest in all arms of the immune system equally (Martin et al. 2006a, Lee 2006). Some attention has focused on the relationship between an animal's "pace of life" and its investment in different components of immunity. "Slow-living" organisms which have long periods of development and long lifespans are predicted to invest more in immune response (Martin et al. 2006b), and also to invest in specific, inducible defenses (e.g. antibody production). Such defenses are costly to develop, but less costly to use, and for organisms that invest a great deal in development, the cost may be outweighed by the benefits later in life of improving survival, minimizing damage to self, and conserving energy to be invested in future reproduction. For "faster-living" species with lower survival rates, the cost of developing such defenses may not be worth the benefit, and thus they are expected to rely more on innate defenses (Martin et al. 2006a, Lee 2006).

For a given species, then, some optimal combination of immune defenses exists. What defenses make up such an optimal response will depend on life history consideration as well as the pathogens to which an organism may be exposed (Schmid-Hempel and Ebert 2003, Lee 2006). This idea has been neglected in the sexual selection literature, but it may have profound implications for the role of immune response in mate choice. If females select mates based on the strength of the male's immune response, the types of response that are most important for that species should also be most important in the mating decision. For instance, in a long-lived species, one might anticipate that females select males with a stronger humoral immune response, regardless of innate

immunity or cell-mediated immunity. Such clear-cut results, of course, may be difficult to find, as the world cannot always be divided into fast-living and slow-living species. Obviously, an animal's pace of life is expected to fit somewhere along a gradient rather than being simply fast or slow. However, by looking at an organism's pace of life, researchers may be able to make predictions about the types of immune response that the organism should invest most heavily in, and these defenses may also play an important role in mate choice.

For researchers interested in how different branches of the immune system interact with one another as well as with sexual signaling, caution must be used when choosing how to measure immune response. To really delve deeper into the issue of trade-offs among different parts of the immune system and optimal levels of different immune defenses, one must consider what the immune tests chosen for a particular study are really measuring. Some of the immune tests that are popular in the literature do not measure the type of immune response they were originally thought to measure. The primary example of this is the PHA response, in which phytohaemagglutinin is injected into the animal and the swelling response is measured. This swelling response was thought to indicate T-cell mediated immunity. However, a recent study by Martin et al. (2006c) found that other immune cells may take part in the reaction, and thus PHA response may indicate "cutaneous" immune response rather than a purely T-lymphocyte mediated response. This is not to say that the PHA test is useless to researchers interested in immunity, especially as evidence suggests that it may be related to survival (Gonzalez et al. 1999). However, it does mean that researchers seeking to study the

strength of cell-mediated immunity independent of other types of immunity should probably use a different test.

*Resistance vs. Tolerance*

Virtually every model predicting the effect of parasites on sexual signals has assumed that females should select mates that have the lowest parasite loads. The Hamilton-Zuk hypothesis, the transmission avoidance model, and the resource provisioning model all share this prediction (Clayton 1991). However, as recent work by Råberg et al. (2007) points out, resistance is not the only method which animals may use to deal with parasites. Rather than fighting off a parasitic infection, animals may simply tolerate it by minimizing the damage done by the parasites (Råberg et al. 2007; Boots 2008). This strategy would allow the animal to stay in better condition than its similarly infected fellows (Fig. 1.2).

The idea of parasite tolerance rather than resistance has long been recognized by plant biologists, but its applications to animals have often been neglected (Råberg et al. 2009). However, if tolerance plays a role in mate choice, its inclusion in models of sexual selection could drastically alter the expected results. Most studies assume that there should be a negative relationship between parasite load and the quality of sexual signals, but this may not be so if females can also choose for resistance. Under such a scenario, higher-quality mates might not differ from the rest of the population in terms of overall parasite load, but they would be better able to tolerate such infection intensities. Individuals or groups that can better tolerate parasites also show reduced resistance

(Råberg et al. 2007). Thus, under the scenario described above, preferred mates might show a lower immune response relative to the rest of the population because they are better able to tolerate parasites but less able to mount an effective response against them. These preferred mates might also have similar or higher parasite loads relative to the other males in the population. Some studies of wild populations have found results consistent with this idea (Clayton 1991).

While the idea of tolerance opens up some intriguing new ideas about the nature of female preference and parasites, it is difficult to study. To demonstrate that tolerance exists, one must look at the effects of different parasite intensities on different host genotypes. If one genotype shows relatively less decline in condition as parasite intensity increases (i.e. if there is a significant interaction between genotype and parasite intensity), then one may conclude that that genotype is more tolerant than the others (Råberg et al. 2009). Given these requirements, demonstrating the effects of tolerance as it relates to mate choice in field studies would be extremely difficult. Laboratory experiments, however, may have more success at teasing apart these interactions. Thus, we encourage researchers with access to laboratory populations to explore this topic further. We second the appeal made by Boots (2008) for more research to demonstrate the existence of tolerance in animal populations, and we further encourage researchers to examine how tolerance may be shaped by sexual selection. Are males that can tolerate infection as attractive to females as those that can resist infection? Can females even distinguish between tolerant and resistant individuals, and would there be any benefit to

doing so? Answering such questions may provide a better understanding of how sexual selection may help shape host-parasite interactions.

**Conclusion and Future Directions**

While the field of ecological immunology and sexual selection has expanded greatly during the past two decades, progress has been hampered by a lack of clear predictions about the relationship between immunity and sexual signaling. Different studies have obtained opposite results, but still interpreted them as supporting the same hypothesis, making the testing and falsification of such hypotheses difficult to impossible. The immunocompetence handicap hypothesis had been particularly prone to this problem (Braude et al. 1999). This difficulty may arise in part because none of the hypotheses currently popular in the field of sexual selection fully explains the complicated link between immune response and sexual signal quality.

We suggest that before scientists delve into the question of which hormones or antioxidants affect the strength of the immune system, we should first strive for a better understanding of how the immune system works, how differences in genetic backgrounds and life history strategies can affect immune response, and how these differences may be important in mate choice. For example, it is not enough to simply claim that testosterone suppresses immunity when not all branches of the immune response may react in the same way. We need a better understanding of how the various components of the immune system relate to each other and to signaling before we focus on the detailed mechanisms controlling those interactions. Most importantly, we urge researchers to go

in with clear predictions about what the relationship between immune response and sexual signaling will be based on a given model, rather than coming up with a post hoc explanation for why the results obtained support that model.

To derive accurate predictions from the models discussed in this review, we may first need to obtain further information. For example, if one studies the Hamilton-Zuk hypothesis, making predictions about the relationship between sexual signals and immune response may be extremely difficult without knowing more about the genes and/or mechanisms underlying resistance. In some cases, resistance may be conferred by differences in immune response (Adamo 2004b). If these differences in immune response are heritable, females could select resistant mates with high immunity, and one would predict a positive relationship between immune response and sexual signaling. In other cases, however, having genes that confer parasite resistance may allow individuals to mount a weaker immune response, at least in some branches of the immune system (Kurtz et al. 2004). Under this scenario, one would predict a negative relationship between the quality of the sexual signal and the level of immune response.

Given this current gap in our knowledge, one avenue researchers should explore further is the relationship between genetic resistance and various components of the immune system. Theoretically, having genetic resistance may enable some individuals to get away with mounting a comparatively weaker immune response (Zuk 1994; Westneat and Birkhead 1998). However, depending on the genes involved, not all branches of the immune system may respond in the same manner. For instance, the MHC plays a role in recognition of antigens by T-lymphocytes, and individuals that have MHC alleles that

confer resistance to pathogens may be able to mount weaker innate immune responses (Kurtz et al. 2004). These alleles may facilitate the T-cell mediated response, although this idea has received mixed support so far (Bonneaud et al. 2005, Bonneaud et al. 2009). Researchers should also consider the possibility that tolerance, rather than resistance, could be an important trait in mate choice. Possessing genes for tolerance may also influence the strength of an individual's immune response. Given that immune response is energetically costly, individuals that tolerate parasites rather than resisting them should not waste resources on mounting a strong immune response. Thus, we would expect that tolerance should be correlated with a lower than average level of immune response. Reduced immune response may even benefit such individuals by reducing the immunopathology that often occurs in the presence of parasites (Graham et al. 2005).

Researchers interested in how energetic trade-offs determine signal honesty may also profit from trying to disentangle the effects of condition on immunity and vice versa. Many studies suggest that immune response depends, at least in part, on condition (Gonzalez et al. 1999, Jacot et al. 2004, Aguilera and Amat 2007 – but see Ohlsson et al. 2002). This condition-dependence is in keeping the view of immune response as a life history trait. However, if the strength of an immune response and sexual signal quality both increase with condition, one may argue that the sexual signal itself evolved to convey information about condition to prospective mates, and that any information about immunity is simply secondary. Modeling suggests that females may benefit more by paying attention to indicators of condition rather than immune response itself (Adamo and Spiteri 2009). However, immune response may also influence condition if it

decreases parasite load. This feedback loop may be critical to the question of how important parasite resistance truly is in mate choice. If immune response is dependent on condition but the reverse is not true, females selecting highly ornamented males could be getting genes related to condition (e.g. genes for foraging ability) that have nothing to do with immune response. Any observed differences in immune response would simply be a product of differences in condition, and therefore not heritable. The Hamilton-Zuk hypothesis cannot operate under such a scenario.

In conclusion, the field of ecological immunology and sexual selection has become tangled in a focus on mechanisms that don't truly explain the results obtained to date. To solve this problem, we propose that more work should focus on how different parts of the immune system interact with each other and how these interactions relate to disease resistance and/or tolerance. This does not mean that we believe that all researchers should become immunologists, but rather that it is important to have a clearer idea of what the immune system does, what our measurements of immunity actually tell us, and how immunity and resistance contribute to an animal's fitness. Such an understanding will help us determine what exactly females gain when they select their mates, and will prove invaluable to those studying the evolution of immunity as well as mate choice.

**References**


Adamo S.A. (2004a) How should behavioural ecologists interpret measurements of immunity? *Animal Behaviour* 68:1443-1449.

Adamo S.A. (2004b) Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. *Journal of Insect Physiology* 50:209-216.

Adamo S.A. and Parsons N.M. (2006) The emergency life-history stage and immunity in the cricket *Gryllus texensis*. *Animal Behaviour* 72:235-244.

Adamo S.A. and Spiteri R.J. (2009) He's healthy, but will he survive the plague? Possible constraints on mate choice for disease resistance. *Animal Behaviour* 77:67-78.

Aguilera E. and Amat J.A. (2007) Carotenoids, immune response and the expression of sexual ornaments in male greenfinches (*Carduelis chlorisi). Naturwissenschaften* 94:895-902.

Ahtiainen J.J., Alatalo R.V., Kortet R., and Rantala M.J. (2004) Sexual advertisement and immune function in an arachnid species (Lycosidae). *Behavioral Ecology* 15:602-606.

Alonso-Alvarez C., Bertrand S., Faivre B., Chastel O., and Sorci G. (2007) Testosterone and oxidative stress: the oxidation handicap hypothesis. *Proceedings of the Royal Society B* 274:819-825.

Alonso-Alvarez C., Pérez-Rodríguez L., Garcia J.T., and Viñuela J. (2009) Testosterone-mediated trade-offs in the old age: a new approach to the immunocompetence handicap hypothesis and carotenoid-based signalling. *Proceedings of the Royal Society B* 276:2093-2101.

Ardia D.R. (2005) Individual quality mediates trade-offs between reproductive effort and immune function in tree swallows. *Journal of Animal Ecology* 74:517-524.

Arriero E. (2009) Rearing environment effects on immune defence in blue tit *Cyanistes caeruleus* nestlings. *Oecologia* 159:697-704.

Biard C., Hardy C., Montreuil S., and Moreau J. (2009) Dynamics of PHA-induced immune response and plasma carotenoids in birds: should we have a closer look? *Journal of Experimental Biology* 212:1336-1343.

Blount J.D., Metcalfe N.B., Birkhead T.R., and Surai P.F. (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. *Science* 300:125-127.

Bonneaud C., Richard M., Faivre B., Westerdahl H., and Sorci G. (2005) An *Mhc* class I allele associated to the expression of T-dependent immune response in the house sparrow. *Immunogenetics* 57:782-789.

Bonneaud C., Sinsheimer J.S., Richard M., Chastel O., and Sorci G. (2009) Mhc polymorphisms fail to explain the heritability of phytohaemagglutinin-induced skin swelling in a wild passerine. *Biology Letters* 5:784-787.

Boots M. (2008) Fight or learn to live with the consequences? *Trends in Ecology and Evolution* 23:248-250.

Braude S., Tang-Martinez Z., and Taylor G.T. (1999) Stress, testosterone, and the immunoredistribution hypothesis. *Behavioral Ecology* 10:345-350.

Buchanan K.L., Evans M.R., and Goldsmith A.R. (2003) Testosterone, dominance signaling and immunosuppression in the house sparrow, *Passer dmoesticus*. *Behavioral Ecology and Sociobiology* 55:50-59.

Clayton D.H. (1991) The influence of parasites on host sexual selection. *Parasitology Today* 7:329-334.

Clotfelter E.D., Ardia D.R., and McGraw K.J. (2007) Red fish, blue fish: trade-offs between pigmentation and immunity in *Betta splendens*. *Behavioral Ecology* 18:1139-1145.

Constantini D. and Møller A.P. (2009) Does immune response cause oxidative stress in birds? A meta-analysis. *Comparative Biochemistry and Physiology A* 153:339-344.

Contreras-Garduño J., Córdoba-Aquilar A., Lanz-Mendoza H., and Rivera A.C. (2009) Territorial behaviour and immunity are mediated by juvenile hormone: the physiological basis of honest signaling? *Functional Ecology* 23:157-163.

Cotter S.C., Myatt J.P., Benskin C.M.H., and Wilson K. (2008) Selection for cuticular melanism reveals immune function and life-history trade-offs in *Spodoptera littoralis*. *Journal of Evolutionary Biology* 21:1744-1754.

Cox R.M., and John-Alder H.B. (2007) Increased mite parasitism as a cost of testosterone in male striped plateau lizards *Sceloporus virgatus*. *Functional Ecology* 21:327-334.

Dowling D.K. and Simmons L.W. (2009) Reactive oxygen species as universal constraints in life-history evolution. *Proceedings of the Royal Society B* 276:1737-1745.

Drury J.P. (2010) Immunity and mate choice: a new outlook. *Animal Behaviour* 79:539-545.

Duffy D.L. and Ball G.F. (2002) Song predicts immunocompetence in male European starlings (*Sturnus vulgaris*). *Proceedings of the Royal Society B* 269:847-852.

Dunn P.O., Garvin J.C., Whittingham L.A., Freeman-Gallant C.R., and Hasselquist D. (2010). Carotenoid and melanin-based ornaments signal similar aspects of male quality in two populations of the common yellowthroat. *Functional Ecology* 24:149-158.

Evans M.R., Goldsmith A.R., and Norris S.R.A. (2000) The effects of testosterone on antibody production and plumage coloration in male house sparrows (*Passer domesticus*). *Behavioral Ecology and Sociobiology* 47:156-163.

Faivre B., Préault M., Salvadori F., Théry M., Gaillard M., and Cézilly F. (2003) Bill colour and immunocompetence in the European blackbird. *Animal Behaviour* 65:1125-1131.

Folstad I. and Karter A.J. (1992) Parasites, bright males, and the immunocompetence handicap hypothesis. *American Naturalist* 139:603-622.

Forsman A.M., Vogel L.A., Sakaluk S.K., and Thompson C.F. (2008) Immune-challenged house wren broods differ in the relative strengths of their responses among different axes of the immune system. *Journal of Evolutionary Biology* 21:873-878.

French S.S., Moore M.C., and Demas G.E. (2009) Ecological immunology: the organism in context. *Integrative and Comparative Biology* 49:246-253.

Garvin J.C., Dunn P.O., Whittingham L.A., Steeber D.A., and Hasselquist D. (2008) Do male ornaments signal immunity in the common yellowthroat? *Behavioral Ecology* 19:54-60.

Getty T. (2002) Signaling health versus parasites. *American Naturalist* 159:363-371.

Gonzalez G., Sorci G., Møller A.P., Ninni P., Haussy C., and de Lope F. (1999) Immunocompetence and condition-dependent sexual advertisement in male house sparrows (*Passer domesticus*). *Journal of Animal Ecology* 68:1225-1234.

Graham A.L., Allen J.E., and Read A.F. (2005) Evolutionary causes and consequences of immunopathology. *Annual Review of Ecology, Evolution, and Systematics* 36:373-397.

Grasman K.A. (2002) Assessing immunological function in toxicological studies of avian wildlife. *Integrative and Comparative Biology* 42:34-42.

Griggio M., Zanollo V., and Hoi H. (2010) UV plumage color is an honest signal of quality in male budgerigars. *Ecological Research* 25:77-82.

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

Hanssen S.A., Hasselquist D., Folstad I., and Erikstad K.E. (2008). A label of health: a previous immune challenge is reflected in the expression of a female plumage trait. *Biology Letters* 4:379-381.

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

Jacot A., Scheuber H., and Brinkhof M.W.G. (2004) Costs of an induced immune response on sexual displays and longevity in field crickets. *Evolution* 58:2280-2286.

Johnsen T.S. and Zuk M. (1999) Parasites and tradeoffs in the immune response of female red jungle fowl. *Oikos* 86:487-492.

Johnstone R.A. and Norris K. (1993) Badges of status and the cost of aggression. *Behavioral Ecology and Sociobiology* 32:127-134.

Kennedy M.W. and Nager R.G. (2006) The perils and prospects of using phytohaemagglutinin in evolutionary ecology. *Trends in Ecology and Evolution* 21:653-655.

Keyser A.J. and Siefferman L.M. (2005) Viability selection against highly ornamented males. *Evolutionary Ecology Research* 14:855-861.

Kotiaho J.S. (2001) Costs of sexual traits: a mismatch between theoretical considerations and empirical evidence. *Biological Reviews* 76:365-376.

Kurtz J., Kable M., Aeschlimann P.B., Häberli M.A., Wegner K.M., Reusch T.B.H., and Milinski M. (2004) Major histocompatibility complex diversity influences parasite resistance and innate immunity in sticklebacks. *Proceedings of the Royal Society B* 271:197-204.

Kurtz J., Wegner K.M., Kable M., Reusch T.B.H., Schaschl H., Hasselquist D., and Milinski M. (2006) MHC genes and oxidative stress in sticklebacks: an immuno-ecological approach. *Proceedings of the Royal Society B* 273:1407-1414.

Lawniczak M.K.N., Barnes A.I., Linklater J.R., Boone J.M., Wigby S., and Chapman T. (2007) Mating and immunity in invertebrates. *Trends in Ecology and Evolution* 22:48-55.

Lee K.A. (2006) Linking immune defense and life history at the levels of the individual and the species. *Integrative and Comparative Biology* 46:1000-1015.

Leman J.C., Weddle C.B., Gershman S.N., Kerr A.M., Ower G.D., St. John J.M., Vogel L.A., and Sakaluk S.K. (2009) Lovesick: immunological costs of mating to male sagebrush crickets. *Journal of Evolutionary Biology* 22:163-171.

Lindström K. and Lundström J. (2000) Male greenfinches (*Carduelis chloris*) with brighter ornaments have higher virus infection clearance rate. Behavioral Ecology and Sociobiology 48:44-51.

López P. and Martín J. (2005) Female Iberian wall lizards prefer male scents that signal a better cell-mediated immune response. *Biology Letters* 1:404-406.

López P., Gabirot M., and Martín J. (2009) Immune challenge affects sexual coloration of male Iberian wall lizards. *Journal of Experimental Zoology* 311A:96-104.

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

Martín J., Civantos E., Amo L., and López P. (2007) Chemical ornaments of male lizards *Psammodromus algirus* may reveal their parasite load and health state to females. *Behavioral Ecology and Sociobiology* 62:173-179.

Martin L.B., Weil Z.M., and Nelson R.J. (2006a) Refining approaches and diversifying directions in ecoimmunology. *Integrative and Comparative Biology* 46:1030-1039.

Martin L.B., Hasselquist D., and Wikelski M. (2006b) Investment in immune defense is linked to pace of life in house sparrows. *Oecologia* 147:565-575.

Martin L.B., Han P., Lewittes J., Kuhlman J.R., Klasing K.C., and Wikelski M. (2006c) Phytohemagglutinin-induced skin swelling in birds: histological support for a classical immunoecological technique. *Functional Ecology* 20:290-299.

Martin L.B., Weil Z.M., and Nelson R.J. (2007) Immune defense and reproductive pace of life in *Peromyscus* mice. *Ecology* 88:2516-2528.

Marzal A., Reviriego M., de Lope F., and Møller A.P. (2007) Fitness cost of an immune response in the house martin (*Delichon urbica*). *Behavioral Ecology and Sociobiology* 61:1573-1580.

Maynard Smith J. (1991) Theories of sexual selection. *Trends in Ecology and Evolution* 6:146-151.

McGraw K.J. (2006) Mechanics of carotenoid-based coloration. In: Hill G.E. and McGraw K.J. eds. *Bird Coloration: Mechanisms and Measurements*. Cambridge, MA: Harvard University Press.

McKean K.A. and Nunney L. (2008) Sexual selection and immune function in *Drosophila melanogaster*. *Evolution* 62:386-400.

Milinski M. (2006) The major histocompatibility complex, sexual selection, and mate choice. *Annual Review of Ecology, Evolution, and Systematics* 37:159-186.

Millet S., Bennett J., Lee K.A., Hau M., and Klasing K.C. (2007) Quantifying and comparing constitutive immunity across avian species. *Developmental and Comparative Immunology* 31:188-201.

Møller A.P. (1987) Social control of deception among status signalling house sparrows *Passer domesticus*. *Behavioral Ecology and Sociobiology* 20:307-311.

Møller A.P. (1990) Effects of a haematophagous mite on the barn swallow (*Hurundo rustica*). *Evolution* 44:771-784.

Møller A.P. (1994) *Sexual Selection and the Barn Swallow*. Oxford: Oxford University Press.

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

Møller A.P. and Petrie M. (2002) Condition dependence, multiple sexual signals, and immunocompetence in peacocks. *Behavioral Ecology* 13:248-253.

Monaghan P., Metcalfe N.B., and Torres R. (2009) Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecology Letters* 12:75-92.

Mougeot F. (2008) Ornamental comb color predicts T-cell mediated immune response in male red grouse *Lagopus lagopus scoticus*. *Naturwissenschaften* 95:125-132.

Mougeot F., Martinez-Padilla J., Webster L.M.I., Blount J.D., Pérez-Rodríguez L., and Piertney S.B. (2009) Honest sexual signaling mediated by parasite and testosterone effects on oxidative balance. *Proceedings of the Royal Society B* 276:1093-1100.

Nolan P.M., Hill G.E., and Stoehr A.M. (1998) Sex, size, and plumage redness predict house finch survival in an epidemic. *Proceedings of the Royal Society B* 265:961-965.

Norris K. and Evans M.R. (2000) Ecological immunology: life history trade-offs and immune defense in birds. *Behavioral Ecology* 11:19-26.

Nur N. and Hasson O. (1984) Phenotypic plasticity and the handicap principle. *Journal of Theoretical Biology* 110:275-297.

Ohlsson T., Smith H.G., Råberg L., and Hasselquist D. (2002) Pheasant sexual reflect nutritional conditions during early growth. *Proceedings of the Royal Society B* 269:21-27.

Olson V.A. and Owens I.P.F. (1998) Costly sexual signals: are carotenoids rare, risky or required? *Trends in Ecology and Evolution* 13:510-514.

Owen J.P., Delany M.E., and Mullens B.A. (2008) MHC haplotype involvement in avian resistance to an ectoparasite. *Immunogenetics* 60:621-631.

Owen-Ashley N.T., Hasselquist D., and Wingfield J.C. (2004) Androgens and the immunocompetence handicap hypothesis: unraveling direct and indirect pathways of immunosuppression in song sparrows. *American Naturalist* 164:490-505.

Pérez-Rodríguez L. (2009) Carotenoids in evolutionary ecology: re-evaluating the antioxidant role. *BioEssays* 31:116-1126.

Råberg L., Grahn M., Hasselquist D., and Svensson E. (1998) On the adaptive significance of stress-induced immunosuppression. *Proceedings of the Royal Society B* 265:1637-1641.

Råberg L., Nilsson J., Ilmonen P., Stjernman M., and Hasselquist D. (2000) The cost of an immune response: vaccination reduces parental effort. *Ecology Letters* 3:382-386.

Råberg L., Sim D., and Read A.F. (2007) Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. *Science* 318:812-814.

Råberg L., Graham A.L., and Read A.F. (2009) Decomposing health: tolerance and resistance to parasites in animals. *Philosophical Transactions of the Royal Society B* 364:37-49.

Rantala M.J., Jokinen I., Kortet R., Vainikka A., and Suhonen J. (2002) Do pheromones reveal male immunocompetence? *Proceedings of the Royal Society B* 269:1681-1685.

Rantala M.J., Vainikka A., and Kortet R. (2003) The role of juvenile hormone in immune function and pheromone production trade-offs: a test of the immunocompetence handicap hypothesis. *Proceedings of the Royal Society B* 270:2257-2261.

Roberts M.L., Buchanan K.L., and Evans M.R. (2004) Testing the immunocompetence handicap hypothesis: a review of the evidence. *Animal Behaviour* 68:227-239.

Roberts M. and Peters A. (2009) Is testosterone immunosuppressive in a condition-dependent manner? An experimental test in blue tits. *Journal of Experimental Biology* 212:1811-1818.

Ros A.F.H., Correia M., Wingfield J.C., and Oliveira R.F. (2009) Mounting an immune response correlates with decreased androgen levels in male peafowl, *Pavo cristatus*. *Journal of Ethology* 27:209-214.

Ryder J.J. and Siva-Jothy M.T. (2000) Male calling song provides a reliable signal of immune function in a cricket. *Proceedings of the Royal Society B* 267:1171-1175.

Saino N. and Møller A.P. (1996) Sexual ornamentation and immunocompetence in the barn swallow. *Behavioral Ecology* 7:227-232.

Salvante K.G. (2006) Techniques for studying integrated immune function in birds. *Auk* 123:575-586.

Schmid-Hempel P. (2003) Variation in immune defense as a question of evolutionary ecology. *Proceedings of the Royal Society B* 270:357-366.

Schmid-Hempel P. and Ebert D. (2003) On the evolutionary ecology of specific immune defence. *Trends in Ecology and Evolution* 18:27-32.

Sheldon B.C. and Verhulst S. (1996) Ecological immunity: costly parasite defense and trade-offs in evolutionary ecology. *Trends in Ecology and Evolution* 11:317-321.

Simmons L.W., Zuk M., and Rotenberry J.T. (2005) Immune function reflected in calling song characteristics in a natural population of the cricket *Teleogryllus commodus*. *Animal Behaviour* 69:1235-1241.

Siva-Jothy M.J. (2000) A mechanistic link between parasite resistance and expression of a sexually selected trait in a damselfly. *Proceedings of the Royal Society B* 267:2523-2527.

Skarstein F., and Folstad I. (1996) Sexual dichromatism and the immunocompetence handicap: an observational approach using arctic charr. *Oikos* 76:359-367.

Tella J.L., Lemus J.A., Carrete M., and Blanco G. (2008) The PHA test reflects acquired T-cell mediated immunocompetence in birds. *PLoS One* 3:e3295.

Verhulst S., Dieleman S.J., and Parmentier H.K. (1999) A trade-off between immunocompetence and sexual ornamentation in domestic fowl. *Proceedings of the National Academy of Sciences USA* 96:4478-4481.

Viney M.E., Riley E.M., and Buchanan K.L. (2005) Optimal immune responses: immunocompetence revisited. *Trends in Ecology and Evolution* 20:665-669.

Vinkler M. and Albrecht T. (2010) Carotenoid maintenance handicap and the physiology of carotenoid-based signalization of health. *Naturwissenschaften* 97:19-28.

von Schantz T., Bensch S., Grahn M., Hasselquist D., and Wittzell H. (1999) Good genes, oxidative stress and condition-dependent sexual signals. *Proceedings of the Royal Society B* 266:1-12.

Wedekind C. and Folstad I. (1994) Adaptive or nonadaptive immunosuppression by sex hormones? *American Naturalist* 143:936-938.

Weir D.M. and Stewart J. (1993) *Immunology*. 7th ed. New York: Churchhill Livingstone.

Westerdahl H., Waldenström J., Hansson B., Hasselquist D., von Schantz T., and Bensch S. (2005) Associations between malaria and MHC genes in a migratory song bird. *Proceedings of the Royal Society B* 272:1511-1518

Westneat D.F. and Birkhead T.R. (1998) Alternative hypotheses linking the immune system and mate choice for good genes. *Proceedings of the Royal Society B* 265:1065-1073.

Whitaker S. and Fair J. (2002) The costs of immunological challenge to developing mountain chickadees, *Poecile gambeli*, in the wild. *Oikos* 99:161-165.

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

Zahavi A. (1977) The cost of honesty (further remarks on the handicap principle). *Journal of Theoretical Biology* 67:603-605.

Zala S.M., Chan B.K., Bilbo S.D., Potts W.K., Nelson R.J., and Penn D.J. (2008) Genetic resistance to infection influences a male's sexual attractiveness and modulation of testosterone. *Brain, Behavior, and Immunity* 22:381-387.

Zuk M. (1990) Reproductive strategies and disease susceptibility: an evolutionary viewpoint. *Parasitology Today* 6:231-233.

Zuk M. (1992) The role of parasites in sexual selection: current evidence and future directions. *Advances in the Study of Behavior* 21:39-68.

Zuk M. (1994) Immunology and the evolution of behavior. In: Real, LA ed. *Behavioral Mechanisms in Evolutionary Ecology*. Chicago: Chicago University Press.

Zuk M., Thornhill R., Ligon J.D., and Johnson K. (1990) Parasites and mate choice in red jungle fowl. *American Zoologist* 30:235-244.

Zuk M., Johnson T.S., and Maclarty T. (1995) Endocrine-immune interactions, ornaments and mate choice in red jungle fowl. . *Proceedings of the Royal Society B* 260:205-210.

Zuk M., Bryant M.J., Kolluru G.T., and Mirmovitch V. (1996) Trade-offs in parasitology, evolution, and behavior. *Parasitology Today* 12:46-47.

Zuk M. and Johnson T.S. (1998) Seasonal changes in the relationship between ornamentation and immune response in red jungle fowl. . *Proceedings of the Royal Society B* 265:1631-1635.

Zuk M. and Stoehr A.M. (2002) Immune defense and host life history. *American Naturalist* 160:S9-S22.

**Table 1.1: A summary of correlational studies examining the relationship between immune response and sexual signal quality.**

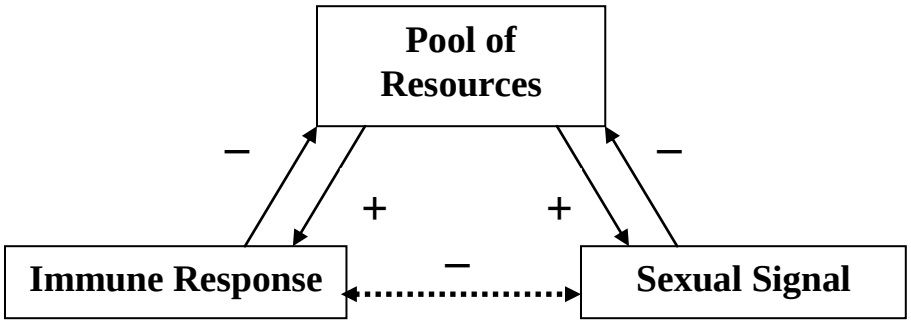
| Species | Measure of Immunity | Signal Considered | Relationship Between Signal Quality and Immune Response | Sources |
|---|---|---|---|---|
| **Arthropods** | | | | |
| ***Hygrolycosa rubrofasciata*** (wolf spider) | Encapsulation Response | Drumming Rate | Positive | Ahtiainen et al., 2004 |
| ***Hygrolycosa rubrofasciata*** (wolf spider) | Lytic Activity | Male Mobility | Positive | Ahtiainen et al., 2004 |
| ***Calopteryx splendens xanthostoma*** (damselfly) | Increase in phenoloxidase levels after a challenge | Darkness and homogeneity of wing pigmentation | Negative | Siva-Jothy, 2000 |
| ***Teleogryllus commodus*** (field cricket) | Encapsulation response | Calling song syllable duration | Positive | Simmons et al., 2005 |
| ***Acheta domesticus*** (house cricket) | Haemocyte load | Number of syllables per chirp in calling song | Positive | Ryder and Siva-Jothy, 2000 |
| ***Tenebrio molitor*** (mealworm beetle) | Phenoloxidase activity | Pheromone scent | Positive | Rantala et al., 2002 |
| ***Tenebrio molitor*** (mealworm beetle) | Encapsulation response | Pheromone scent | Positive | Rantala et al., 2002 |
| | | | | |
| **Fish** | | | | |
| ***Salvelinus alpines*** (Arctic charr) | Lymphocyte count | Degree of red coloration | Negative | Skarstein and Folstad, 1996 |

| | | | | |
|---|---|---|---|---|
| **Reptiles** | | | | |
| ***Podarcis hispanica*** (Iberian wall lizard) | PHA* response | Chemical composition of femoral secretions | Positive | López and Martín, 2005 |
| ***Psammodromus algirus*** (large Psammodromus) | PHA* response | Chemical composition of femoral secretions | Positive | Martín et al., 2007 |
| | | | | |
| **Birds** | | | | |
| ***Gallus gallus*** (jungle fowl) | Lymphocyte count | Comb size | Negative | Zuk et al., 1995 |
| ***Phasianus colchicus*** (pheasant) | PHA* response | Spur length | Positive | Ohlsson et al., 2002 |
| ***Lagopus lagopus scoticus*** | PHA* response | Comb color | Positive | Mougeot 2008 |
| ***Pavo cristatus*** (peafowl) | PHA* response | Male train length | Positive | Møller and Petrie, 2002 |
| ***Pavo cristatus*** (peafowl) | Antibody response to sheep red blood cells | Male train length | Negative | Møller and Petrie, 2002 |
| ***Melopsittacus undulates*** (budgerigar) | PHA* Response | UV-chroma of feathers | Positive | Griggio et al., 2010 |
| ***Turdus merula*** (European blackbird) | PHA* Response | Bill coloration | Positive | Faivre et al., 2003 |
| ***Turdus merula*** (European blackbird) | Secondary antibody response | Bill coloration | Negative | Faivre et a., 2003 |
| ***Sialia mexicana*** (western bluebird) | Ability to survive an epidemic | Blue coloration | Negative | Keyser and Siefferman, 2005 |
| ***Sternus vulgaris*** (European starling) | PHA* response | Song rate | Positive | Duffy and Ball 2002 |
| ***Sternus vulgaris*** (European starling) | Antibody response to keyhole limpet haemocyanin | Song rate | Positive | Duffy and Ball, 2002 |

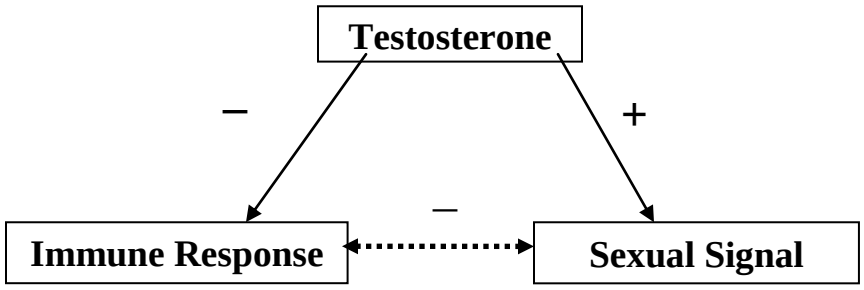
| | | | | |
|---|---|---|---|---|
| ***Carpodacus mexicanus*** (house finch) | Ability to survive an epidemic | Red coloration | Positive | Nolan et al., 1998 |
| ***Geothlypis trichas*** (common yellowthroat) | IgG levels | Size of facial mask | Positive | Garvin et al., 2008 |
| ***Geothlypis trichas*** (common yellowthroat) | PHA* response | Size of facial mask | Negative | Garvin et al., 2008 |
| ***Carduelis chloris*** (greenfinch) | Viral clearance rate | Male tail patch size | Positive | Lindström and Lundström, 2000 |
| ***Carduelis chloris*** (greenfinch) | Antibody titers in response to challenge with *Brucella abortis* | Male breast coloration (chroma) | Negative | Aguilera and Amat, (2007) |

*PHA=phytohemagglutinin response, a common assay of cell-mediated immunity in which phytohemagglutinin is injected and the swelling response is measured. Greater swelling is considered a sign of a stronger response (and therefore a stronger immune system).

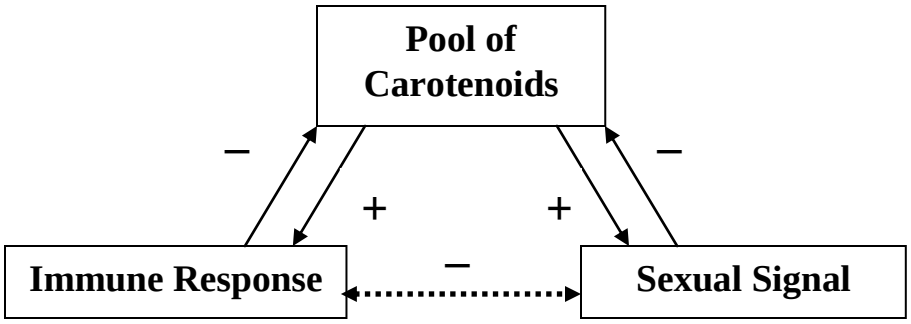
Figure 1.1



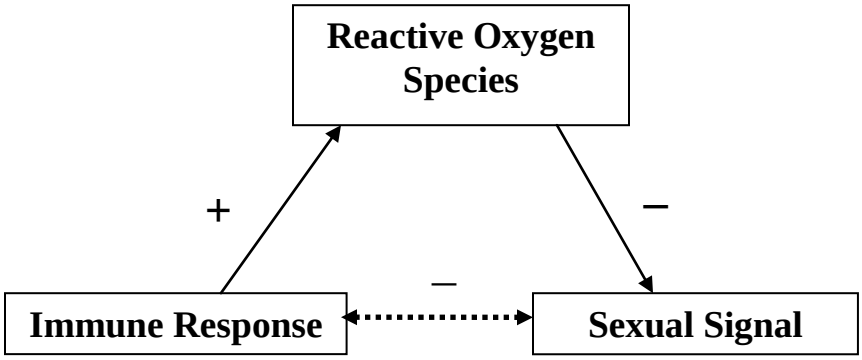

a) Life-history theory




b) Immunocompetence handicap hypothesis



c) Carotenoids

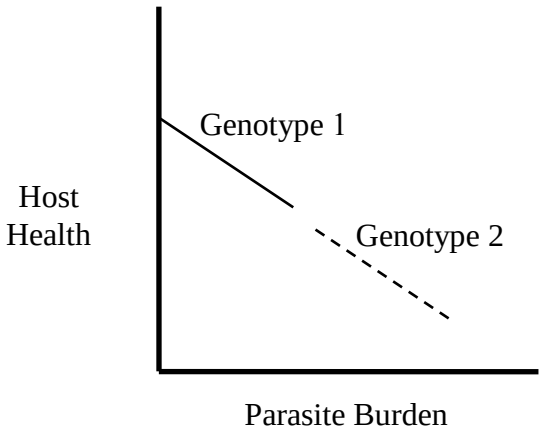



d) Oxidative Stress

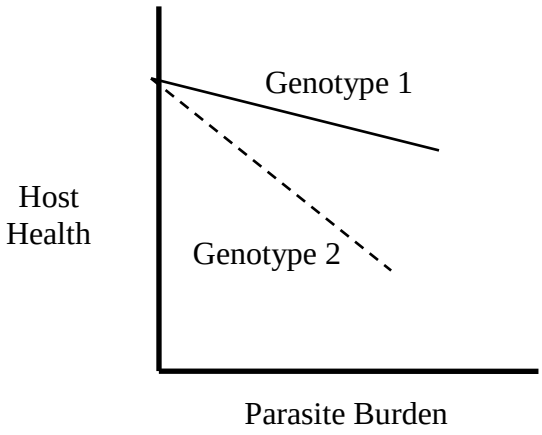
**Figure 1.1: A summary of the mechanisms linking immunity and sexual signal quality.** In all cases, there is a negative relationship predicted between sexual signaling and immune response. a) The life history theory of immunity predicts that the trade-off between immune response is due to limited energy or resources that can be allocated either to sexual signaling or to immune response. b) The immunocompetence handicap hypothesis proposes that testosterone is simultaneously increases sexual signaling while depressing immune response. c) Carotenoids are thought to be a limited resource important both in signaling and immune function. Some have hypothesized that allocation of carotenoids either to sexual signals or the immune system may be responsible for the trade-off between immunity and signaling. d) The oxidative stress hypothesis predicts that immune responses generate reactive oxygen species, which then cause oxidative stress and lower the quality of sexual ornaments.

Figure 1.2

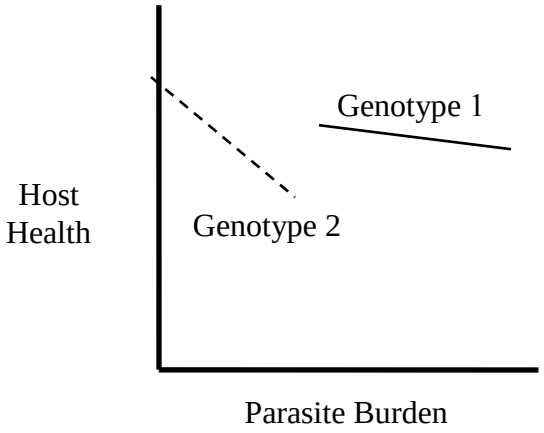
a)


Genotype 1
Host
Health
Genotype 2
Parasite Burden

b)


Genotype 1
Host
Health
Genotype 2
Parasite Burden

c)


Genotype 1
Host
Health
Genotype 2
Parasite Burden

d)

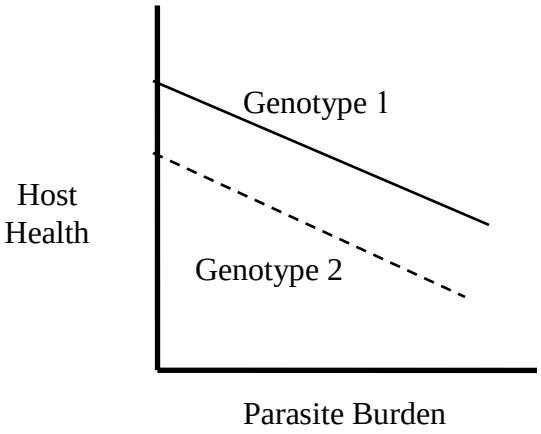

Genotype 1
Host
Health
Genotype 2
Parasite Burden

**Figure 1.2: The relationship between host health and parasite burden for two different host genotypes.** a) Genotype 1 is more resistant than Genotype 2 and therefore maintains a lower parasite burden. There is no difference in tolerance. b) Genotype 1 is more tolerant than Genotype 2, but there is no difference in resistance. c) Genotype 1 is more tolerant than Genotype 2, but less resistant. d) There are no differences in either resistance or tolerance between the two genotypes, but Genotype 1 has greater vigor overall. Figure adapted from Råberg et al. (2007).

# Chapter 2

## Coloration, Paternity, and Assortative Mating in Western Bluebirds

**Abstract**

Coloration in birds can act as an important sexual signal in males, yet in many species both sexes display bright colors. Mutual mate choice may account for this pattern, as males investing a great deal of parental care in the offspring should be choosy about their social mates. It is less clear whether this pattern of mate choice can apply to extra-pair partners as well. Here, we examined western bluebirds (*Sialia mexicana*) to determine if there is assortative mating in which more colorful individuals tend to pair with one another, both in social pairs and between females and their extra-pair partners. Both male and female western bluebirds display both UV-blue structural plumage and a melanin-based chestnut breast patch, although females are duller than males. Social pairs mated assortatively with regard to UV-blue brightness, but not chestnut coloration. There was no evidence that extra-pair partners mated assortatively, but males with brighter UV-blue coloration had fewer extra-pair offspring in their nests. Older males tended to be more successful at siring extra-pair offspring, despite displaying no differences in coloration compared to younger males. Coloration did not play a role in determining extra-pair male success. These results suggest that coloration plays a role in female mate choice for social mates, but not extra-pair partners.

## Introduction

Although most work in sexual selection to date has focused on ornaments in males; in many species, females display some level of ornamentation as well. Several hypotheses for why such ornaments exist in females have been proposed. These include genetic correlation, which suggests female coloration arises as a result of sharing genes for bright plumage with males, in whom plumage coloration evolves under sexual selection (Amundsen 2000, Cardoso and Mota 2010). Another idea suggests that female coloration signals competitive ability to conspecifics (Clutton-Brock 2009, Tobias et al. 2012). One prominent hypothesis states that mutual mate choice can account for the presence of elaborate ornaments in females (Amundsen 2000). Overall, however, the mechanisms driving the evolution of female ornamentation are not well understood (Amundsen 2000, Clutton-Brock 2009).

Typically, females are thought of as the choosy sex while males compete for female attention and are less choosy about their mates (Andersson 1994). However, in situations in which males invest a lot of resources in a given mating, males should also be choosy (Trivers 1972). This is most clearly illustrated in sex role-reversed species, such as pipefish and polyandrous birds, in which males provide the majority of the care for the offspring and females compete for mates (Andersson 1994). However, in socially monogamous species in which males help the females provide costly parental care, such as in many passerine birds, males may also benefit from being choosy (Griggio et al. 2005). Males may benefit from selecting high-quality females to rear their offspring. If males are choosy as well as females, this leads to the prediction that males and females

should pair assortatively, with high-quality males attracting and selecting higher-quality females (Griggio et al. 2005).

Males in socially monogamous species are expected to be selective about their social mates, with whom they share the workload of provisioning the offspring. Whether males should also show a preference when selecting extra-pair mates is less clear. On the one hand, males may not gain much by being choosy about extra-pair partners. If individual copulations are cheap, a male does not invest much in each extra-pair partner, and thus has little reason to be selective (Trivers 1972). However, if opportunities for extra-pair copulations are limited, and if females differ markedly in their ability to rear offspring successfully, one might predict that males will show preferences when selecting extra-pair partners as well. Females, in contrast, should be choosy about all potential mates, be they extra-pair or social. In some species, plumage coloration can act as an indicator of condition and provisioning effort in both males and females (Siefferman and Hill 2005a,b); individuals could thus assess a prospective partner's plumage when selecting a mate.

We tested these predictions by examining coloration and paternity success in western bluebirds (*Sialia mexicana*). Like many species of passerine, western bluebirds are socially monogamous with reasonably high rates of extra-pair paternity (Griffith et al. 2002, Dickinson 2003). In some populations, as many as 45% of nests contain at least one extra-pair offspring (Dickinson 2003). Coloration has been implicated as an important sexual signal in many species of birds (Hill 2006), including eastern bluebirds (*Sialia sialis*; Siefferman and Hill 2005a,b, but see Liu et al. 2007, 2009), and mountain

bluebirds (*Sialia currocoides*; Balenger et al. 2009). Evidence in western bluebirds suggests that females control extra-pair copulations and only mate with preferred males (Dickinson 2001, Ferree and Dickinson 2011).

We predicted that if western bluebirds exhibit mutual mate choice, more brightly colored males would pair socially with more brightly colored females. Assortative mating has been documented in other passerines (Andersson et al. 1998, Griggio et al. 2005, Bitton et al. 2008, Rowe and Weatherhead 2011), but few studies have attempted to determine whether these patterns also apply to extra-pair paternity. Here, we examine rates of extra-pair paternity in relation to male and female coloration. We predict that, if males are selective about extra-pair partners, then assortative mating by color will occur between females and their extra-pair mates as well. We also predict that bright females that are "mismatched" with their social mates (i.e. bright females paired with dull males) will have greater numbers of extra-pair offspring in their nests.

## Materials and Methods

### *Study species and field methods*

Western bluebirds are 23–30 g passerines. They have feathers that reflect in the UV-blue part of the electromagnetic spectrum on their head, back, rump, wings, and tail; these get their color from the feather microstructure (Siefferman and Hill 2003). In addition, they have a melanin-based chestnut patch on the breast (McGraw et al. 2004). Both males and females display similar coloration, although males are brighter (Fig. 2.1).

Bluebirds are secondary cavity nesters, and will readily use artificial nestboxes (Guinan et al. 2008), allowing easy access to nestlings for paternity assessment.

We conducted all field work on the Pajarito Plateau in Los Alamos, New Mexico. The canyons and mesa tops in the Plateau have a series of sites with nestboxes set up on trees. These wooden boxes are placed roughly 1-2 meters off the ground on trees such as Ponderosa pines (*Pinus ponderosa*) at the higher elevations and piñon pines (*Pinus edulis*) at the lower elevations. For further details on the sites and nestboxes, see Fair et al. (2003) and Jacobs et al. (2013).

We conducted the project during the breeding season (May through August) in 2010, 2011, and 2012. During that time, we recorded which nestboxes were occupied, which species lived there, the number of eggs laid, the hatching date of the eggs, and the number of nestlings that successfully fledged. Due to the number of and distance between field sites, we were not able to check sites daily, but we visited each site at least once every two weeks. To determine the hatch date for a given clutch, we estimated the age of the nestlings and counted backwards. Bluebird nestlings hatch synchronously, so we assigned a single hatch date to each nest.

After nestlings had hatched, we captured the adults by using mist nets or trapping them in the box. Nets were placed directly in front of boxes, and primarily captured individuals attempting to enter the nestbox (pers. observation). Thus we assumed that the adults captured in front of a given box were the social parents of any nestlings in that box. For each individual, we recorded sex and measured wing chord (±1 mm) with a ruler, tarsus length (± 0.1 mm) using digital calipers, and mass (± 0.1 g) using a portable

electronic balance, and fitted the bird with a numbered US Fish and Wildlife Service aluminum band. We aged adult males, classifying them as either second year (SY – one year old) or after second year (ASY – two or more years old) based on the presence or absence of molt limits (Shizuka and Dickinson 2005). In many cases, we lacked the experience to positively age females, in which the molt limits can appear less distinct than they do in males (D. Shizuka, pers. comm.), and in a few cases, males began molting near the end of the breeding season and we could not age them reliably. We classified such birds as after hatch year (AHY) and excluded them from analyses using age as a factor. We collected feather samples from the breast (chestnut coloration) and the rump (UV-blue coloration) for all adults. A total of nine feathers were collected from each of these regions and stored in opaque envelopes until color analysis. We then used a sterile needle to puncture the brachial vein and collect 10-50 µL of blood on an FTA® (Whatman Ltd.) card for DNA extraction.

Once nestlings had reached nine days of age or older, we banded them and took a small (10-50 µL) blood sample from each one, which we stored on FTA® cards until DNA extraction and analysis. We then monitored nests until all the nestlings had fledged. Bluebirds in the population typically fledge at age 19-21 days, but can fledge as early as 16 days (Fair and Myers 2002, Guinan et al. 2008). If we found nests empty after the nestlings had reached 16 days of age, we counted the nestlings as fledged. If we found a nest empty before that time, we counted it as depredated. In a few instances, we found banded nestlings dead in the boxes after the rest of the nestlings had fledged; we recorded these deaths, but did not determine what caused them. In our population, a small

percentage of birds produce two successful broods during a single breeding season. For this study, we did not distinguish between first and second broods, but included all nestlings from a given set of social parents for that year in the analysis.

*Coloration Measurements*

We taped nine feathers from each body region on a black, non-reflective background in an overlapping pattern to mimic how they would sit on the birds (Siefferman and Hill 2003). We then measured reflectance from 300-700 nm using an Ocean Optics USB4000 spectrophotometer (range: 200-1100 nm; Ocean Optics Inc., Dunedin, Florida, USA) and a xenon light source. We measured the reflectance of each sample relative to a white standard (Ocean Optics). For each reading, the probe was encased in a black sheath to exclude light and keep the probe at a 90º angle relative to the feathers for all readings. We took five readings of each region by lifting up the probe and then replacing it. These readings were averaged together.

To quantify individual coloration, we used three measures: hue, chroma, and brightness. To measure brightness, we took the average reflectance value across a 300-700 nm spectrum. This was done for both breast brightness (chestnut) and rump brightness (UV-blue). Other papers examining UV-blue coloration in bluebirds have measured the intensity, or the peak reflectance value, instead of brightness (Siefferman and Hill 2003, Balenger et al. 2009). We chose to use average brightness because this measure could easily be calculated the same way for both UV-blue and chestnut plumage. Our measurements of overall average brightness for the UV-blue feathers were highly

correlated with the measure of blue intensity ($n = 141$, $r = 0.95$, $p < 0.001$). To measure chroma, we summed the reflectance values over the area of interest (300-500 for UV-blue region, 500-700 for chestnut region) and divided that by the total summed reflectance over the whole spectrum (Siefferman and Hill 2003). For UV-blue feathers, we calculated the hue as the wavelength of peak reflectance. The chestnut feathers exhibited no obvious peak, and reflectance values increased steadily beyond 700 nm (Fig. 2.1). To calculate hue for this region, we took the wavelength that corresponded to the median reflectance between 300 and 700 nm, or $\lambda[(R_{max}+R_{min})/2)]$ (Rowe and Weatherhead 2011).

*Molecular Analyses*

DNA was eluted from the FTA® cards according to the manufacturer's instructions. We checked DNA concentration using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE); all samples contained 10-150 ng/µL of DNA. To assign paternity, we used highly variable microsatellite loci previously described for western bluebirds and closely-related eastern bluebirds. We amplified fragments from five loci: Smex5, Smex9, and Smex14 (Ferree et al. 2008) and Sialia36 and Sialia37 (Faircloth et al. 2006). PCR products were sized using an ABI 3100 Genetic Analyzer, and we confirmed product size visually using GeneMapper v4.1. We calculated allele frequencies and assigned paternity to individual males using the program CERVUS 3.0 (Kalinowski et al. 2007). The microsatellites we used had an average of 11.2 alleles per locus, and none of the allele frequencies deviated from Hardy-Weinberg equilibrium. The combined probability of non-exclusion was 0.008 when the mother was known. In cases where

offspring showed no mismatches in alleles relative to the social father, we classified them as within-pair offspring. We assigned offspring to extra-pair sires if CERVUS assigned paternity to a given male with 95% confidence or if CERVUS assigned a male with 80% confidence and the putative father was from the same field site or a field site in close proximity to the nest. We used this criterion as, during the breeding season, most conspecific intruders and extra-pair males come from adjacent territories (Dickinson 2001, Akçay et al. 2012).

In addition to assessing paternity, we also used DNA samples to determine if adults were infected with avian malaria (*Plasmodium spp.* and *Haemoproteus spp.*). To do this, we used a nested PCR reaction following the methods described in Waldenström et al. (2004) to amplify a section of mitochondrial DNA in the parasites. We ran PCR products on a gel and determined infection by the presence or absence of bands. For more details on the detection of avian malaria, see chapter 3.

*Statistical Analyses*

We performed all analyses in SAS v9.2 (SAS Institute 2008). We checked data for normality using a Shapiro-Wilk test and transformed non-normal variables as needed. Males and females have different coloration, with females displaying duller, lighter plumage than males (Fig. 2.1). Because of this, when color data for both sexes were included together in the analysis, data exhibited a bimodal, non-normal distribution; thus we analyzed coloration in each sex separately. Data on UV-blue coloration did not vary between years, and so we pooled data from all years for the analysis. However, we found

a significant effect of year on breast coloration variables. To control for this, we standardized data for breast hue, chroma, and brightness within each year, setting the mean to zero and the standard deviation to one.

Color variables (hue, chroma, and brightness) taken from the breast region were highly correlated with each other (Table 2.1); we performed a principal components analysis to reduce the number of variables. A separate principal components analysis was run on the breast color variables for each sex. The first principal component from each analysis was retained using the Kaiser-Guttman criterion (eigenvalues greater than one, Table 2.2). Hue, chroma, and brightness for the UV-blue coloration were not highly correlated with each other in males (Table 2.1) or females, and thus we could not easily reduce these variables using a principal components analysis (Tabachnick and Fidell 2007). Instead, we chose to focus on a single measure, brightness, because this measure exhibited the greatest variation between individuals, as demonstrated by a coefficient of variation that was almost an order of magnitude higher than the coefficients for hue and chroma (in males: UV-blue hue CV = 0.027, UV-blue chroma CV = 0.052, UV-blue brightness CV = 0.206).

Over the course of the study, we captured and banded 129 adult birds; of these, 21 (16%) were recaptured in multiple years. If they stayed mated to the same partner in both years (4 pairs), we only included the data for that pair from the first year in which they were captured. If an individual acquired a new social mate in subsequent years, we viewed this as an independent pairing event and included data from both pairs in the analysis. We used a correlation analysis to detect assortative mating between males and

females. To determine the effects of coloration on rates of extra-pair paternity, we used a general linear model with the proportion of within pair young in a nest as the response variable and the coloration of both social parents as predictor variables. We tested for an interaction between social father coloration and social mother coloration to determine if "mismatched" pairs had higher rates of extra-pair paternity.

To see if coloration acted as an indicator of male quality, we used a general linear model to test for relationships between male color variables and other characteristics, such as mass, tarsus length, breeding date (as estimated by the hatch date of the offspring), and infection with avian malaria. To test whether coloration affected a male's ability to sire offspring in another nest, we used a logistic regression, classifying males as either successful or unsuccessful at fathering extra-pair young. We included mass, male age, breast, and rump coloration, and tarsus length (a measure of body size) in these analyses. We compared coloration of cuckolded males (males with extra-pair young in their nests) and extra-pair males using paired t-tests. In one instance we detected offspring from two extra-pair sires within the same nest. We averaged the coloration scores of these two extra-pair males. In some cases, we collected data from individuals whose partners we were unable to catch or whose nests were depredated before we could take samples from the nestlings. Because of this, sample sizes vary.

**Results**

We retained the first principal component from the analyses of breast feather coloration in males and females. In both sexes, the first component had high positive

loadings on breast hue and breast chroma, and high negative loadings on breast brightness (Table 2.2). Higher PC1 scores correspond to individuals that have darker, redder, more saturated chestnut feathers on their breast patches. Neither the brightness of the UV-blue plumage nor PC1 of breast plumage differed significantly between second year and after second year males (UV-blue coloration: $F_{1,56} = 1.06$, $p = 0.31$, chestnut PC1: $F_{1,57} = 0.03$, $p = 0.87$).

*Assortative Mating*

We obtained coloration data from 48 social pairs in which we captured both the male and the female. Within social pairs, bluebirds mated assortatively with respect to UV-blue coloration, with brighter males pairing with brighter females ($r = 0.31$, $n = 48$, $p = 0.03$, Fig. 2.2). However, the males and females of social pairs did not show any evidence of assortative mating by breast coloration ($r = 0.08$, $n=48$, $p = 0.57$). The total number of offspring fledged from a nest did not depend on male coloration, female coloration, or the interaction between the two, either when considering rump coloration ($F_{3,45} = 0.52$, $p = 0.67$) or breast coloration ($F_{3,45} = 1.34$, $p = 0.27$).

We genotyped 324 nestlings over three years, 114 of which we classified definitively as extra-pair offspring (35.2%). Of the 76 nests for which we genotyped nestlings, 51 of them (67%) contained at least one extra-pair offspring. In many cases, we were unable to confidently assign paternity to nestlings. However, we were able to assign 31 extra-pair offspring to 19 different fathers. One male entered the analysis twice as an extra-pair sire because he fathered extra-pair young in two different years. We had

complete coloration data for 20 pairs of females and their extra-pair partners. In one case, a female had extra-pair offspring sired by more than one male; we included both sires in the analysis, but removing one did not substantially affect our results. We found no evidence of assortative mating by coloration between females and their extra-pair partners either when considering UV-blue plumage ($r = 0.17$, $n = 20$, $p = 0.47$) or chestnut plumage ($r = -0.07$, $n = 20$, $p = 0.77$).

*Coloration and Extra-pair Paternity*

The interactions between male and female plumage coloration were not significant, so we removed them from the model. When we did this, we found a marginally non-significant effect on the proportion of within-pair young in a nest ($F_{4,43} = 2.31$, $p = 0.07$), but only male UV-blue brightness significantly predicted how well a male maintained paternity ($F_{1,43} = 6.46$, $p = 0.02$), with brighter males siring a greater proportion of the offspring in their own nests (Fig. 2.3). When we looked at the characteristics of males that successfully sired extra-pair offspring, the overall model was significant ($\chi^2_{5,43} = 13.02$, $p = 0.02$). However, the only variable that significantly predicted success was male age ($\chi^2_{1,43} = 6.72$, $p = 0.01$), with tarsus length as a nearly significant predictor ($\chi^2_{1,43} = 3.63$, $p = 0.06$). Males that sired extra-pair offspring tended to be older and larger (Table 2.3). We found no difference in coloration between extra-pair males and the males they cuckholded either in UV-blue brightness ($t_{11} = -0.27$, $p = 0.79$) or chestnut coloration ($t_{11} = 0.65$, $p = 0.53$). Male coloration did not show any

relationship with mass, body size (tarsus length), infection status, or breeding date (all $p > 0.2$).

**Discussion**

Social pairs of bluebirds had more similar plumage coloration, as has been found in several other studies of passerines (Andersson et al. 1998, Griggio et al. 2005, Bitton et al. 2008, Rowe and Weatherhead 2011). Liu et al. (2009) found no evidence of assortative mating in the closely related eastern bluebird, but had a smaller sample size. Brighter UV-blue coloration also appeared to play a role in social mate selection in our population, and brighter males maintained a higher proportion of paternity within their own nests. Based on these findings, one might also expect that brighter females paired to dull males might be more likely to seek out extra-pair copulations, but this was not the case. In fact, our data do not suggest any role of female coloration in determining the rates of extra-pair paternity within a nest.

Females may prefer to mate with brighter males because coloration provides information about direct benefits. For example, male eastern bluebirds with brighter UV-blue coloration provisioned incubating females more than duller males (Siefferman and Hill 2005b). Thus, females mated to brighter males may gain direct benefits in terms of food and possibly parental care for their offspring. We were unfortunately unable to measure nest provisioning by males in this study; however, a similar mechanism could be operating in our population. If so, females would benefit more from having a brighter

social mate, but would not necessarily benefit from extra-pair copulations with brighter males.

Explanations other than mutual mate choice may account for the pattern of assortative mating we found. For example, coloration may play a role in social selection (West-Eberhard 1983). Western bluebirds are secondary cavity nesters, and as such, access to nest sites may be limited, causing competition both with conspecifics (Guinan et al. 2008) and other cavity-nesting bird species (Duckworth and Badyaev 2007). In bluebirds, both males and females defend the nest site (Guinan et al. 2008). Female coloration may act as a signal of female social status and competitive ability to potential competitors more often than it functions in mutual mate choice (Tobias et al. 2012). Male coloration may act in a similar manner, signaling fighting status to competitors and functioning in male-male competition for territories. In pied flycatchers (*Ficedula hypoleuca*), another secondary cavity nester, Alatalo et al. (1986) demonstrated that male coloration was not directly used in mate choice by females, but that females instead selected the male with the best territory, resulting in the appearance of female preference for color. A similar mechanism could operate in western bluebirds, and if females then compete with each other for males with the best territories, this could produce the pattern we found in social pairs.

Eastern bluebird females did not prefer more brightly colored males in either aviary or field experiments, implying that coloration itself is not important in mate choice in this species (Liu et al. 2007, 2009). However, coloration does correlate with a male's ability to secure a territory early in the breeding season (Siefferman and Hill 2005c).

These results lend support to the idea that coloration functions primarily as a social signal in bluebirds, and that assortative mating patterns may result from brighter males and females defending better territories rather than mate choice on ornaments per se. We could not distinguish between these alternatives in our study. If bright coloration acts as a signal in territorial male-male competition, perhaps brighter males can better exclude intruders from their territories. This might explain why brighter males maintain a greater share of within-pair paternity. However, we consider male exclusion of competitors to be an unlikely explanation for maintenance of within-pair paternity, as highly aggressive male bluebirds do not have lower numbers of extra-pair offspring in their nests (Duckworth 2006). If brighter pairs hold better territories, one might also predict that such pairs would successfully fledge more offspring. We did not find any evidence that this was the case. However, predation accounts for a large number of nest failures in our study, and the probability that a nest will be depredated may not depend on individual brightness in this species.

Our results on how coloration affects patterns of extra-pair paternity are almost opposite the patterns found in mountain bluebirds. There, UV-blue coloration predicts male success at obtaining extra-pair copulations, but brighter males do not have fewer extra-pair offspring in their own nests (Balenger et al. 2009). Mountain bluebirds are congeners of western bluebirds, and one might predict that sexual selection should operate similarly in these species. However, coloration in this species does not indicate parental effort (Balenger et al. 2007), unlike in eastern bluebirds (Siefferman and Hill 2003, 2005a). Thus coloration may not play an important role in signaling direct benefits

in mountain bluebirds. Moreover, this study did not take male age into account as a confounding factor. It is possible that in mountain bluebirds, coloration is affected by age, as is true for eastern bluebirds (Siefferman et al. 2005). Female western bluebirds prefer older males as extra-pair partners (Dickinson 2001, Ferree and Dickinson 2011); if mountain bluebird females show similar preferences and older males display brighter plumage, this could account for the discrepancy. Extra-pair mate choice for older individuals occurs in our population. Such choice for older malesis common in passerines (Cleasby and Nakagawa 2012), and female choice for overall viability may explain this pattern (Kokko and Lindström 1996).

Our study focuses on ornaments that come from melanin or structural coloration. In birds, many studies have focused on carotenoid-based plumage and its role as a sexual signal. Such studies often infer that carotenoid-based signals are more honest indicators of individual condition and health, and therefore more likely to be used by choosy females for mate choice (Olson and Owens 1998, Badyaev and Hill 2000). This assumption is based on the fact that animals must obtain carotenoids from their diets, and thus carotenoid-based signals may indicate good foraging ability (McGraw 2006). Also, studies have suggested a link between carotenoids and immune response, making carotenoid-based signals good candidates as indicators of health and parasite infection status (Lozano 1994, Blount et al. 2003). Melanin-based signals, in contrast, were considered easy to make and more useful as badges of status, with social interactions required to ensure signal honesty (Møller 1987, Badyaev and Hill 2000). Recently, however, some scientists have asked whether carotenoids truly deserve a special place

above melanin in honest signaling theory (Jawor and Breitwisch 2003, Griffith et al. 2006). While animals can produce melanins themselves, melaning production may be energetically expensive or require the use of limiting metals or amino acids for synthesis, making melanins more costly than previously thought (Jawor and Breitwisch 2003, McGraw 2008). A recent study on yellowthroats (*Geothlypis trichas*) demonstrated that in one population, the color of the carotenoid-based yellow breast best predicted immune response, while in another population, the size of melanin-based black mask best predicted immune response (Dunn et al. 2010). These two plumage traits conveyed essentially the same information about health, despite their different mechanisms of production (Dunn et al. 2010). Structural plumage can, likewise, indicate important information about male quality, such as condition and parental effort (Keyser and Hill 2000, Siefferman and Hill 2003). In our study, however, neither the melanin-based chestnut plumage nor the structural-based UV-blue plumage indicated male size or mass (which we use as a proxy for overall condition; Schamber et al. 2009). Plumage coloration could indicate another quality we did not measure, such as fighting ability or parental effort (Siefferman and Hill 2003, 2005b).

One should note that our study includes data taken primarily from breeding adults. There may well be floater males (and females) in the population that we did not capture. This pattern resulted from logistical limitations; it is extremely difficult to capture birds reliably without a known point (such as a nest) to which one knows they will return. However, this could bias our results; if brighter males are more likely to hold territories and attract mates, such males would be more likely to be sampled in our

dataset. This limitation may also help explain why we found no effects of age on plumage color, in contrast to other studies on bluebirds (Siefferman et al. 2005, Budden and Dickinson 2009). Perhaps only brightly colored second year males can secure territories, in which case our data on second year males would be biased in favor of brighter individuals. Another shortcoming is that there may have been pairs of bluebird nesting in natural cavities (rather than nestboxes) at our sites that we did not catch, as well as males whose nests were depredated before we could capture them. This would not necessarily affect the data on coloration, as we have no particular reason to think that such males would differ in their coloration from the ones we sampled. However, it makes assigning paternity more difficult. We were unable to assign paternity to a large number of extra-pair offspring, indicating that there were many males present in the population that we did not catch. We have no way of knowing whether these missing males were floaters, males nesting in natural cavities, or males in nestboxes whose nests were depredated before we sampled them. If the latter, the males in our study could have fathered extra-pair offspring in these nests that we would not have detected, leading to an underestimate of the reproductive success of the males in our study. Also, the limited number of extra-pair sires assigned might have affected our ability to detect assortative mating between females and their extra-pair partners due to reduced sample sizes.

In conclusion, we found evidence that coloration plays a role in social pair formation and within-pair paternity rates in western bluebirds, but that male age, rather than coloration, determines success at siring extra-pair offspring. This implies that plumage coloration acts more as a signal of direct benefit in this species, possibly related

to territory quality. Such signals would prove useful for females selecting social mates, but not extra-pair partners. Future work should elucidate which signals females use to select extra-pair mates in this species, including the mechanisms by which females discriminate between older and younger males, and the benefits, if any to choosing older males as extra-pair sires.

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**References**


Akçay Ç., Searcy W.A., Campbell E., Reed V.A., Templeton C.N., Hardwick K.M., and Beecher M.D. (2012) Who initiates extrapair mating in song sparrows? *Behavioral Ecology* 23:44-50.

Alatalo R.V., Lundberg A., and Glynn C. (1986) Female pied flycatchers chose territory quality and not male characteristics. *Nature* 323:152-153.

Amundsen T. (2000) Why are female birds ornamented? *Trends in Ecology and Evolution* 15:149-155.

Andersson M. (1994) *Sexual Selection*. Princeton: Princeton University Press.

Andersson S., Ornborg J., and Andersson M. (1998) Ultraviolet sexual dimorphism and assortative mating in blue tits. *Proceedings of the Royal Society B* 265:445-450.

Badyaev A.V. and Hill G.E. (2000) Evolution of sexual dichromatism: contributions of carotenoid- versus melanin-based coloration. *Biological Journal of the Linnean Society* 69:153-172.

Balenger S.L., Johnson L.S., Brubaker J.L., and Ostlind E. (2007) Parental effort in relation to structural plumage coloration in the mountain bluebird (*Sialia currucoides*). *Ethology* 113:838-846.

Balenger S.L., Johnson L.S., and Masters B.S. (2009) Sexual selection in a socially monogamous bird: male color predicts paternity success in the mountain bluebird. *Behavioral Ecology and Sociobiology* 63: 403-411.

Bitton P., Dawson R.D., and Ochs C.L. (2008) Plumage characteristics, reproductive investment and assortative mating in tree swallows *Tachycineta bicolor*. *Behavioral Ecology and Sociobiology* 62:1543-1550.

Blount J.D., Metcalfe N.B., Birkhead T.R., and Surai P.F. (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. *Science* 300:125-127.

Budden A.E. and Dickinson J.L. (2009) Signals of quality and age: the information content of multiple plumage ornaments in male western bluebirds *Sialia mexicana*. *Journal of Avian Biology* 40:18-27.

Cardoso G.C. and Mota P.G. (2010) Evolution of female carotenoid coloration by sexual constraint in *Carduelis* finches. *BMC Evolutionary Biology* 10:82.

Clearsby I.R. and Nakagawa S. (2012) The influence of male age on within-pair and extra-pair paternity in passerines. *Ibis* 154:318-324.

Clutton-Brock T. (2009) Sexual selection in females. *Animal Behaviour* 77:3-11.

Dickinson J.L. (2001) Extrapair copulations in western bluebirds (*Sialia mexicana*): female receptivity favors older males. *Behavioral Ecology and Sociobiology* 50:423-429.

Dickinson J.L. (2003) Male share of provisioning is not influenced by actual or apparent loss of paternity in western bluebirds. *Behavioral Ecology* 14: 360-366.

Duckworth R.A. (2006) Behavioral correlations across breeding contexts provide a mechanism for a cost of aggression. *Behavioral Ecology* 17:1011-1019.

Duckworth R.A. and Badyaev A.V. (2007) Coupling of dispersal and aggression facilitates the rapid range expansion of a passerine bird. *Proceedings of the National Academy of Sciences USA* 104:15017-15022.

Dunn P.O., Garvin J.C., Whittingham L.A., Freeman-Gallant C.R., and Hasselquist D. (2010) Carotenoid and melanin-based ornaments signal similar aspects of male quality in two populations of the common yellowthroat. *Functional Ecology* 24:149-158.

Fair, J. M., and O. B. Myers. 2002. The ecological and physiological costs of lead shot and immunological challenge to developing Western Bluebirds. Ecotoxicology 11:199-208.

Fair J.M., Myers O.B., and Ricklefs R.E. (2003) Immune and growth response of western bluebirds and ash-throated flycatchers to soil contaminants. *Ecological Applications* 13: 1817-1829.

Faircloth B.C., Keller G.P., Nairn J., Palmer W.E., Carroll J.P., and Gowaty P.A. (2006) Tetranucleotide microsatellite loci from eastern bluebirds *Sialia sialis*. *Molecular Ecology Notes* 6: 646-649.

Ferree E.D., Dickinson J.L., Kleiber D., Stern C.A., Haydock J., Stanback M.T., Schmidt V., Eisenberg L., and Stolzenburg C. (2008) Development and cross-species testing of western bluebird (*Sialia mexicana*) microsatellite primers. *Molecular Ecology Resources* 8: 1348-1350.

Ferree E.D. and Dickinson J.L. (2011) Natural extrapair paternity matches receptivity patterns in unguarded females: evidence for importance of female choice. *Animal Behaviour* 82:1167-1173.

Griffith S.C., Owens I.P.F., and Thuman K.A. (2002) Extra-pair paternity in birds: a review of interspecific variation and adaptive function. *Molecular Ecology* 11: 2195-2212.

Griffith S.C., Parker T.H., and Olson V.A. (2006) Melanin- versus carotenoid-based sexual signals: is the difference really so black and red? *Animal Behaviour* 71:749-763.

Griggio M., Valera F., Casas A., and Pilastro A. (2005) Males prefer ornamented females: a field experiment of male choice in the rock sparrow. *Animal Behaviour* 69:1243-1250.

Guinan J.A., Gowaty P.A., and Eltzroth E.K. (2008) Western Bluebird (Sialia mexicana),. In A. Poole, Ed. *The Birds of North America Online* Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online: http://bna.birds.cornell.edu/bna/species/510doi:10.2173/bna.510

Hill G.E. (2006) Female mate choice for ornamental coloration. In: Hill G.E. and McGraw K.J. ed. *Bird Coloration: Function and Evolution* (Vol. 2). Cambridge: Harvard University Press.

Jacobs A.C., Reader L.L., and Fair J.M. (2013) What determines the rates of double brooding in western bluebirds? *Condor* 115:386-393.

Jawor J.M. and Breitwisch R. (2003) Melanin ornaments, honesty, and sexual selection. *Auk* 120:249-265.

Kalinowski S.T., Taper M.L., and Marshall T.C. (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* 16:1099-1006.

Keyser A.J. and Hill G.E. (2000) Structurally based plumage coloration is an honest signal of quality in male blue grosbeaks. *Behavioral Ecology* 11:202-209.

Kokko H. and Lindström J. (1996) Evolution of female preference for old mates. *Proceedings of the Royal Society B* 263:1533-1538.

Liu M., Siefferman L., and Hill G.E. (2007) An experimental test of female choice relative to male structural coloration in eastern bluebirds. *Behavioral Ecology and Sociobiology* 61:623-630.

Liu M., Siefferman L., Mays H., Steffen J.E., and Hill G.E. (2009) A field test of female mate preference for male plumage coloration in eastern bluebirds. *Animal Behaviour* 78:879-885.

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

McGraw K.J. (2006) Mechanics of carotenoids-based coloration. In: Hill G.E. and McGraw K.J. ed. *Bird Coloration: Mechanisms and Measurements* (Vol. 1). Cambridge: Harvard University Press.

McGraw K.J. (2008) An update on the honesty of melanin-based color signals in birds. *Pigment Cell Melanoma Research* 21:133-138.

McGraw K.J., Wakamatsu K., Ito S., Nolan P.M., Jouventin P., Dobson F.S., Austic R.E., Safran R.J., Siefferman L.M., Hill G.E., and Parker R.S. (2004) You can't judge a pigment by its color: carotenoid and melanin content of yellow and brown

feathers in swallows, bluebirds, penguins, and domestic chickens. *Condor* 106:390-395.

Møller A.P. (1987) Social control of deception among status signalling house sparrows *Passer domesticus*. *Behavioral Ecology and Sociobiology* 20:307-311.

Olson V.A. and Owens I.P.F. (1998) Costly sexual signals: are carotenoids rare, risky, or required? *Trends in Ecology and Evolution* 13:510-514.

Rowe K.M.C. and Weatherhead P.J. (2011) Assortative mating in relation to plumage traits shared by male and female American robins. *Condor* 113:881-889.

SAS Institute. 2008. *SAS/STAT user's guide*. Version 9, 2nd ed. Cary, NC: SAS Institute Inc.

Schamber J.L., Esler D., and Flint P.L. (2009) Evaluating the validity of using unverified indices of body condition. *Journal of Avian Biology* 40:49-56.

Shizuka D. and Dickinson J.L. (2005) Using molt limits to age western bluebirds. *Journal of Field Ornithology* 76:193-196.

Siefferman L. and Hill G.E. (2003) Structural and melanin coloration indicate parental effort and reproductive success in male eastern bluebirds. *Behavioral Ecology* 14: 855-861.

Siefferman L.M. and Hill G.E. (2005a) Evidence for sexual selection on structural plumage coloration of female eastern bluebirds (*Sialia sialis*). *Evolution* 59: 1819-1828.

Siefferman L.M. and Hill G.E. (2005b) Blue structural coloration of male eastern bluebirds *Sialia sialis* predicts incubation provisioning to females. *Journal of Avian Biology* 36:488-493.

Siefferman L.M. and Hill G.E. (2005c). UV-blue structural coloration and competition for nestboxes in male eastern bluebirds. *Animal Behaviour* 69:67-72.

Siefferman L., Hill G.E., and Dobson F.S. (2005) Ornamental plumage coloration and condition are dependent on age in eastern bluebirds *Sialia sialis*. *Journal of Avian Biology* 36:428-435.

Tabachnick B.G. and Fidell L.S. (2007) *Using Multivariate Statistics, 5th Ed.* Boston: Pearson Education, Inc.

Tobias J.A., Montgomerie R., and Lyon B.E. (2012) The evolution of female ornaments and weaponry: social selection, sexual selection and ecological competition. *Proceedings of the Royal Society B* 367:2274-2293.

Trivers R.L. (1972) Parental investment and sexual selection. In: Campbell B., ed. *Sexual Selection and the Descent of Man, 1871-1971*. Chicago: Aldine Publishing Company.

Waldenström J., Bensch S., Hasselquist D., and Östman Ö. (2004) A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. *Journal of Parasitology* 90:191-194.

West-Eberhard M.J. (1983) Sexual selection, social competition, and speciation. *Quarterly Review of Biology* 58:155-183.

**Table 2.1: Spearman correlation coefficients between hue, chroma, and brightness from the breast region (chestnut) and the rump (UV-blue) in male western bluebirds (n=71).** * indicates $p < 0.05$, ** indicates $p < 0.001$

| | Rump Hue | Rump Chroma | Rump Brightness | Breast Hue | Breast Chroma | Breast Brightness |
|---|---|---|---|---|---|---|
| Rump Hue | 1.0 | -0.0001 | -0.025 | **-0.446**** | **-0.264*** | **0.371*** |
| Rump Chroma | | 1.0 | -0.011 | 0.089 | 0.026 | -0.128 |
| Rump Brightness | | | 1.0 | 0.219 | 0.147 | -0.170 |
| Breast Hue | | | | 1.0 | **0.492**** | **-0.753**** |
| Breast Chroma | | | | | 1.0 | **-0.585**** |
| Breast Brightness | | | | | | 1.0 |

**Table 2.2: Results of the principal components analysis performed on chestnut breast coloration variables (hue, chroma, and brightness) in both males and females.** We retained the first principal component in each case; all others had eigenvalues less than one.

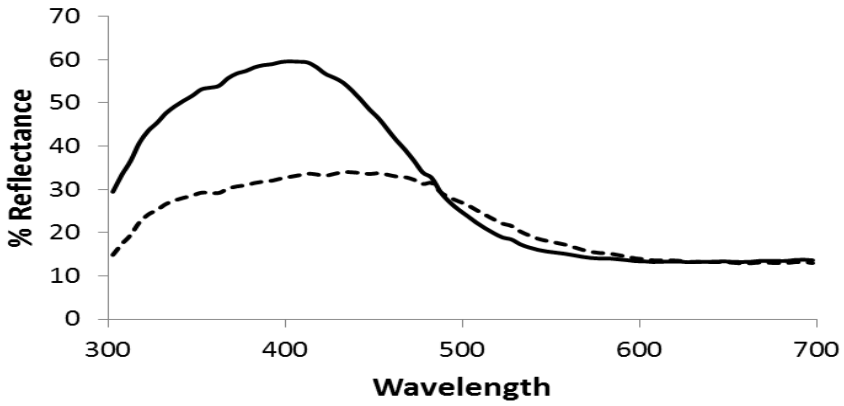
| | **Male PC1 loadings** | **Female PC1 loadings** |
|---|---|---|
| Hue | 0.87 | 0.90 |
| Chroma | 0.73 | 0.89 |
| Brightness | -0.89 | -0.89 |
| **Total Variation Explained by PC1** | **69.5%** | **80.4%** |

**Table 2.3: Results of the logistic regression performed to determine whether coloration and individual traits affected the probability that a male would sire extra-pair offspring.** We considered mass, body size (tarsus length), UV-blue brightness, breast coloration (PC1), and age as predictor variables. The model describes the probability that a male will not sire extra-pair offspring.

| | | | | | 95% Confidence Interval for Odds Ratio | |
|---|---|---|---|---|---|---|
| Variable | β | Wald Chi-Square | P-value | Odds Ratio | *Lower* | *Upper* |
| Mass | -0.16 | 0.599 | 0.439 | 0.854 | 0.572 | 1.274 |
| Tarsus Length | -0.75 | 3.634 | **0.057** | 0.474 | 0.220 | 1.021 |
| UV-blue Brightness | 0.04 | 0.375 | 0.540 | 1.039 | 0.920 | 1.173 |
| Breast Coloration | -0.17 | 0.1875 | 0.6650 | 0.840 | 0.381 | 1.852 |
| Age | -3.18 | 6.717 | **0.010** | 0.042 | 0.004 | 0.461 |

Figure 2.1

A)




B)

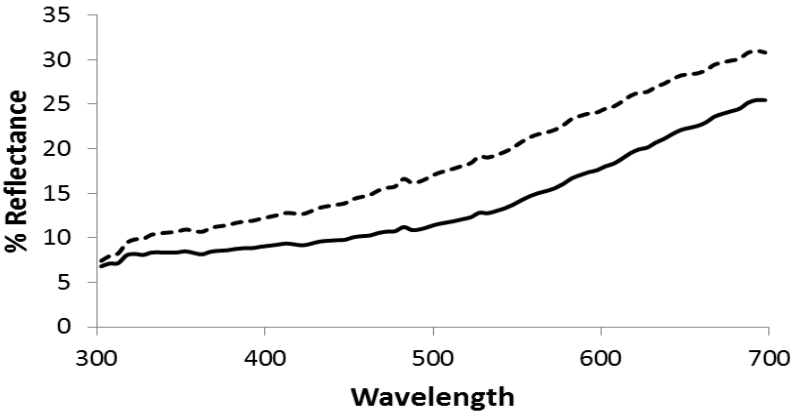



**Figure 2.1: The reflectance curves averaged from birds captured in 2010.** The solid line represents the average for males (n = 18) and the dashed line represents the average for females (n = 20). A) The reflectance curve generated by the UV-blue rump feathers. B) The reflectance curve generated by the chestnut breast feathers.

Figure 2.2

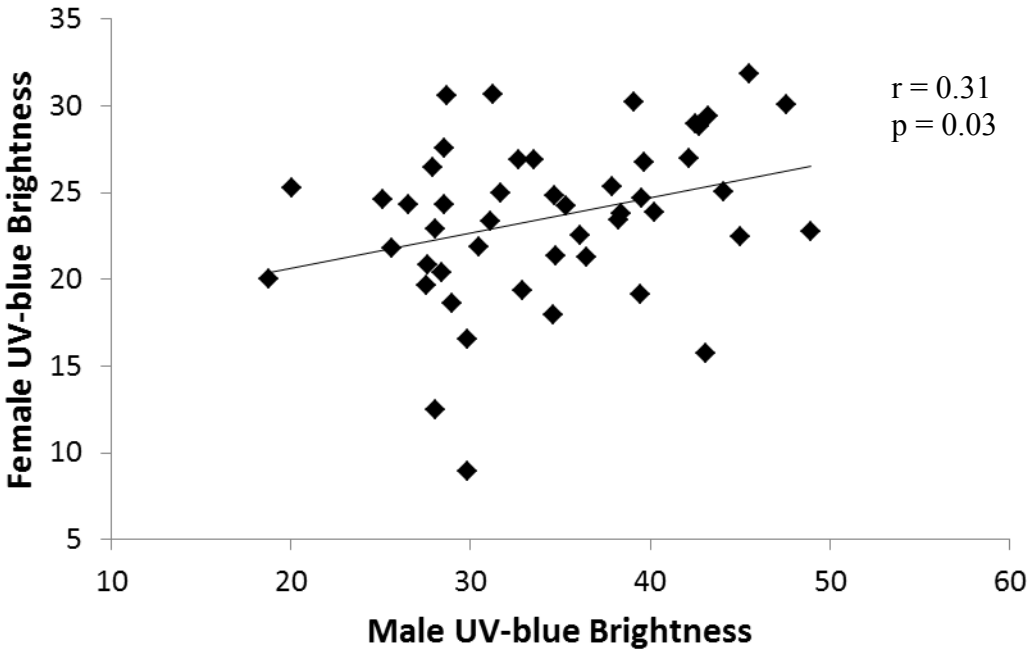



**Figure 2.2: The relationship between brightness of the UV-blue rump feathers in males and females of a social pair.**

Figure 2.3

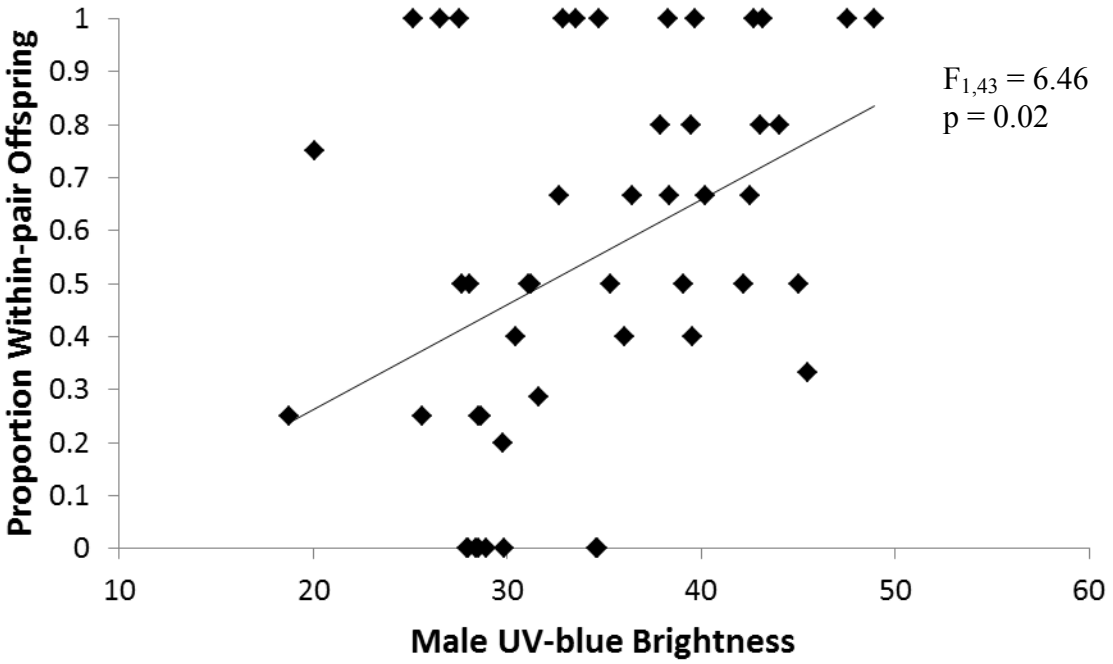



**Figure 2.3: The relationship between male UV-blue brightness and the proportion of nestlings he sired in his own nest.**

# Chapter 3

## Parasite infection, but not immune response, influences paternity in western bluebirds

**Abstract**

Parasites can impose heavy costs on their hosts, and immune response is a crucial component of an animal's defense against parasites and pathogens. Females may benefit from selecting mates that are parasite resistant and/or have a stronger immune response, and trade-offs between immune response and sexual signaling have been proposed as a mechanism to ensure signal honesty. Much of the work on sexual signaling and immune response does not consider parasites directly and thus cannot confirm whether a stronger immune response necessarily results in lower parasite burdens. Also immunity is costly, and these costs can lower the overall fitness of individuals with too strong of an immune response. Males with immune responses of intermediate strength are therefore expected to be preferred by females and have the highest reproductive success. We tested whether immune response and parasite loads relate to sexual signaling and mating preferences in western bluebirds (*Sialia mexicana*). Immunity did not predict male reproductive success when considering either within-pair or extra-pair offspring, although a stronger immune response was correlated with lower parasite loads. However, uninfected males were more likely to sire extra-pair offspring than males infected with avian malaria. Thus females were more likely to mate with uninfected males, but not necessarily males with a stronger immune response. Our results are consistent with the idea that females select parasite-resistant males as mates in order to gain resistance genes for their offspring.

## Introduction

Parasites are ubiquitous and can affect many aspects of their hosts' biology. Sexual selection is one such aspect, and sexual signals may have evolved, at least in part, to convey information to prospective mates about an individual's ability to resist parasites (Hamilton and Zuk 1982). Females selecting males with the most elaborate sexual signals may improve offspring fitness by gaining genes for parasite resistance, an idea known as the Hamilton-Zuk hypothesis (Hamilton and Zuk 1982). Numerous studies have tested this hypothesis by examining immune response in lieu of parasites (Møller et al. 1999). Immunity constitutes one of the main lines of defense that organisms have against pathogens. However, immune responses may be costly, and these costs may create trade-offs between immunity and other aspects of an organism's life history, such as reproduction (Sheldon and Verhulst 1996). Trade-offs between the immune system and reproductive effort have been invoked often in the context of sexual signaling. Under this scenario, sexual signals work as handicaps (Zahavi 1975, 1977, Getty 2002), and only high-quality individuals can afford to pay the costs of producing a sexual signal while maintaining an appropriate immune response (Folstad and Karter 1992, Sheldon and Verhulst 1996).

One critical assumption that many studies examining immunity and sexual signaling make is that stronger immunity results in lower parasite burdens and therefore greater parasite resistance. However, such studies rarely test the validity of this assumption. Immunity may or may not determine parasite loads, depending upon the parasites involved and the method(s) with which one measures immunity (Adamo 2004).

Thus it is critically important to also examine parasite levels and how these relate to immune response and fitness (Graham et al. 2011). This allows one to better interpret immune measures, as higher measures of immunity may not always result in better parasite resistance and higher survival/fitness (Adamo 2004, Graham et al. 2011).

Another problem that has arisen in studies examining the relationship between immunity and sexual signals is that they often find conflicting results, with some reporting that individuals with a stronger immune response have better sexual signals, while others report the opposite pattern (Jacobs and Zuk 2012). One possible explanation for these discrepancies is that immunity and sexual signal quality may exhibit a non-linear relationship. Ecoimmunology studies that examine sexual signaling often implicitly assume that a stronger immune response is better for an individual. However, the costs of immunity may create a situation in which individuals benefit most by having an intermediate level of immunity (Viney et al. 2005). Too weak of an immune response would result in the individual succumbing to pathogens, but too strong of an immune response would be costly, both in terms of energy (Sheldon and Verhulst 1996, Schmid-Hempel 2003) and potential damage to the host's own tissues (Graham et al. 2005). These costs can have serious consequences; for example, breeding female eiders (*Somateria mollissima)* that mounted an immune response to a novel antigen had lower survival rates (Hanssen et al 2004). Given that the birds were not infected with actual parasites, the reduced survival must have come from the costs of the immune response itself, indicating that a strong immune response can have negative fitness consequences for the host.

Some empirical work on wild birds supports the idea that an intermediate level of immunity results in higher fitness. Stjernman et al. (2008) found that blue tits (*Cyanistes caeruleus*) infected with intermediate levels of the blood parasite *Haemoproteus* had higher survivorship than individuals with very high or very low levels of infection. This implies that there is a cost for individuals that mount an immune response strong enough to drive parasite levels very low. Similarly, Råberg and Stjernman (2003) found evidence of stabilizing, rather than directional, selection on primary antibody response to a novel pathogen in blue tits, implying that there is some intermediate, optimal level of immunity.

Here we examine the relationship between parasite load, immune response, sexual signaling, and reproductive success in the western bluebird (*Siala mexicana*). To determine parasite infection, we focus on Haemosporidian blood parasites such as *Plasmodium* spp. and closely-related *Haemoproteus* spp. (hereafter referred to generally as avian malaria; Pérez-Tris et al. 2005). Haemosporidian parasites cause chronic, long-term infections in many of the birds they parasitize, and such chronic infections are ideal for testing predictions of the Hamilton-Zuk Hypothesis (Hamilton and Zuk 1982). Moreover, such parasites are common in wild birds, and infection with these parasites can result in lower fitness (Atkinson and Van Riper 1991, Merino et al. 2000, Asghar et al. 2011).

Western bluebirds, like many species of passerines, are socially monogamous (Dickinson 2001, Griffith et al. 2002). Males will socially pair with a single female to raise the chicks, but will also engage in extra-pair copulations with females at neighboring territories. Rates of extra-pair paternity vary by population: in a California,

45% of nests contained extra-pair offspring (Dickinson 2003), while in Montana the rate was much lower, with only 27% of nests containing extra-pair young (Duckworth 2006). In the population studied here, 67% of nests contained extra-pair offspring (see chapter 2). Extra-pair paternity can increase overall male reproductive success (O'Brien and Dawson 2011), and also increases the variance of male reproductive success and the strength of sexual selection on males (Albrecht et al. 2009, Balenger et al. 2009a). To properly measure male reproductive success, one must take such extra-pair offspring into account. In this species, plumage coloration may act as a sexual signal and play a role in social pair formation (see chapter 2). If an immune response of intermediate strength does confer the highest fitness, we expect females to prefer those males that have such a response. Therefore we predicted that coloration would show a non-linear relationship with immune response. We also predicted that individuals with a stronger immune response would have lower parasite burdens, and that males with an intermediate immune response would be preferred as mates and sire the most extra-pair offspring.

## Materials and Methods

### *Study Species and Field Methods*

Western bluebirds are medium-sized (23 – 30 g) passerines that nest in secondary cavities and readily inhabit human-made nestboxes (Guinan et al. 2008). In our population, they breed from May until August, and most pairs produce a single clutch of 4 – 5 eggs per season (range 2 – 7 eggs per clutch). Double brooding occurs rarely, with only around 5% of pairs producing second clutches (Jacobs et al. 2013). Both male and

female western bluebirds display UV-blue coloration on the back, head, wings, and tail, and a patch of chestnut coloration on the breast. Males, however, display brighter plumage than females (see chapter 2).

All field work was conducted at the Los Alamos National Laboratory (LANL) in Los Alamos, New Mexico. Los Alamos is located on the Pajarito Plateau at approximately 2,200 m (7,300 ft) in the Jemez Mountains in northern New Mexico. A network of nestboxes was established around the Plateau in 1997 and has been monitored every breeding season since then. This network consists of over 400 wooden nestboxes mounted on trees in ponderosa pine forests or piñon-juniper woodlands located in 37 sites (Fair and Myers 2002a, Jacobs et al. 2013). Boxes are mounted approximately 1-2 meters above the ground, primarily on ponderosa pines (*Pinus ponderosa*) or piñon pines (*Pinus edulis)*. Western bluebirds are the most common occupants of these boxes. During the course of this study (2010 – 2012), a large wildfire occurred in the area. In late June, 2011, the Las Conchas fire burned over 150,000 acres in the Jemez Mountains (New Mexico Incident Information System 2011). Although this fire did not burn any of our field sites, it forced an evacuation of the area and disrupted field work for several weeks. For further details on field sites and box construction, see Fair et al. (2003) and Jacobs et al. (2013).

All parasite and coloration data were collected during the breeding seasons in 2010 through 2012. Immune measurements were carried out in the 2011 and 2012 seasons. During the breeding season, we monitored nestboxes and recorded all active nests, or nests in which we found eggs. For each box, we determined the species nesting

there, the clutch size, the number of eggs that hatched, the hatching date, and the number of offspring fledged. In many cases, we did not record the date of clutch initiation because we found nests after the clutch had been completed. However, we use the Julian hatching date of the nestlings as our measure of the timing of breeding.

We captured adult birds at their boxes using either box traps or mist nets. This method primarily captures individuals coming to the box to feed nestlings (pers. obs.), and we presumed that adults captured in front of a given box were the social parents of any nestlings inside that box. Upon capture, we fitted each individual with a U.S. Fish and Wildlife Service numbered aluminum leg band and we measured the wing chord (±1 mm), tarsus length (±0.1 mm) and mass (±0.1 g) for each individual and took feather samples from the rump and chest regions for coloration analysis (see below). To determine age, we used the presence or absence of molt limits to classify individuals as second-year (SY) or after second-year (ASY) birds (Shizuka and Dickinson 2005). We also used a sterile needle to collect a blood sample of roughly 100 µL from the brachial vein. We placed a drop of blood on a slide and made a blood film, placed around 10-50 µL on an FTA® (Whatman Ltd.) card for DNA extraction, and took the rest back to the lab for use in the bacteria-killing (BKA) assay (see below).

As part of a different experiment, we captured some birds twice – the first time we inoculate birds with sheep red blood cells (SRBCs; see below). We also collected feather samples from adults during this capture event. A week later, we recaptured birds to take blood for immune assays (see below). For these birds, we had three total measures of immune response: bacteria killing ability (BKA) of the plasma, antibody response to

SRBCs, and leukocyte profiles (see below). For all other adults in the study, our immune measurements consisted of leukocyte profiles and BKA response. In some cases, we failed to recapture focal individuals injected with SRBCs at the end of the second week; we had DNA and feather samples for these birds, but no immune data or data on infection intensity (see below). Many of these individuals were birds we captured during the summer of 2011, when the Las Conchas wildfire forced an evacuation of our study areas and disrupted our sampling efforts.

When nestlings reached nine days of age, we banded them and took a small (10-50 µL) blood sample from them for use in paternity analyses. We continued to monitor nests until all nestlings had fledged, at age 16 days or older (Fair and Myers 2002b). If we found nests empty before nestlings would have reached fledging age, we considered them depredated. In some cases, individual nestlings within a given nest disappeared before reaching fledging age; we counted them as depredated as well. In a few cases, we found banded nestlings dead after their siblings had fledged; we collected and recorded these, but did not attempt to determine the cause of death. All work was done in accordance with the Guidelines to the Use of Wild Birds in Research (Fair et al. 2010) and with the approval of the LANL Institutional Animal Care and Use Committee.

*Bacteria-killing Assay*

To measure immune function, we used a bacteria killing assay (BKA) measuring the ability of the plasma to kill bacteria *in vitro*, primarily via natural antibodies and complement (Millet et al. 2007, Liebl and Martin 2009). We used *Escherichia coli*

(ATCC #8739) supplied in pellets containing $1x10^6$ to $1x10^7$ microorganisms per pellet (Epower Microorganisms, MicroBioLogics, St. Cloud, MN). Each pellet was reconstituted in 40 mL of sterile 1X phosphate buffered saline (PBS) at 37°C. This stock solution was then diluted down to make a working solution of $2x10^5$ microorganisms per mL. We made a fresh stock solution every week and kept all bacterial solutions at 4ºC at all times. We followed the procedures described in Liebl and Martin (2009). Briefly, 1.5 μL of plasma was mixed with 34.5 μL PBS and 12.5 μL of the bacterial working solution. This mixture was then incubated for 30 minutes at 37°C. After the initial incubation, we added 250 μL of sterile tryptic soy broth. We also prepared a sterile blank, which contained 48.5 μL of PBS with 250 μL of tryptic soy broth, and a positive control, which contained 36 μL PBS, 12.5 μL of the bacterial working solution, and 250 μL of tryptic soy broth. All samples were incubated for 12 hours at 37°C after the addition of the tryptic soy broth. We used a Tecan Infinite 200 (Tecan Group Ltd., Männedorf, Switzerland) to measure the absorbance of the samples at 300 nm. We calculated the antimicrobial activity of the plasma as 1 – (absorbance of sample/absorbance of positive control). We ran all samples in triplicate when we had sufficient plasma volumes and in duplicate when we did not and we averaged the results from each replicate to get a single value for each individual.

*Antibody Response*

To measure the ability of an individual to mount an antibody response to a novel antigen, we challenged birds using sheep red blood cells (SRBCs). We captured adults at

a select set of nests shortly after the nestlings had hatched as part of another study on how supplemental feeding affects immune response in the wild. Captured birds were banded to ensure individual identification and then injected intraperitoneally with 0.075 mL of a 10% SRBC solution in 10x PBS(Sigma-Aldrich Company, St. Louis, MO). Basic measurements, feather samples, and blood samples for paternity were also taken at this time. Birds were then released, and one week following the initial injection, we recaptured the birds and collected a 20-50 μL sample of blood in an unheparinized microhematocrit tube. We also collected blood for the bactericide assay and slides at this time. Blood samples were taken from the wing vein using sterile methods. We successfully recaptured and collected blood from 18 individuals (10 females and 8 males).

We spun the blood down and froze the resulting serum sample at -70°C until we could perform the assay. Before beginning the assay, we first heated the serum samples to 56°C for 30 minutes in a hot water bath. We then serially diluted serum samples and added SRBCs to each well, then recorded the highest dilution in which we observed agglutination (Fair et al. 2003). We report values as the $\log_2$ of the reciprocal of the highest dilution with agglutination.

*Leukocyte and Parasite Counts*

A single drop of blood was placed on a slide and spread to form a blood film. Once the blood had air dried, we fixed the slide in 100% methanol and stained it using Wright-Geimsa stain (Dein 1984). We examined all blood films under 1000x

magnification. For each slide, we conducted two counts of 100 leukocytes, classifying these as lymphocytes, heterphils, monocytes, eosinophils, or basophils. We then averaged the results of both counts and took the ratio of heterophils:lymphocytes (H/L ratio).

Each slide was examined for a minimum of a half hour to look for parasites. If we detected an infection, we counted a minimum of 5,000 red blood cells (RBCs) and quantified the number of these that contained parasites. One person (ACJ) conducted all cell and parasite counts to minimize variation between observers. We could not readily distinguish between *Haemoproteus* and *Plasmodium* infections in the slides, but subsequent molecular analyses revealed that both genera were present (see below). We did not detect any infections with *Leucocytozoan*. Aside from malaria infections, the only other parasite detected in the blood films was an infection by an unidentified filarial nematode in one individual; we did not include this in the analysis.

*Molecular Analyses: Detection of Malaria*

We eluted DNA from the FTA cards according to the manufacturer's instructions and confirmed that all samples contained sufficient DNA using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE). Each DNA sample contained 10-150 ng/µL of DNA. To determine whether birds were infected with avian malaria, we used a nested PCR reaction following the methods described in Waldenström et al. (2004). In cases where the PCR gave us different results from the slides (e.g. if the PCR indicated that the bird was infected, but no parasites were detected on the slide), we ran the reaction twice

to confirm our results. If the second reaction confirmed the presence of the parasite, we counted the bird as infected.

We took products from the nested PCR reaction from five birds and sequenced them to determine the strains of parasite infecting the population. Once we had obtained sequences, we used the Basic Local Alignment Search Tool (BLAST) to locate similar sequences. We determined that the parasites fell into two general categories: some birds were infected with a strain of *Plasmodium*, while others were infected with a strain of *Haemoproteus*. This corresponds with other studies looking at avian malaria in western bluebirds (Ricklefs and Fallon 2002, Martinsen et al. 2008). MalAvi, the database for information on avian haemosporidian parasites (Bensch et al. 2009), indicates that two strains of avian malaria have been detected in this species. One strain belongs to the genus *Haemoproteus*, and also infects the closely related eastern bluebird (Ricklefs and Fallon 2002). The other strain is classified as a variety of *Plasmodium relictum* (Martinsen et al. 2008).

*Paternity Analyses*

To assign paternity, we used highly variable microsatellite loci previously described for bluebirds. We used the loci Smex5, Smex9, and Smex14 (Ferree et al. 2008) and Sialia36 and Sialia37 (Faircloth et al. 2006). PCR products were sized using an ABI 3100 Genetic Analyzer, and we confirmed product size visually using GeneMapper v4.1. To assign extra-pair offspring to their genetic fathers, we used CERVUS 3.0 (Kalinowski et al. 2007). For further details on the paternity analysis, see chapter 2.

*Coloration Analyses*

We collected nine feathers from each region on the body we measured. Feathers came from the rump, which exhibits UV-blue, structural-based coloration (Siefferman and Hill 2003) and the breast, which exhibits rusty-red, melanin-based coloration (McGraw et al. 2004). We taped feathers to a black, non-reflective background in an overlapping fashion to mimic how they would lie on the birds (Siefferman and Hill 2003). We then measured reflectance relative to a white standard using an Ocean Optics USB4000 spectrophotometer (range: 200-1100 nm; Ocean Optics Inc., Dunedin, Florida, USA) with a xenon light source. For each region, we took five readings by lifting up and replacing the probe; these readings were subsequently averaged together.

To quantify individual coloration, we used three measures: hue, chroma, and brightness. To measure brightness, we took the average reflectance value across the 300-700 nm spectrum. This was done for both breast brightness (chestnut) and rump brightness (UV-blue). Other papers examining UV-blue coloration in bluebirds have measured the intensity, or the peak reflectance value, instead of brightness (Siefferman and Hill 2003, Balenger et al. 2009b). We chose to use average brightness because this measure could easily be calculated the same way for both UV-blue and chestnut plumage. Our measurements of overall average brightness for the UV-blue feathers were highly correlated with the measure of blue intensity ($n = 141$, $r = 0.95$, $p < 0.001$). To measure chroma, we summed the reflectance values over the area of interest (300-500 for UV-blue region, 500-700 for chestnut region) and divided that by the total summed reflectance

over the whole spectrum. For UV-blue feathers, we calculated the hue as the wavelength of peak reflectance. The chestnut feathers exhibited no obvious peak, and reflectance values increased steadily beyond 700 nm. To calculate hue for this region, we took the wavelength that corresponded to the median reflectance between 300 and 700 nm, or $\lambda[(R_{max}+R_{min}/2)]$ (Rowe and Weatherhead 2011).

*Statistical Analyses*

We performed all analyses in SAS v9.2 (SAS Institute 2008). In some cases, we captured the same individual in multiple years. We included each individual in the analysis only once to avoid pseudoreplication. When selecting which points to include, we chose to include the data for the year from which we had collected the most information (e.g. all immune assay data). In cases where we had complete data on the same individual for both years, we randomly selected one year to include in the analysis. Some birds were part of a food supplementation experiment; however, as neither immune response nor parasitic infection differed between fed and control birds (MANOVA $F_{3,13}$ = 2.26, p = 0.13), we pooled data from both treatments and used them in our analyses.

When examining coloration, we chose to focus on male UV-blue brightness, as measured by the overall brightness of the rump feathers. This measure is variable between individuals and plays a role in assortative mating and male paternity (see chapter 2). To determine whether infection with avian malaria affected other measures of breeding and condition, we performed a MANOVA (proc glm) with infection status (infected versus uninfected) as the predictor variable and mass, breeding date, and H/L

ratio as response variables. We also ran a series of general linear models to determine the effects of immune response (BKA), mass, infection status, age, and H/L ratio on male coloration, within-pair paternity, and overall reproductive success (including all surviving within-pair and extra-pair young sired by that male). We also included $BKA^2$ in our model to test for non-linear effects of immune response. We included year in these models as a random effect. We used a logistic regression to test whether infection status (infected versus not), age, mass, breeding date, or immune response (BKA) affected a male's probability of siring extra-pair young and of losing paternity in his own nest.

In cases of uninfected birds, we could not distinguish between birds that did not show an infection with malaria because they had successfully fought off an infection and those that were not infected because they had never been exposed. Therefore, we did not consider uninfected individuals to be informative. For the analyses on the intensity of infection, we considered only birds we knew to be infected and excluded uninfected individuals from the analysis. In some cases, we had DNA samples but no slide data for infected individuals – we excluded these individuals from all analyses on parasitemia. We identified 8 individuals as infected using molecular methods, but had no quantification of parasite load because we were unable to detect parasites using the slides. We arbitrarily assigned these individuals an infection rate of 1/15,000 RBCs. We used this value because it is greater than zero, thus indicating the presence of an infection, but low enough that we probably would not have detected an infection in the slides.

Even when considering infected individuals only, parasite intensity data were non-normally distributed, with a few of the hosts harboring high parasite loads while

most carried only light infections. Transformation of the data did not improve the distribution. We used a Wilcoxon Rank Sum test to detect differences in infection intensity between the sexes. To determine if immune measures correlated with lower parasite loads, we used Spearman rank correlations. Due to the small sample sizes for the SRBC antibody assay, we analyzed these data separately to avoid reducing sample sizes for all analyses. We used a t-test to compare the antibody response of infected versus uninfected individuals.

## Results

### *Parasite Infection*

We were able to obtain parasite data using molecular methods for 124 individual birds, some of which we caught in multiple years. Of these, 50 (40.3%) were infected with avian malaria, 25 females and 25 males. In a two cases, recaptured individuals that had been infected showed no infection when caught in subsequent years, indicating a clearance of the infection. Infected individuals did not differ from uninfected individuals in terms of mass, BKA response, or H/L ratio ($F_{3,64} = 0.03$, $p = 0.83$).

We found no differences between the sexes in terms of BKA, H/L ratio, or infection ($F_{3,64} = 1.63$, $p = 0.19$). However, when we focused on the proportion of parasitized RBCs within infected individuals, we found a non-significant trend for males to have lower infection levels than females ($z = 1.72$, $p = 0.09$). Individuals with a stronger BKA response tended to have lower levels of infection ($r_s = -0.47$, $n = 30$, $p = 0.009$, Fig. 3.1). We found a marginally non-significant difference in antibody response

to SRBCs between infected and uninfected individuals ($t_{14} = 1.97$, $p = 0.07$); uninfected individuals tended to have stronger responses. We found no association between SRBC response and parasitemia ($r_s = -0.40$, $n = 7$, $p = 0.37$), but this might be due to small sample sizes (7 infected individuals for whom we had both SRBC data and RBC parasitemia estimates).

*Coloration and Immune Response*

UV-blue brightness in males was unrelated to infection status, BKA, mass, or breed date (overall model $F_{5,32} = 0.71$, $p = 0.62$, all $p > 0.1$). We found no polynomial relationship between immune response (BKA) and coloration, as indicated by a non-significant effect of BKA squared ($F_{1,32} = 2.26$, $p = 0.14$).

*Male Reproductive Success, Immune Response, and Parasites*

We genotyped 324 nestlings over three years, 114 of which we classified definitively as extra-pair offspring (35.2%). Of the 76 nests for which we genotyped nestlings, 51 of them (67%) contained at least one extra-pair offspring. In many cases, we were unable to confidently assign paternity to nestlings. However, we were able to assign 31 extra-pair offspring to 19 different fathers. Six nestlings (five within-pair and one extra-pair) disappeared or died of unknown causes after we had collected DNA samples but before fledging. We included data from these nestlings in calculating the proportion of within-pair young in a given nest, but did not include them when determining a male's overall reproductive success for a given year.

We found no evidence that the likelihood of a male losing paternity in his own nest was affected by male age, mass, breeding date, immune response (BKA) or infection with avian malaria ($\chi^2_{6,33} = 4.08$, $p = 0.67$). However, we did find an effect of infection on the probability that a male would sire extra-pair offspring in another nest ($\chi^2_{6,31} = 13.43$, $p = 0.04$). Males that were more likely to sire extra-pair offspring tended to be uninfected (Wald $\chi^2_{1,31} = 4.88$, $p = 0.03$; Fig. 3.2) and heavier (Wald $\chi^2_{1,31} = 3.93$, $p = 0.05$; Table 3.1). When we considered the difference between second-year and after second-year individuals, males that sired extra-pair young tended to be older, but this trend did not reach significance (Wald $\chi^2_{1,31} = 3.14$, $p = 0.08$). We found no effects of mass, breed date, year, infection or immune response on how well a male maintained paternity within his own nest, as determined by the proportion of within-pair young in the nest (overall model $F_{6,31} = 0.40$, $p = 0.87$, all individual $p > 0.30$). Additionally, we failed to identify any factors that influenced overall male reproductive success (total offspring fledged: $F_{6,31} = 0.85$, $p = 0.55$).

**Discussion**

Male bluebirds infected with avian malaria were less likely to sire extra-pair offspring. This suggests that females may prefer unparasitized males as extra-pair partners. Females did not, however, prefer unparasitized males as social mates as well perhaps because social mate choice is constrained by other factors, such as nest site availability. Females may also seek other characteristics in social mates that are more indicative of direct benefits such a good territory or a high level of paternal care. Since

females gain no direct benefits from extra-pair mates, they could engage in extra-pair matings to obtain good genes for their offspring (Griffith et al. 2002, but see Akçay and Roughgarden 2007). Our findings are consistent with the idea of females selecting extra-pair mates that may carry genes for parasite resistance, as suggested by the Hamilton-Zuk hypothesis (Hamilton and Zuk 1982). Females might prefer uninfected mates to avoid becoming infected themselves (Clayton 1991, Able 1996), but this is unlikely to apply to the spread of avian malaria, which requires a vector, usually a biting fly (Atkinson and van Riper 1991). Given the transmission route of the parasite, engaging in extra-pair copulations with an infected individual likely does not increase the risk of infection.

Female western bluebirds exert active control over extra-pair copulations and will reject the advances of non-preferred males (Dickinson 2001). However, females in this species do not seem to actively seek out males for copulations, unlike other species of passerines (Chiver et al. 2008). Rather, males from neighboring territories make forays into the female's territory and solicit copulations from her (Dickinson et al. 2000, Dickinson 2001). Our finding that uninfected males sire more extra-pair offspring could indicate that females are actively choosing such males. Conversely, this pattern could also arise if infected males are less likely to make forays into their neighbors' territories.

The strength of the immune response did not predict male sexual signaling or reproductive success, despite being related to lower parasite burdens. Possibly the relationship between immune response and lower parasite burdens is too weak for any effect of immunity to be detected in our study. Interestingly, we also did not find any influence of immune response or infection on male plumage coloration. This contradicts

other studies, which have found that infection with parasites reduces the quality of sexual signals (Møller 1990,1991, Zuk et al. 1990) or that individuals with better plumage-based sexual signals can better survive or cope with infection (Nolan et al. 1998, Lindstöm and Lundström 2000). However, in western bluebirds, UV-blue coloration plays a role in social pair formation, but does not affect extra-pair paternity (chapter 2). Thus coloration may be more useful for indicating direct benefits in this species, such as male parental investment (Siefferman and Hill 2003, 2005). If coloration acts as an indicator of direct benefits in this species, females have no reason to select brighter males as extra-pair partners, as they gain no direct benefits from such partners.

The lack of association between plumage coloration and infection implies that if females are using male infection status as a criterion for extra-pair mate choice, they must have some other way of assessing male health. It is unlikely that they use overall male condition, as in our population we found no differences in mass between infected and uninfected individuals. Possibly behavioral differences exist between males infected with malaria and uninfected males; females could then use these differences when choosing extra-pair partners. Higher parasite loads can reduce other aspects of male display, such as song rate (Møller 1991), and females may use these displays when selecting mates. Dickinson et al. (2000) documented a display done by male western bluebirds when seeking extra-pair copulations from females. However, they also reported that this display did not influence male success in gaining copulations, making it an unlikely candidate for female choice.

We found no effects of immune response or parasite infection on overall male reproductive success. This is surprising, given that uninfected males tend to sire more extra-pair offspring. Reproductive success, of course, is determined by many factors unrelated to parasites and immunity. For example, predation accounts for most of the nest failures in our population (unpub. data) and other populations of western bluebirds (Kozma and Kroll 2010). Predation of nestlings is unlikely to depend on the parasite status of the adults. Also, we may have underestimated male reproductive success if males sired extra-pair offspring in nests we did not sample. While we collected data on most nestlings, some nests were depredated before the nestlings were old enough for us to obtain samples. Finally, males may have been nesting in nearby natural cavities whose nestlings we did not sample.

While immune assays can provide useful information about an individual's health, it is critical to also look at parasites and how they interact with the immune system (Adamo 2004, Graham et al. 2011). Our findings that birds that have a stronger bacteria-killing ability of the blood tended to have lower numbers of parasites in the blood, and that uninfected birds tended to have stronger antibody responses to SRBCs may have resulted from a stronger immune response suppressing parasite levels. There is indirect evidence that the bacteria-killing ability of the blood can help birds fight infection in the wild. For example, Florida scrub-jays (*Aphelocoma coerulescens*) with a stronger bacteria-killing response were more likely to survive an epidemic (Wilcoxen et al. 2010). However, we cannot say for certain whether the trend we observed for BKA resulted from as the activity of complement and natural antibodies, which are measured

by the BKA response (Millet et al. 2007), or whether BKA correlates with some other mechanism of immunity which acts against the malaria parasites. Various components of the immune system may interact and trade off with one another (Forsman et al. 2008, Palacios et al. 2012), and it is possible that a strong response in BKA correlates with a strong response in a different branch of the immune system that may help to fight off malaria. Also, we cannot say for certain whether the relationships we observed are due to the effects of the immune system on parasites or due to parasites altering the immune system. Parasites can interact with the immune system in a variety of ways, including immune evasion and blocking of certain reactions within the immune system (Schmid-Hempel 2008). Thus we may have detected differences in immune response because more heavily infected individuals have reduced immune defenses as a result of parasitism.

When birds become infected with avian malaria, they pass through two phases of the infection. During the first, acute phase, parasitemia levels spike, and many infected individuals die (Atkinson and van Riper 1991, Zehtindjiev et al. 2008, Asghar et al. 2012). This is followed by a much longer, chronic infection period, during which the parasites persist in the host at low, but fairly steady, levels (Atkinson and van Riper 1991, Asghar et al. 2012) and have more subtle, but still detrimental, effects on host fitness (Merino et al. 2000, Asghar et al. 2011). The birds in our study population were harboring chronic malaria infections, as indicated by low levels of parasitemia. These values may still provide us with some indication of how heavily infected individuals were during the acute phase, as the intensity of infection within an individual is strongly

correlated between these two phases (Asghar et al. 2012). However, detecting infection during the chronic phase can be difficult. In many cases, individuals with very low parasite counts may be categorized as uninfected when using slides, leading to an underestimation of the true parasite prevalence in the population. We encountered this problem in our study when using slide data. Of the fifty individuals that we identified as infected using molecular methods, there were eight (16%) for which we did not detect any infection using slides.

Molecular methods have shown that birds from our population are infected with a strain of *Haemoproteus* (Ricklefs and Fallon 2002). Our sequence data confirm that this parasite is present, and that some birds are infected with a strain of *Plasmodium*. However, we were unable to distinguish between these species using slide data, and thus cannot say which birds were infected with *Plasmodium* and which were infected with *Haemoproteus*. This is a potential weakness of our study, as different lineages of avian malaria can impose different costs on their hosts. For example, Asghar et al. (2011) found that infection a specific lineage of *Haemoproteus payevskyi* (lineage GRW1) was associated with later arrival of female great reed warblers (*Acrocephalus arundinaceus*) at the breeding site, while the other lineages investigated did not show this pattern. Thus further, detailed analysis of exactly which lineages infected which birds could prove fruitful. Another possible shortcoming of our study is that we only sampled breeding individuals. If heavily infected birds are less likely to hold territories and secure mates, we may have missed them in our sampling. This could lead to an underestimation of the true prevalence and intensity of infection in our population.

In conclusion, we found evidence that uninfected males are more successful at siring extra-pair offspring, but immune response did not influence male paternity, despite being correlated with lower parasite burdens. This implies that parasite infection plays a larger role than immune response in sexual selection in this species. In future, to properly tease out the effects of parasites on mate choice and the relationships between parasite load and immunity, studies employing experimental infections would prove useful. Such manipulations would allow us to determine if individuals with stronger immunity can truly fight off infection better than those with a weaker immune response.

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## References


Able D.J. (1996) The contagion indicator hypothesis for parasite-mediated sexual selection. *Proceedings of the National Academy of Sciences* 93:2229-2233.

Adamo S.A. (2004) Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. *Journal of Insect Physiology* 50:209-216.

Akçay E. and Roughgarden J. (2007) Extra-pair paternity in birds: review of the genetic benefits. *Evolutionary Ecology Research* 9:855-868.

Albrecht T., Vinkler M., Schnitzer J., Poláková R., Munclinger P., and Bryja J. (2009) Extra-pair fertilizations contribute to selection on secondary male ornamentation in a socially monogamous passerine. *Journal of Evolutionary Biology* 22:2020-2030.

Asghar M., Hasselquist D., and Bensch S. (2011) Are chronic avian haemosporidian infections costly in wild birds? *Journal of Avian Biology* 42:530-537.

Asghar M., Westerdahl H., Zehtindjiev P., Ilieva M. Hasselquist D., and Bensch S. (2012) Primary peak and chronic malaria infection levels are correlated in experimentally infected great reed warblers. *Parasitology* 139:1246-1252.

Atkinson C.T. and van Riper C. (1991) Pathogenicity and epizootiology of avian haematozoa: *Plasmodium*, *Leucocytozoon*, and *Haemoproteus*. In: Loye J.E. and Zuk M. eds. *Bird-Parasite Interactions: Ecology, Evolution, and Behaviour*. Oxford: Oxford University Press.

Balenger S.L., Johnson L.S., Mays H.L., and Masters B.S. (2009a) Extra-pair paternity in the socially monogamous mountain bluebird *Sialia currucoides* and its effect on the potential for sexual selection. *Journal of Avian Biology* 40: 173-180.

Balenger S.L., Johnson L.S., and Masters B.S. (2009b) Sexual selection in a socially monogamous bird: male color predicts paternity success in the mountain bluebird. *Behavioral Ecology and Sociobiology* 63: 403-411.

Bensch S., Hellgren O., and Pérez-Tris J. (2009) MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. *Molecular Ecology Resources* 9:1353-1358.

Chiver I., Stutchbury B.J.M., and Morton E.S. (2008) Do male plumage and song characteristics influence female off-territory forays and paternity in the hooded warbler? *Behavioral Ecology and Sociobiology* 62: 1981-1990.

Clayton D.H. (1991) The influence of parasites on host sexual selection. *Parasitology Today* 7:329-334.

Dein F.J. (1984) *Laboratory Manual of Avian Hematology*. East Northport, NY: Association of Avian Veterinarians.

Dickinson J.L. (2001) Extrapair copulations in western bluebirds (*Sialia mexicana*): female receptivity favors older males. *Behavioral Ecology and Sociobiology* 50:423-429.

Dickinson J.L. (2003) Male share of provisioning is not influenced by actual or apparent loss of paternity in western bluebirds. *Behavioral Ecology* 14: 360-366.

Dickinson J.L., Kraaijeveld K., and Smit-Kraaijeveld F. (2000) Specialized extrapair mating display in western bluebirds. *Auk* 117:1078-1080.

Duckworth R.A. (2006) Behavioral correlations across breeding contexts provide a mechanism for a cost of aggression. *Behavioral Ecology* 17:1011-1019.

Fair J. M. and Myers O.B. (2002a) Early reproductive success of western bluebirds and ash-throated flycatchers: a contaminated landscape perspective. *Environmental Pollution* 118:321-330.

Fair, J. M., and Myers O.B. (2002b) The ecological and physiological costs of lead shot and immunological challenge to developing Western Bluebirds. Ecotoxicology 11:199-208.

Fair J.M., Myers O.B., and Ricklefs R.E. (2003) Immune and growth response of western bluebirds and ash-throated flycatchers to soil contaminants. *Ecological Applications* 13: 1817-1829.

Fair J. M., Paul E., Jones J., Clark A.B., Davie C., and Kaiser G. (20100 *Guidelines to the Use of Wild Birds in Research.* The Ornithological Council, Washington D.C.

Faircloth B.C., Keller G.P., Nairn J., Palmer W.E., Carroll J.P., and Gowaty P.A. (2006) Tetranucleotide microsatellite loci from eastern bluebirds *Sialia sialis*. *Molecular Ecology Notes* 6: 646-649.

Ferree E.D., Dickinson J.L., Kleiber D., Stern C.A., Haydock J., Stanback M.T., Schmidt V., Eisenberg L., and Stolzenburg C. (2008) Development and cross-species testing of western bluebird (*Sialia mexicana*) microsatellite primers. *Molecular Ecology Resources* 8: 1348-1350.

Folstad I. and Karter A.J. (1992) Parasites, bright males, and the immunocompetence handicap hypothesis. *American Naturalist* 139:603-622.

Forsman A.M., Vogel L.A., Sakaluk S.K., and Thompson C.F. (2008) Immune-challenged house wren broods differ in the relative strengths of their responses among different axes of the immune system. *Journal of Evolutionary Biology* 21:873-878.

Getty T. (2002) Signaling health versus parasites. *American Naturalist* 159:363-371.

Graham A.L., Allen J.E., and Read A.F. (2005) Evolutionary causes and consequences of immunopathology. *Annual Review of Ecology Evolution and Systematics* 36: 373-397.

Graham A.L., Shuker D.M., Pollitt L.C., Auld S.K.J.R., Wilson A.J., and Little T.J. (2011) Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology. *Functional Ecology* 25:5-17.

Griffith S.C., Owens I.P.F., and Thuman K.A. (2002) Extra-pair paternity in birds: a review of interspecific variation and adaptive function. *Molecular Ecology* 11: 2195-2212.

Guinan J.A., Gowaty P.A., and Eltzroth E.K. (2008) Western Bluebird (Sialia mexicana),. In A. Poole, Ed. *The Birds of North America Online* Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online: http://bna.birds.cornell.edu/bna/species/510doi:10.2173/bna.510

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

Hanssen S.A., Hasselquist D., Folstad I., and Erikstad K.E. (2004) Costs of immunity: immune responsiveness reduces survival in a vertebrate. *Proceedings of the Royal Society: B* 271:925-930.

Jacobs A.C. and Zuk M. (2012) Parasites and Sexual Selection: Do Mechanisms Matter? In: Demas G.E. and Nelson R.J. eds. *Eco-Immunology*. New York: Oxford University Press.

Jacobs A.C., Reader L.L., and Fair J.M. (2013) What determines the rates of double brooding in western bluebirds? *Condor* 115:386-393.

Kalinowski S.T., Taper M.L., and Marshall T.C. (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* 16:1099-1006.

Kozma J.M. and Kroll A.J. (2010) Nest survival of western bluebirds using tree cavities in managed ponderosa pine forests of central Washington. *Condor* 112:87-95.

Liebl A.L. and Martin L.B. (2009) Simple quantification of blood and plasma antimicrobial capacity using spectrophotometry. *Functional Ecology* 23:1091-1096.

Lindström K. and Lundström J. (2000) Male greenfinches (*Carduelis chloris*) with brighter ornaments have higher virus infection clearance rate. *Behavioral Ecology and Sociobiology* 48:44-51.

Martinsen E.S., Perkins S.L., and Schall J.J. (2008) A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): Evolution of life-

history traits and host switches. *Molecular Phylogenetics and Evolution* 47:261-273.

McGraw K.J., Wakamatsu K., Ito S., Nolan P.M., Jouventin P., Dobson F.S., Austic R.E., Safran R.J., Siefferman L.M., Hill G.E., and Parker R.S. (2004) You can't judge a pigment by its color: carotenoid and melanin content of yellow and brown feathers in swallows, bluebirds, penguins, and domestic chickens. *Condor* 106:390-395.

Merino S., Moreno J., Sanz J.J., and Arriero E. (2000) Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). *Proceedings of the Royal Society of London B* 267: 2507-2510.

Millet S., Bennett J., Lee K.A., Hau M.., and Klasing K.C. (2007) Quantifying and comparing constitutive immunity across avian species. *Developmental and Comparative Immunology*. 31:188-201.

Møller A.P. (1990) Effects of a haematophagous mite on the barn swallow (*Hurundo rustica*). *Evolution* 44:771-784.

Møller A.P. (1991) Parasite load reduces song output in a passerine bird. *Animal Behaviour* 41:723-730.

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

New Mexico Incident Information System [online]. (2011) *Las Conchas*. <http://www.inciweb.org/incident/2385/> (May 11 2012).

Nolan P.M., Hill G.E., and Stoehr A.M. (1998) Sex, size, and plumage redness predict house finch survival in an epidemic. *Proceedings of the Royal Society: B* 265:961-965.

O'Brien E.L. and Dawson R.D. (2011) Plumage color and food availability affect male reproductive success in a socially monogamous bird. *Behavioral Ecology* 22:66-72.

Palacios M.G., Cunnick J.E., Winkler D.W., and Vleck C.M. (2012) Interrelations among immune defense indices reflect major components of the immune system in a free-living vertebrate. *Physiological and Biochemical Zoology* 85:1-10.

Pérez-Tris J., Hasselquist D., Hellgren O., Krizanauskiene A., Waldenström J., and Bensch S. (2005) What are malaria parasites? *Trends in Parasitology* 21:209-211.

Råberg L. and Stjernman M. (2003) Natural selection on immune responsiveness in blue tits *Parus caeruleus*. *Evolution* 57:1670-1678.

Ricklefs R.E. and Fallon S.M. (2002) Diversification and host switching in avian malaria parasites. *Proceedings of the Royal Society B* 269:885-892.

Rowe K.M.C. and Weatherhead P.J. (2011) Assortative mating in relation to plumage traits shared by male and female American robins. *Condor* 113:881-889.

SAS Institute. 2008. *SAS/STAT user's guide*. Version 9, 2nd ed. Cary, NC: SAS Institute Inc.

Schmid-Hempel P. (2003) Variation in immune defense as a question of evolutionary ecology. *Proceedings of the Royal Society: B* 270:357-366.

Schmid-Hempel P. (2008) Parasite immune evasion: a momentous molecular war. *Trends in Ecology and Evolution* 23:318-326.

Sheldon B.C. and Verhulst S. (1996) Ecological immunity: costly parasite defense and trade-offs in evolutionary ecology. *Trends in Ecology and Evolution* 11:317-321.

Shizuka D. and Dickinson J.L. (2005) Using molt limits to age western bluebirds. *Journal of Field Ornithology* 76:193-196.

Siefferman L. and Hill G.E. (2003) Structural and melanin coloration indicate parental effort and reproductive success in male eastern bluebirds. *Behavioral Ecology* 14: 855-861.

Siefferman L.M. and Hill G.E. (2005) Blue structural coloration of male eastern bluebirds *Sialia sialis* predicts incubation provisioning to females. *Journal of Avian Biology* 36:488-493.

Stjernman M., Råberg L., and Nilsson J. (2008) Maximum host survival at intermediate parasite infection intensities. *PLoS one* 3: article e2463.

Viney M.E., Riley E.M., and Buchanan K.L. (2005) Optimal immune responses: immunocompetence revisited. *Trends in Ecology and Evolution* 20:665-669.

Waldenström J., Bensch S., Hasselquist D., and Östman Ö. (2004) A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. *Journal of Parasitology* 90:191-194.

Wilcoxen T.E., Boughton R.K., and Schoech S.J. (2010) Selection on innate immunity and body condition in Florida scrub-jays throughout an epidemic. *Biology Letters* 6: 552-554.

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

Zahavi A. (1977) The cost of honesty (further remarks on the handicap principle). *Journal of Theoretical Biology* 67:603-605.

Zehtindjiev P., Ilieva M., Westerdahl H., Hansson B., Valkiūnas G., and Bensch S. (2008) Dynamics of parasitemia of malaria parasites in a naturally and experimentally infected migratory songbird, the great reed warbler *Acrocephalus arundinaceus*. *Experimental Parasitology* 119:99-110.

Zuk M., Thornhill R., Ligon J.D., and Johnson K. (1990) Parasites and mate choice in red jungle fowl. *American Zoologist* 30:235-244.

**Table 3.1: Results of the logistic regression performed to determine whether individual traits affected the probability that a male would sire extra-pair offspring.** We considered mass, breeding date (as indicated by the hatch date of the nestlings), infection status (0=uninfected, 1=infected), immune response (bactericidal capacity of the blood/BKA), and age as predictor variables.

| | | | | | 95% Confidence Interval for Odds Ratio | |
|---|---|---|---|---|---|---|
| Variable | β | Wald Chi-Square | P-value | Odds Ratio | *Lower* | *Upper* |
| Mass | -0.82 | 3.93 | **0.047** | 0.439 | 0.194 | 0.990 |
| Breed Date | 0.01 | 0.04 | 0.844 | 1.008 | 0.933 | 1.088 |
| Infection (0 vs. 1) | -1.65 | 4.88 | **0.027** | 0.037 | 0.002 | 0.689 |
| BKA | -1.19 | 2.72 | 0.099 | 0.303 | 0.008 | 11.233 |
| Age (AHY vs SY) | -0.36 | 0.17 | 0.681 | 0.120 | 0.003 | 5.066 |
| Age (ASY vs. SY) | -1.41 | 3.14 | 0.077 | 0.042 | 0.001 | 1.461 |

Figure 3.1

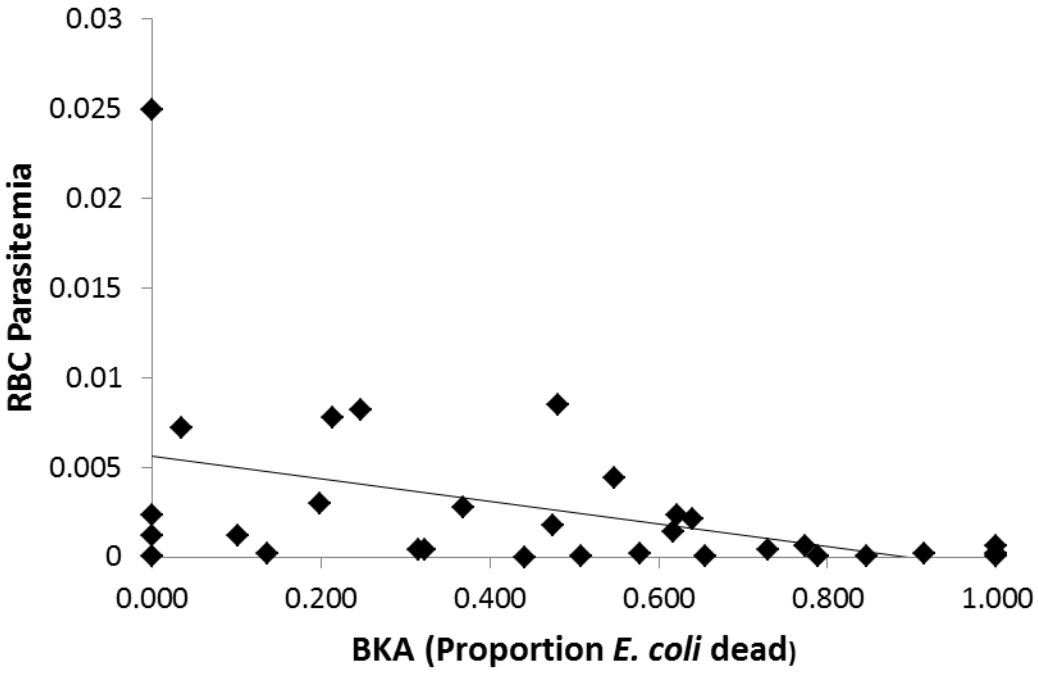


**Figure 3.1: The relationship between immune response (as measured by the bacteria-killing assay) and the proportion of RBCs infected with avian malaria, excluding uninfected individuals.**

Figure 3.2

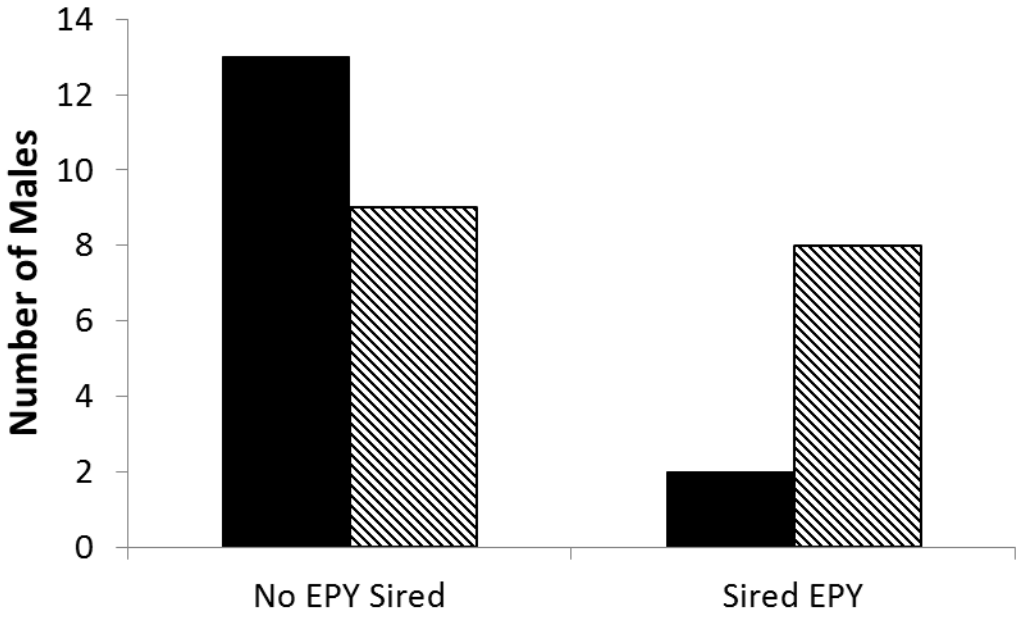


**Figure 3.2: The number of males who successfully sired extra-pair offspring versus those that did not.** Solid bars indicate individuals infected with malaria, cross-hatched bars indicate uninfected individuals.

# Chapter 4

## What determines the rates of double brooding in Western Bluebirds?

**Abstract**

Double brooding (producing a second brood after successfully rearing a first) is a common reproductive strategy in many bird species that can greatly increase reproductive output. However, the proportion of breeding pairs that produce a second brood varies considerably between years. Here, we examine how environmental factors such as weather patterns and individual factors such as clutch size, female body mass, and breeding date affect the rates of double brooding in a population of Western Bluebirds (*Sialia mexicana*) in New Mexico. More pairs tended to produce second broods in years in which freezing ended earlier and there was warm, dry weather during the breeding season, but this pattern was only apparent when we excluded from the analysis years in which wildfires occurred. In addition, pairs that bred earlier were more likely to produce a second brood, and females in these pairs tended to be heavier. This indicates that both the length of the breeding season and the weather during that time influence rates of double brooding, and that individual condition can affect whether or not an individual produces a second brood. Knowledge of how weather patterns influence bird reproductive effort can help scientists predict how bird populations will respond to changing weather in the future.

## Introduction

In birds, one way for individuals to increase their reproductive output is by producing more than one brood per season (Lack 1954). In temperate birds, which only breed for a short period during the year, this ability may be constrained by the amount of time available during the breeding season. Despite this, double brooding has been documented in a variety of temperate bird species, mostly passerines (Geupel and DeSante 1990, Moore and Morris 2005, Monroe et al. 2008), and can allow an individual to dramatically increase its reproductive success in a given season (Morrison and Bolger 2002, Monroe et al. 2008).

Given the obvious benefits of double brooding, one might expect it to be a common strategy. However, in many species, not all breeding pairs produce two broods in a given season (Geupel and DeSante 1990, Verboven and Verhulst 1996, Nagy and Holmes 2005a, Monroe et al. 2008). Moreover, the frequency of pairs double brooding within a given population often changes from year to year, implying a strong effect of environment on the propensity to double brood (Nagy and Holmes 2005a, Husby et al. 2009). Pinpointing specific environmental factors and examining their influence on double brooding rates can tell us how birds respond to changes in their environment, and how these changes can influence breeding effort and future population size.

Many studies have examined why some individual pairs of birds double brood and others do not (Verboven and Verhulst 1996, Verboven et al. 2001, Nagy and Holmes 2005a). Food availability is thought to be an important determinant (Morrison and Bolger 2002, Moore and Morris 2005, Nagy and Holmes 2005a, 2005b), although other factors,

such as the length of the breeding season (Svensson 1995, Monroe et al. 2008), the microhabitat of the nest site (Grübler et al. 2010), and the amount of parental care provided to the first brood (Moore and Morris 2005, Grüebler and Naef-Daenzer 2008), may also influence double brooding. Most studies to date have examined double brooding rates over a period of less than five years (but see Nagy and Holmes 2005a, Husby et al. 2009). These shorter-term studies have examined what factors determine whether individual pairs of birds will produce a second brood or not. However, the shorter timeframe has not allowed researchers to properly assess the role of yearly variation in weather in determining population rates of double brooding. Very few longer-term studies have addressed this issue (Husby et al. 2009).

Here, we use data taken over fifteen years as part of a long-term environmental monitoring study to examine the factors influencing rates of double brooding in Western Bluebirds (*Sialia mexicana*). The long-term nature of our data allows us to test hypotheses about how variation in annual weather conditions influences the rates of double brooding. In addition, we look at how individual pairs that produce second broods differ from those that do not in terms of mass, breeding date, clutch size, and investment in nestlings. We predict that individuals producing second broods would nest earlier and be heavier (i.e. in better condition), but may have smaller clutches, particularly in first broods, implying a cost to producing a second brood. In addition, if parents have a limited amount of energy and resources to invest in reproduction, parents of double broods may have to sacrifice nestling quality for nestling quantity. Thus, we predict that nestlings from double broods will weigh less than nestlings from single broods. Knowing

the environmental and individual factors linked to rates of double brooding may help scientists better understand how changing weather patterns caused by climate change can impact the reproductive output of wild birds and, potentially, population size and persistence. Additionally, accurate and continual measuring of double brooding rates has important implications for conservation, as it can alter the estimates of reproductive output used in models of population growth trajectories (Friesen et al. 1999, Friesen et al. 2000).

**Methods**

We conducted our work on Western Bluebirds, which are medium sized (23-30g) passerines. Western Bluebirds are secondary cavity nesters and readily use human-made nest boxes (Guinan et al. 2008). They are socially monogamous, and both males and females defend the nest and provision the nestlings. Each pair produces, on average, 4-5 eggs in a given clutch, although clutch sizes can range from two to seven eggs in our population. Double brooding has been reported in other populations of this species (Brawn 1991, Dickinson et al. 1996, Keyser et al. 2004).

All field work was conducted at sites on and around the Los Alamos National Laboratory (LANL), in Los Alamos, New Mexico. Los Alamos is located on the Pajarito Plateau at approximately 2,200 m (7,300 ft) in the Jemez Mountains in northern New Mexico. Over 400 nest boxes on LANL property have been monitored since 1997 as part of the Avian Nestbox Network, intended to monitor the effects of contaminants on avian breeding biology (Fair and Myers 2002a, Fair et al. 2003). We used wooden nest boxes

(measurements 18.4 cm by 15.5 cm by 30.5 cm) with a 3.8 cm diameter hole to allow the bluebirds to enter while excluding larger competitors. Boxes could be opened via the front panel to allow monitoring of nest contents. We cleared old nests out of the boxes at the end of each season.

Nest boxes were placed on trees 1-2 meters off the ground at locations dominated by ponderosa pine (*Pinus ponderosa*) forests at the higher elevations and by piñion-juniper (*Pinus edulis – Juniperus* spp.) woodlands at lower elevations. Our study area was broken into 37 different sites spread out over the mesa tops and canyons that make up the Pajarito Plateau (Fair et al. 2003). These sites are located across the Laboratory property, which consists of approximately 111 $km^2$ of land (Fair and Myers 2002a). We also had two sites adjacent to the town of Los Alamos, one at the golf course and one at the cemetery. Each site had, on average, 20 nest boxes placed in a line or along a loop, with each box approximately 50-75 m away from its nearest neighbor. Over time, some sites have been abandoned and the nest boxes removed, while new sites have been established, so that the overall number of nest boxes actively monitored has only increased slightly since 1997. Western Bluebirds are by far the most common species in this system, with occupancy rates around 15-25% for most years of the study. The lowest occupancy rates occurred early in the study, with approximately 5% of the boxes occupied by bluebirds in 1997 (unpublished data). Other species commonly nesting in the boxes include Ash-throated Flycatchers (*Myiarchus cinerascens*), Violet-green Swallows (*Tachycineta thalassina*), and House Wrens (*Troglodytes aedon*).

Nest boxes were monitored every year during the breeding season, which begins in early to mid-May and ends in early August. Due to the abundance of and distance between sites, each individual site was checked at least once every two weeks. We considered a nest box occupied if it contained a nest with at least one egg. For each nest, we recorded the clutch size, the proportion of eggs that hatched, the hatch date, and the number of young known or presumed to have fledged. We did not record the date of clutch initiation, as many nests were found after the female had finished laying her clutch; however, we consider that the hatch date of the nestlings provides an adequate measure of the timing of breeding. We did not always visit nests on the day the nestlings hatched; however, we used the developmental stage of the nestlings to estimate their age within a day and determined the hatch date by counting backwards. In this species, eggs hatch synchronously; thus all nestlings in a given nest were the same age. Nestlings were assumed to have fledged if the nest was empty after nestlings had reached 16 days, after which time fledging begins (Fair and Myers 2002b). If we found a nest empty before nestlings would have been old enough to fledge, we counted it as depredated.

We captured adults after their eggs had hatched using box traps or mist nets placed directly in front of the boxes. Each captured adult was fitted with a U.S. Fish and Wildlife Service numbered aluminum leg band, and we recorded the bird's sex, wing chord, tarsus length, and mass to the nearest 0.1 g. Nestlings were banded when they were nine days old or older. Return rates are relatively low in our population, and less than 5% of the individuals banded over the course of the study were recaptured in subsequent years. During 1997 through 2006, nestling mass was taken at various ages as

part of other projects on the effects of contaminants and drought on nestling immune response (Fair et al. 2003, Fair and Whitaker 2008); we use these data to compare masses of nestlings from single versus double broods. For this study, we consider nestling mass at day 15, as this was the age closest to fledging for which we had consistent data. Nestling mass was used as a proxy for parental investment in the nestlings. All work was conducted in accordance with the Guidelines to the Use of Wild Birds in Research (Fair et al. 2010) and with the approval of the LANL Institutional Animal Care and Use Committee.

To determine the number of double brooding pairs, we only counted pairs that successfully fledged at least one nestling from the first brood before renesting. In some cases, pairs that lost their first brood to predation would begin a second clutch; we did not consider these pairs to be double-brooded. Most birds remained at one nest box throughout the breeding season; however, in a few cases, birds would fledge their first brood from one nest box, and then move to another box nearby to lay the second clutch. We counted these attempts as double broods if the same female was captured at both boxes. We calculated the percentage of pairs that produced a second clutch out of all pairs of that attempted breeding in a given year.

During the fifteen years of the study, two large wildfires burned areas on or adjacent to some of the field sites. In 2000, the Cerro Grande fire started in early May (Kokaly et al. 2007, Kotliar et al. 2007), when the bluebirds were setting up territories and beginning to breed. It burned over 40,000 acres and destroyed roughly one third of the existing nest boxes. In many cases, we put up new boxes in the burned areas later that

summer. In 2011, the Las Conchas fire started on June 26 when most bluebirds had nestlings or had already successfully fledged a first brood (38% of the occupied nests had eggs or nestlings on this date).. This fire burned over 150,000 acres (New Mexico Incident Information System 2011). While this fire did not destroy any nest boxes, it burned areas adjacent to several sites, preventing regular monitoring of the breeding pairs, and the region was considerably smoky for over two weeks. Because both of these fires occurred during or immediately prior to the breeding season, the fires themselves may have had an impact on the propensity of the birds to double brood and/or our ability to record second broods accurately. For these reasons, we chose to exclude fire years from many of the analyses involving yearly rates of double brooding (see below).

*Arthropod Abundances*

We obtained data on arthropod abundances to use as a proxy for annual food availability from a report on the Long Term Studies of Ground Dwelling Arthropod Biodiversity at Bandelier National Monument (Brantley 2011). This study used pitfall traps to assess the number and species of ground-dwelling arthropods from 1992 through 2010. Abundances were given as the mean number of arthropods per trap for a given year; data on intra-annual variation was not available for most years. The study surveyed three habitat types: piñion-juniper woodlands, ponderosa pine forests, and mixed conifer forests. We used only the data from the ponderosa pine forests, as the bluebirds at our sites predominantly use this habitat type (unpublished data). Bandelier National Monument is located less than 10 miles away and is adjacent to our study areas; thus we

consider these data to be a serviceably accurate reflection of the abundance of ground-dwelling arthropods at our field sites.

*Weather Variables*

Weather data were obtained from LANL's "Weather Machine" from the weather station at Technical Area-6, which is near the center of our entire study area. To gain an idea of yearly variation in climate, we looked at the average daily temperature of a given year, the average maximum daily temperature, and the total precipitation from October through the beginning of May. Almost all of the snowfall in Los Alamos occurs during these months (Bowen 1992). Thus, we chose this to measure the relevant "bioyear" precipitation (Rotenberry and Wiens 1991) rather than using the total precipitation for a calendar year, as a calendar year may not capture variation in precipitation during biologically relevant growth periods (Kempes et al. 2008).

We also looked at weather variables during the breeding season to determine how smaller-scale weather variation within a year affects double brooding rates. We chose to look at the average of the daily maximum temperature and the total precipitation from the beginning of May through the end of July. Data on average daily temperatures for individual months were not available prior to 2003; thus we used average maximum temperature instead. We did not include weather data from August in this analysis because most birds have either finished breeding or are rearing their final broods by August. Thus, the decision to double brood or not has already been made before that point. In addition to temperature and precipitation data, we looked at minimum daily

temperatures in spring to determine the date of the last two consecutive freezes (two consecutive nights in which the temperature dropped below 0ºC), which we used as an indicator of the earliest time birds could begin breeding for a given season. We chose to look at two consecutive freezes because a single freeze event could be caused by a short, unpredictable cold snap. Such cold snaps can occur after the breeding season has already started, and thus are unlikely to affect the decision to breed early (personal observation).

*Statistical Analyses*

Data were checked for normality using a Shapiro-Wilks test. We performed a principal components analysis on the weather data to reduce the number of variables in the analysis. We performed two separate analyses, one on the annual weather variables (annual average temperature, annual average maximum temperature, and bioyear precipitation), and one on breeding season weather variables (average maximum temperature, total season precipitation, date of last freezes). The first principal component from each analysis was retained using the Kaiser-Guttman criterion (eigenvalues greater than one). We then performed a correlation analysis to determine the relationship between PC1 scores and the percentage of pairs double brooding. Arthropod data were non-normally distributed and could not be transformed to meet normality assumptions. We performed a Spearman Rank Correlation to test for a relationship between yearly arthropod abundances and rates of double brooding.

To examine parental investment in nestlings, we compared nestling masses at day 15 from single and double broods. Nestlings within a single nest are likely to be more

similar to one another than to nestlings from a different nest due to differences in parental care and rearing environment. To account for these differences, we analyzed nestling mass using a nested ANOVA with nest of origin as a factor nested within brood type (single vs. double). For nestlings from double broods, first-brood nestlings ($n$ = 23) and second-brood nestlings ($n$ = 8) did not differ significantly in mass ($t_{29}$ = -1.3, $P$ = 0.19). Thus we pooled nestling masses from these two groups for the analysis.

To determine what individual differences existed between single versus double-brooded pairs, we used a logistic regression with brooding state (single-brooded versus double-brooded) as the dependent variable and clutch size, hatching date, and female mass as predictor variables. We used mass in this analysis rather than a metric of body condition (e.g. mass divided by tarsus length) because such metrics may actually be less reliable indicators of an individual's fat and protein stores than mass alone (Schamber et al. 2009). For double broods, we used data from the first brood only. Nests that had been abandoned or depredated before eggs hatched were excluded from the analysis, as clutches from these nests were often incomplete or their sizes were not recorded. We did not consider nestling mass in this analysis as these data were only collected during certain years and were thus not available for most nests. All statistical analyses were performed using SAS (SAS Institute 2008). We used $\alpha = 0.05$ when considering the significance of each test. Values are reported in the Results section as means ± SD.

**Results**

Overall, 5% (55 out of 1031) of all bluebird pairs produced second broods over the fifteen years of study. Rates of double brooding varied among years, ranging from a high of 14% in 2005 to 0 in 2001, which was a drought year. The highest rates of double brooding occurred from 2002 through 2007 (Fig. 4.1).

PC1 from the principal components analysis on annual weather explained 74% of the variation; this component had high positive loadings for average annual temperature and annual maximum temperature and a high negative loading for bioyear precipitation (Table 1). Thus, PC1 describes warm, dry years. However, PC1 was not a good predictor of rates of double brooding when looking at all years ($n$ = 15, $r$ = 0.15, $P$=0.59) or when excluding fire years ($n$ = 13, r = 0.29, $P$ = 0.34). Arthropod abundances were also not correlated with rates of double brooding ($n$ = 14, $r_s$ = 0.15, $P$ = 0.61).

For weather data during the breeding season, PC1 explained 66% of the variation and was used in subsequent analyses. PC1 was positively correlated with the amount of precipitation during the breeding season and the date of the last freezes, and negatively correlated with the average maximum temperature during the breeding season (Table 1). Thus, high PC1 scores indicate short, cool, wet summers. When all years were included in the analysis, weather during the breeding season was not a significant predictor of rates of double brooding ($n$ = 15, $r$ = -0.36, $P$ = 0.19). However, when fire years were removed from the analysis, the relationship became significant ($n$ = 13, $r$ = -0.57, $P$ = 0.04). PC1 was negatively correlated with the rate of double brooding, indicating that double broods

are more common during years when freezes end early and the breeding season is warmer and drier (Fig. 4.2).

We had masses for 393 nestlings from 108 different nests at day 15. Thirty-one of those individuals were from double broods, and 362 were from single broods. The average mass of nestlings from single broods was 25.6 ± 2.9 g and the average mass of nestlings from double broods was 24.9 ± 2.4 g. Nestling masses differed depending upon nest of origin ($F_{105,286} = 11.7$, $P < 0.001$), but there was no difference in mass between nestlings from single versus double broods ($F_{1,286} = 0.5$, $P = 0.47$).

For differences between individuals based on whether they produced a second brood or not, a full logistic regression model with three predictors (hatch date, clutch size, and female mass) was statistically significant ($\chi^2_{3,264} = 26.0$, $P < 0.001$). However, only hatch date significantly predicted whether or not a given female would produce a second brood ($\chi^2_{1,264} = 23.7$, $P < 0.001$), with female mass being a marginally significant predictor ($\chi^2_{1,264} = 3.3$, $P = 0.07$, Table 2). Pairs that produced second broods tended to breed earlier and the females in double-brooded pairs were heavier (Table 3). However, neither of these variables greatly improved the power of the model to predict single versus double-brooded pairs, as indicated by an odds ratio close to one (Table 2).

**Discussion**

Rates of double brooding in Western Bluebirds are related to weather patterns during the breeding season in our study. Overall, the 5% rate of double brooding reported in this study is quite low compared to other passerines in which double brooding has been

reported. In a study on Wood Thrushes (*Hylocichla mustelina*), Friesen et al. (2000) reported that 64% to 86% of pairs produced second broods, while in Black-throated Blue Warblers (*Dendroica caerulescens*), on average, 53% of pairs produced second broods (Nagy and Holmes 2005a). The 5% rate of double brooding we found is similar to values reported for Tree Swallows (*Tachycineta bicolor*), a species in which double broods are considered rare (Monroe et al. 2008).

Other studies have recorded rates of double brooding in Western Bluebirds. Dickinson et al. (1996) reported that fewer than 20% of Western Bluebird pairs in California produce second broods, and that second broods only occurred in eight out of the twelve years of study. This would imply that double brooding may be rarer in her population, as we recorded only one year (2001) during which no double brooding occurred. However, Brawn (1991) reported that rates of double brooding ranged from 18% to 34% in a population of Western Bluebirds in northern Arizona. These rates are much higher than the rates in our population, despite the fact that Brawn's (1991) study was conducted at field sites that are similar in elevation and vegetation type to our study area. However, the weather reported by Brawn (1991) was cooler and drier than the weather at our sites during the early breeding season. Thus, weather may play a role in explaining the differences between these populations.

Keyser et al. (2004) also reported second clutches in a population of Western Bluebirds located in Oregon. They counted 312 second clutches over a five year period, roughly 37% of all breeding pairs, which is considerably higher than the rates in our population. However, Keyser et al. (2004) reported that birds began breeding in early

April in their study area, which is a full month earlier than breeding begins for birds in our population. They also reported that breeding continued well through July and that some clutches were still hatching as late as the end of August, whereas most of the birds in our study have finished breeding by early August. Thus, the longer breeding season may allow a larger proportion of pairs to produce second broods, and in some cases even third brood were reported in this population.

Husby et al. (2009) reported that rates of double brooding in Great Tits (*Parus major*) have declined over the last several decades. They attribute this pattern to changes in climate and the timing of greatest food abundance resulting in fewer offspring from second broods surviving to the following year, therefore reducing the benefits of double brooding. While we found no such trend in our data, this may be due to our lower sample size and the length of time considered. Our data cover fifteen years, while the data on Great Tits span over 50 years. Such an overall decline in double brooding rates may be occurring in Western Bluebirds as well, which might help to explain why the rates reported by Brawn (1991) from the 1980s are much higher than ours, despite the fact that Brawn's field sites are similar to ours in elevation and habitat type. However, this could be due to as-yet unknown differences between the populations. Discrepancies in the rates of double brooding found for different populations of the same species have been reported before (Geupel and DeSante 1990).

Weather during the breeding season rather than the year as whole had the greatest influence on whether or not pairs produced second broods. Our results suggest that long, dry summers allow a greater proportion of bluebirds to produce second clutches; this

implies that such weather patterns may result in population growth. However, such dry summers may occur during droughts, which have other detrimental impacts on the population, including negative effects on clutch size, nestling immunity, nestling mass, and fledging success (Fair and Whitaker 2008). The southwestern United States has already experienced several droughts in the last ten years, and this problem may become worse as climate change affects the region (MacDonald 2010). Under such conditions, fledging success from single broods is expected to decrease, and a slight increase in the rates of double brooding may not compensate for the negative effects of drought on the population as a whole. Thus, while weather patterns outside the breeding season may not affect double brooding rates, scientists may need to considered these patterns as well to predict overall reproductive output for a population (Rotenberry and Wiens 1991).

Weather itself is unlikely to directly control whether or not birds double brood, but it may act indirectly on food availability (Lack 1954, Bryant 1973, Bryant 1975). Los Alamos experiences summer rains that typically begin in July and account for most of the precipitation during the breeding season (Bowen 1992). These rains may negatively impact either the insect population or the ability of parents to forage, making food harder to come by in rainy summers (Bryant 1975). Unfortunately, our data on insect abundances do not cover within-year variation, so we are unable to test this assumption. Moreover, we failed to find any relationship between annual arthropod abundances and rates of double brooding. This is unusual, as previous studies reporting annual variation in the percentage of pairs that produce second broods have attributed this variation to differences in food availability. For instance, Nagy and Holmes (2005b) provided

supplemental food to breeding pairs of Black-throated Blue Warblers and found that fed females were more likely to produce second broods. They also reported that unfed pairs did not produce second broods during a year of low food abundance.

Other studies examining how food availability affects double brooding suggest the timing of breeding relative to levels of peak food production may be more important than the overall abundance of food (Verboven et al. 2001). This may explain why we found no relationship between arthropod abundances and rates of double brooding, as we looked only at annual totals and not abundances of arthropods during the breeding season. Also, the arthropod data we used came from pitfall traps, which collect primarily ground-dwelling insects. Bluebirds tend to forage by waiting on a perch for prey and then flying out to catch insects on the ground (Mock 1991), but they can also capture flying insects (personal observation), and thus the number of ground-dwelling insects may not accurately reflect the overall abundance of prey items available to bluebirds.

We found no differences in mass between single versus double brood nestlings. This result conflicts with the findings from previous studies, many of which report that double broods often have lighter nestlings, at least during the first brood. Studies on other species have found that birds with smaller first broods are more likely to produce second broods, implying a trade-off in reproductive effort between the broods (Lindén 1988, Nagy and Holmes 2005a, Monroe et al. 2008). In Barn Swallows (*Hirundo rustica)*, parents that produced two broods fed fledged nestlings from the first brood less, presumably because feeding the first brood longer would have resulted in less time available for the second brood (Grüebler and Naef-Daenzer 2008). Great Tits that

produce second broods tend to start breeding before food abundance has peaked, and nestling mass is closely tied to the level of food (Verboven et al. 2001). Mägi and Mänd (2004) also observed that Great Tit nestlings from second broods were larger than those from first broods, which they attributed to parents maximizing the quality of young from second broods, as size at fledging had a greater influence on fitness late in the breeding season when second broods were fledged. Given these many examples, it is surprising that we did not see any difference in nestling mass. However, our sample size for nestlings from double broods was small (due in part to low rates of double brooding), and thus we may not have had sufficient power to detect a relationship, particularly given the large variation in nestling mass among nests.

Bluebird pairs that produced second broods tended to begin breeding earlier in the season, and many other studies support this finding (Geupel and DeSante 1990, Verboven and Verhulst 1996, Monroe et al. 2008). This result also supports our finding that rates of double brooding increase during years in which freezing temperatures end relatively early. Given that our sites are located at a relatively high altitude, resulting in a shorter breeding season, this result is not surprising. Breeding begins in May and lasts until the beginning of August. In Western Bluebirds, it takes four to five weeks minimum to raise a single brood to fledging: two weeks for incubation, 16 to 21 days from hatching to fledging (Fair and Myers 2002b, Guinan et al. 2008). Given these time constraints, pairs that begin breeding later in the season simply may not have time to rear a second brood. Thus, the shortened breeding season associated with high altitude or high latitude may

constrain rates of double brooding such that only early-breeding individuals can produce a second brood.

Our results suggest that heavier females may be more likely to produce second broods. This could indicate that female condition and/or quality plays a role in double brooding. This may be because females that double brood can do so because they live on better territories with more food resources (Nagy and Holmes 2005a). While other work has found that the timing of breeding seems to influence double brooding more than differences in individual quality (Verboven and Verhulst 1996), our results suggest that both may be important determinants of double brooding.

In our analyses of weather patterns, we excluded years in which large wildfires occurred during the breeding season. However, the fires may have continued to affect bird populations in subsequent years through changes in vegetation structure. Bluebirds are thought to respond positively to fire (Bock and Block 2005), and Western Bluebirds were detected at higher densities in the two years following the Cerro Grande fire (2001 and 2002), particularly in severely burned areas (Kotliar et al. 2007). This coincides with an increase in the rates of double brooding beginning in 2002. The ability of bluebirds to do well after fires may explain the peak in the rates of double brooding that occurred from 2002 through 2007. Particularly striking is the increase in double brooding that occurred in some of the canyons that burned in 2000. In 2005, for example, 60% of the pairs that produced second broods nested in lower Mortandad Canyon, which had burned during the Cerro Grande fire. Double brooding only occurred in this canyon from 2002 through 2005, suggesting that as the vegetation recovered after the fire, the habitat

became less hospitable to the bluebirds. We do not yet have sufficient data to fully explore how fire and weather patterns may interact to affect rates of double brooding. However, if fire does positively affect double brooding, then in future we expect to see an increase in the rates of double brooding at sites adjacent to the Las Conchas Fire burn areas.

In conclusion, weather during the breeding season can affect the proportion of bluebird pairs that produce second broods. Individual factors such as breeding date also play a role in determining whether or not a pair will produce a second brood. The influence of weather is particularly is striking in that we were able to detect a relationship despite using a system in which the rates of double brooding are relatively low. This analysis was only possible because of the long-term nature of our data, thus emphasizing the need for long-term studies when looking for factors affecting wild animal breeding and population dynamics.

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**References**

Bock C.E., and Block W.M. (2005) Fire and birds in the southwestern United States. *Studies in Avian Biology* 30:14-32.

Bowen B.M. (1992) *Los Alamos climatology summary: including latest normals from 1961-1990*. Los Alamos National Laboratory Technical Report LA-12232-MS.

Brantley S.L. (2011) *Long-term studies of ground-dwelling arthropod biodiversity at Bandelier National Monument*. U.S. Geological Survey Report.

Brawn J.D. (1991) Environmental effects on variation and covariation in reproductive traits of Western Bluebirds. *Oecologia* 86:193-201.

Bryant D.M. (1973) The factors influencing selection of food by House Martins (*Delichon urbica* (L.)). *Journal of Animal Ecology* 42:539-564.

Bryant D.M. (1975) Breeding biology of House Martins *Delichon urbica* in relation to aerial insect abundance. *Ibis* 117:180-216.

Dickinson J.L., Koenig W.D., and Pitelka F.A. (1996) Fitness consequences of helping behavior in the western bluebird. *Behavioral Ecology* 7:168-177.

Fair J.M. and Myers O.B. (2002a) Early reproductive success of Western Bluebirds and Ash-throated Flycatchers: a contaminated landscape perspective. *Environmental Pollution* 118:321-330.

Fair J.M. and Myers O.B. (2002b) The ecological and physiological costs of lead shot and immunological challenge to developing Western Bluebirds. *Ecotoxicology* 11:199-208.

Fair J.M., Myers O.B., and Ricklefs R.E. (2003) Immune and growth response of Western Bluebirds and Ash-throated Flycatchers to soil contaminants. *Ecological Applications* 13:1817-1829.

Fair J.M., and Whitaker S.J. (2008) Avian cell-mediated immune response to drought. *Wilson Journal of Ornithology* 120: 813-819.

Fair J.M., Paul E., Jones J., Clark A.B., Davie C., and Kaiser G. (2010) *Guidelines to the Use of Wild Birds in Research*. Washington D.C.: The Ornithological Council.

Friesen L., Cadman M.D., and MacKay R.J. (1999) Nesting success of neotropical migrant songbirds in a highly fragmented landscape. *Conservation Biology* 13:338-346.

Friesen L.E., Wyatt V.E., Cadman M.D., MacKay R.J., Cheskey E.D., Allen M.L., and Ramsay D. (2000) Extent of double-brooding and seasonal movement of nesting females in a northern population of Wood Thrushes. *Wilson Bulletin* 112:505-509.

Geupel G.R., and DeSante D.F. (1990) Incidence and determinants of double brooding in Wrentits. *Condor* 92:67-75.

Grüebler M.U., and Naef-Daenzer B. (2008) Fitness consequences of pre- and post-fledging timing decisions in a double-brooded passerine. *Ecology* 89:2736-2745.

Grüebler M.U., Korner-Nievergelt F., and von Hirschheydt J. (2010) The reproductive benefits of livestock farming in Barn Swallows *Hirundo rustica*: quality of nest site or foraging habitat? *Journal of Applied Ecology* 47:1340-1347.

Guinan J.A., Gowaty P.A., and Eltzroth E.K. [online] (2008) Western Bluebird (*Sialia mexicana)*. *In* A. Poole [ed.] *The Birds of North America Online*. Ithaca, NY: Cornell Lab of Ornithology. <http://bna.birds.cornell.edu/bna/species/510doi:10.2173/bna.510> (6 January, 2012).

Husby A., Loeske E.B., and Visser M.E. (2009) Decline in the frequency and benefits of multiple brooding in great tits as a consequence of changing environment. *Proceedings of the Royal Society B* 276:1845-1854.

Kempes C.P., Myers O.B., Breshears D.D., and Ebersole J.J. (2008) Comparing response of *Pinus edulis* tree-ring growth to five alternate moisture indices using historic meteorological data. *Journal of Arid Environments* 72:350-357.

Keyser A.J., Keyser M.T., and Promislow D.E.L. (2004) Life-history variation and demography in Western Bluebirds (*Sialia mexicana*) in Oregon. *Auk* 121:118-133.

Kokaly R.F., Rockwell B.W., Haire S.L., and King T.V. (2007) Characterization of post-fire surface cover, soils, and burn severity at the Cerro Grande Fire, New Mexico, using hyperspectral and multispectral remote sensing. *Remote Sensing of Environment* 106:305-325.

Kotliar N.B., Kennedy P.L., and Ferree K. (2007) Avifaunal responses to fire in southwestern montane forests along a burn severity gradient. *Ecological Applications* 17:491-507.

Lack D. (1954) *The Natural Regulation of Animal Numbers*. London: Oxford University Press.

Lindén M. (1988) Reproductive trade-off between first and second clutches in the Great Tit *Parus major*: an experimental study. *Oikos* 51:285-290.

MacDonald G.M. (2010) Water, climate change, and sustainability in the southwest. *Proceedings of the National Academy of Sciences USA* 107:21256-21262.

Mägi M., and Mänd R. (2004). Habitat differences in allocation of eggs between successive breeding attempts in Great Tits *Parus major*. *Ecoscience* 11:361-369.

Mock P.J. (1991) Daily allocation of time and energy of Western Bluebirds feeding nestlings. *Condor* 93:598-611.

Monroe A.P., Hallinger K.K., Brasso R.L., and Cristol D.A. (2008) Occurrence and implications of double brooding in a southern population of Tree Swallows. *Condor* 110:382-386.

Moore D.J., and Morris R.D. (2005) The production of second clutches in the Common Tern: proximate effects of timing and food supply. *Waterbirds* 28:458-467.

Morrison S.A., and Bolger D.T. (2002) Variation in a sparrow's reproductive success with rainfall: food and predator-mediated processes. *Oecologia* 133:315-324.

Nagy L.R., and Holmes R.T. (2005a) To double-brood or not? Individual variation in the reproductive effort in Black-throated Blue Warblers (*Dendroica caerulescens*). *Auk* 122:902-914.

Nagy L.R., and Holmes R.T. (2005b) Food limits annual fecundity of a migratory songbird: an experimental study. *Ecology* 86:675-681.

New Mexico Incident Information System [online]. (2011) *Las Conchas*. <http://www.inciweb.org/incident/2385/> (May 11 2012).

Rotenberry J.T., and Wiens J.A. (1991) Weather and reproductive variation in shrubsteppe sparrows: a hierarchical analysis. *Ecology* 72:1325-1335.

SAS Institute. (2008) *SAS/STAT user's guide. Version 9*, 2nd ed. Cary, NC: SAS Institute Inc.

Schamber J.L., Esler D., and Flint P.L. (2009) Evaluating the validity of using unverified indices of body condition. *Journal of Avian Biology* 40:49-56.

Svensson E. (1995) Avian reproductive timing: when should parents be prudent? *Animal Behaviour* 49:1569-1575.

Verboven N., and Verhulst S. (1996) Seasonal variation in the incidence of double broods: the date hypothesis fits better than the quality hypothesis. *Journal of Animal Ecology* 65:264-273.

Verboven N., Tinbergen J.M., and Verhulst S. (2001) Food, reproductive success, and multiple breeding in the Great Tit *Parus major*. *Ardea* 89:387-406.

**Table 4.1: Loadings and eigenvalues for the first principal component for weather variables during the breeding season and the first principal component for annual weather variables.** No other components had eigenvalues greater than 1.

| Breeding Season Weather | | Annual Weather | |
|---|---|---|---|
| Variable | PC1 Loadings | Variable | PC1 Loadings |
| Maximum Temperature | -0.943 | Annual Average Temperature | 0.963 |
| Precipitation | 0.823 | Annual Maximum Temperature | 0.936 |
| Date of Last Freezes | 0.629 | Bioyear Precipitation | -0.638 |
| PC1 Eigenvalue | 1.974 | PC1 Eigenvalue | 2.210 |
| Variation Accounted For | 66% | Variation Accounted For | 74% |

**Table 4.2: Results of the logistic regression to determine whether differences between individuals influenced the probability of producing a second brood.**

Individual differences considered include breeding date (as indicated by the hatch date of the nestlings), female mass, and clutch size. The model describes the probability that a pair will be single rather than double brooded

| | | | | | 95% Confidence Interval for Odds Ratio | |
|---|---|---|---|---|---|---|
| Variable | β | Wald Chi-Square | P-value | Odds Ratio | *Lower* | *Upper* |
| Hatch date | 0.081 | 23.72 | <0.001 | 1.084 | 1.050 | 1.120 |
| Clutch size | -0.028 | 0.01 | 0.93 | 0.972 | 0.528 | 1.789 |
| Female mass | -0.196 | 3.28 | 0.07 | 0.822 | 0.665 | 1.016 |

**Table 4.3: Averages and standard deviations of female mass, clutch size, and nestling hatch date for single and double brooded nests.** Hatch dates are given as the Julian date and female mass is recorded in grams.

| | Single Brood | | Double Brood | |
|---|---|---|---|---|
| Variable | Average | SD | Average | SD |
| Hatch date | 160 | 16.30 | 142 | 12.31 |
| Clutch size | 4.7 | 0.79 | 5.0 | 0.76 |
| Female mass | 24.5 | 2.24 | 25.2 | 2.28 |

Figure 4.1

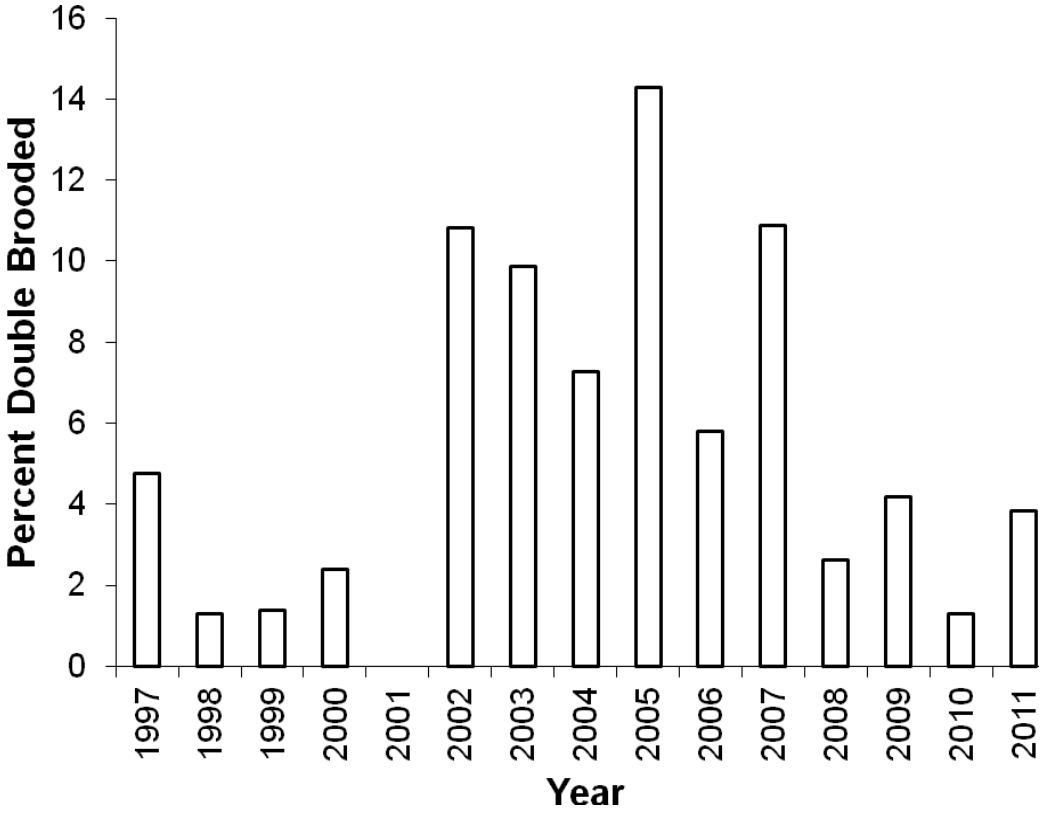


**Figure 4.1: The percentage of breeding Western Bluebird pairs that produced a second brood for each year from 1997 to 2011 (*n* = 1031 pairs total).**

Figure 4.2

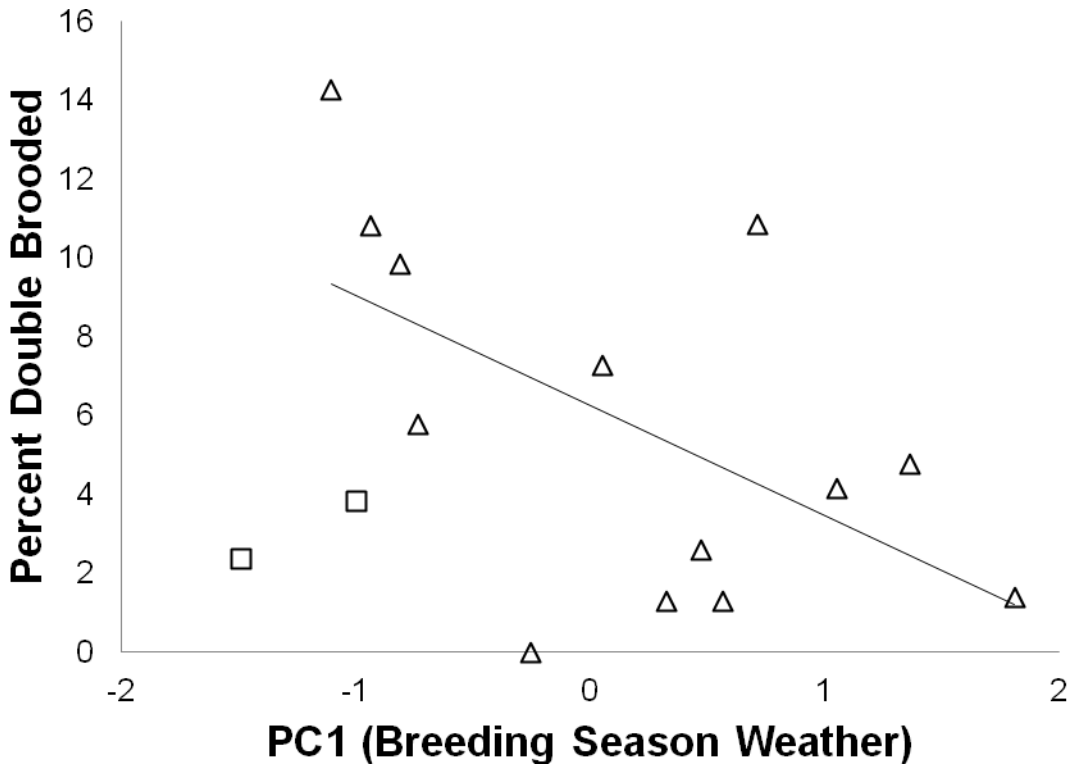


**Figure 4.2: The relationship between weather during the breeding season and the percentage of pairs that double brood.** Higher PC1 scores indicate a later start of the breeding season, more rain, and lower temperatures during the breeding season. Years in which wildfires occurred (2000 and 2011) are excluded.

## Dissertation Conclusions

Sexual signals may act as indicators of male quality, allowing females to discriminate among many possible mates. In birds, plumage coloration often acts as a sexual signal and can indicate many different aspects of male quality (Hill 2006). Here, I found that both coloration and parasites play a role in sexual selection in western bluebirds. However, coloration did not indicate parasite infection status in this species. Instead, these two factors determined different aspects of male reproductive success, with coloration playing a role in social mate choice and parasite infection status determining male extra-pair mating success. The finding that coloration plays a role in social mate choice but not extra-pair mate choice suggests that coloration indicates direct benefits in this species, although we did not test this hypothesis directly. However, this appears to be the case in the closely-related eastern bluebird, where male coloration can provide females with information about territory quality and male parental investment (Siefferman and Hill 2003,2005a,b).

In contrast, female choice of extra-pair mates depends more on male age and infection status, consistent with the idea of female selection for genetic benefits. Age was also important in male success, although why female should prefer older males remains a mystery (Akçay and Roughgarden 2007). Malaria infection did not affect social mate choice or coloration; however, if females select social mates for direct benefits such as parental care, one would predict that malaria infection would be less important in social mate choice, as it does not necessarily interfere with a male's ability to provide for the offspring (Szöllősi et al. 2009). Given the division between mate choice for direct

benefits (social mates) versus mate choice for genetic benefits (extra-pair mates), it is not surprising that we found no relationship between coloration and parasite load. This may also explain why some studies report that males that gain paternity as extra-pair sires are not necessarily better at maintaining paternity within their own nests (Bitton et al. 2007, O'Brien and Dawson 2011).

While many studies have reported a relationship between sexual signal quality and the strength of an immune response (Møller and Petrie 2002, Mougeot 2008, Griggio et al. 2010), I found no such relationship. Rather, parasites seemed to play a prominent role in determining which males successfully sired extra-pair offspring and which did not. This is not surprising, given that much of the initial rationale behind studying immune response and sexual signals came from the assumption that immunity determined parasite loads. Thus most studies examining immune response and sexual signaling tacitly assume that parasites are the major, underlying factor driving the relationship. However, given that we found a negative relationship between parasite intensity and immune response, one might expect that we should also have found a connection between immunity and male mating success. Possibly the relationship between immunity and parasites is too weak for us to find this. These results, however, highlight the importance of including measures or parasite infection in studies examining immunity (Adamo 2004, Graham et al. 2011).

While uninfected males were more successful at siring extra-pair offspring, overall reproductive success, or the number of offspring fledged in a year, did not differ between infected and uninfected individuals. This may be because other factors, such as

predation and environmental conditions, play a role in determining how many offspring successfully fledge. For example, drought can severely reduce fledging success (Fair and Whitaker 2008). Our finding that weather during the breeding season influences double brooding also highlights the importance of environmental conditions in determining reproductive success. Researchers interested in determining male reproductive success must take into account many different factors that influence overall offspring production.

**References**


Adamo S.A. (2004) How should behavioural ecologists interpret measurements of immunity? *Animal Behaviour* 68:1443-1449.

Akçay E. and Roughgarden J. (2007) Extra-pair paternity in birds: review of the genetic benefits. *Evolutionary Ecology Research* 9:855-868.

Bitton P., O'Brien E.L., and Dawson R.D. (2007) Plumage brightness and age predict extrapair fertilization success of male tree swallows, *Tachycineta bicolor*. *Animal Behaviour* 74:1777-1784.

Fair J.M., and Whitaker S.J. (2008) Avian cell-mediated immune response to drought. *Wilson Journal of Ornithology* 120:813-819.

Graham A.L., Shuker D.M., Pollitt L.C., Auld S.K.J.R., Wilson A.J., and Little T.J. (2011) Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology. *Functional Ecology* 25:5-17.

Griggio M., Zanollo V., and Hoi H. (2010) UV plumage color is an honest signal of quality in male budgerigars. *Ecological Research* 25:77-82.

Hill G.E. (2006) Female mate choice for ornamental coloration. In: Hill G.E. and McGraw K.J. ed. *Bird Coloration: Function and Evolution* (Vol. 2). Cambridge: Harvard University Press.

Møller A.P. and Petrie M. (2002) Condition dependence, multiple sexual signals, and immunocompetence in peacocks. *Behavioral Ecology* 13:248-253.

Mougeot F. (2008) Ornamental comb color predicts T-cell mediated immune response in male red grouse *Lagopus lagopus scoticus*. *Naturwissenschaften* 95:125-132.

O'Brien E.L. and Dawson R.D. (2011) Plumage color and food availability affect male reproductive success in a socially monogamous bird. *Behavioral Ecology* 22:66-72.

Siefferman L. and Hill G.E. (2003) Structural and melanin coloration indicate parental effort and reproductive success in male eastern bluebirds. *Behavioral Ecology* 14: 855-861.

Siefferman L.M. and Hill G.E. (2005a) Blue structural coloration of male eastern bluebirds *Sialia sialis* predicts incubation provisioning to females. *Journal of Avian Biology* 36:488-493.

Siefferman L.M. and Hill G.E. (2005b). UV-blue structural coloration and competition for nestboxes in male eastern bluebirds. *Animal Behaviour* 69:67-72.

Szöllősi E., Rosivall B., Hasselquist D., and Török J. (2009) The effect of parental quality and malaria infection on nestling performance in the collared flycatcher (*Ficedula albicollis*). *Journal of Ornithology* 150:519-527.