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TITLE: Comparing Measures of Immunity and Health Status in the Golden-winged Warbler (*Vermivora chrysoptera*) X Blue-winged Warbler (*V. cyanoptera*) Hybridization System

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ABSTRACT

Comparing Measures of Immunity and Health Status in the Golden-winged Warbler (*Vermivora chrysoptera*) X Blue-winged Warbler (*V. cyanoptera*) Hybridization System

I report the results of the first immunological tests in the Golden-winged Warbler (*Vermivora chrysoptera*) X Blue-winged Warbler (*V. cyanoptera*) hybridization complex. The study intended to investigate whether the Blue-winged Warbler or hybrids could have superior immunity over the Golden-winged Warbler, contributing to the rapid displacement of the latter. Blood samples were collected from breeding Golden-winged Warblers, Blue-winged Warblers and their hybrids in Central Wisconsin. I found no significant differences in the index to natural antibodies or hematocrit among these species and their hybrids. Unexpectedly, tick prevalence was higher in the Blue-winged Warblers than in the Golden-winged Warblers or their hybrids. It seems unlikely that the difference in immune quality is a factor for the displacement of the Golden-winged Warbler. Still, the comparable performance of hybrids relative to the parent species may provide additional support to the scenario that hybridization could continue where these species co-occur.

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**Comparing Measures of Immunity and Health Status in the Golden-winged Warbler
(*Vermivora chrysoptera*) X Blue-winged Warbler (*V. cyanoptera*) Hybridization System**

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Abstract

I report the results of the first immunological and hematological tests in the Golden-winged Warbler (*Vermivora chrysoptera*) X Blue-winged Warbler (*V. cyanoptera*) hybridization complex. The study intended to investigate whether the Blue-winged Warbler or hybrids could have superior immunity and health status over the Golden-winged Warbler, contributing to the latter's rapid displacement over much of its breeding range in North America. Blood samples were collected from breeding male Golden-winged Warblers, Blue-winged Warblers, and their hybrids in Central Wisconsin during the summer of 2010. Hybrids were identified by phenotypic characteristics and mitochondrial DNA analysis. No significant differences were observed among the two species and their hybrids in natural antibody levels in the plasma, a measure of innate humoral immune function. Differences in hematocrit (packed cell volume), an indicator of hematological health status, were also not observed. Interestingly, Blue-winged Warblers showed significantly higher tick prevalence as compared to the Golden-winged Warblers and hybrids, yet tick infestation showed no effect on the hematocrit of infested birds. Additionally, Golden-winged Warblers and hybrids showed greater values of mass and wing chord as compared to Blue-winged Warblers, yet it seems unlikely that such physical traits are involved in the displacement of the Golden-winged Warbler. Further work is planned to compare blood parasite (avian malaria) prevalence and differential white blood cell count, but from the limited results thus far, it seems unlikely that immunity or hematological health are involved in the displacement of the Golden-winged Warbler. On the other hand, hybrids do not seem to be at any disadvantage in these measures. This gives additional support to the scenario that hybridization could continue where these species co-occur, and that the pure Golden-winged Warblers may become exceedingly rare in the future.

Introduction

Understanding the mechanisms contributing to population decline is essential to prevent species extinction and loss of biodiversity (Smith et al. 2009; Wikelski and Cooke 2006). The Golden-winged Warbler (GWWA, *Vermivora chrysoptera*, Figure 1A), a small neotropical migrant bird, has been experiencing a rapid decline in numbers over much of its breeding range for the past 40 years (Confer 2006; Buehler et al. 2007). An estimated 64% of the global population has been lost since 1966 (Rich et al. 2004). It is now listed as a species of management concern in Wisconsin and nation wide (Confer 2006). Loss of shrubland habitat alone does not seem to fully explain the species decline; predictable local extinction of GWWA is observed following a period of invasion by Blue-winged Warblers (BWWA, *V. cyanoptera*, Figure 1B) and hybridization (Buehler et al. 2007). It is thought that the two species were put into contact in the 1800s due to human manipulation of the landscape (Gill 1980). Since then, hybridization and the decline of the GWWA seem to have been taking place (Gill 1980). Some causal mechanisms for the displacement are suggested (Confer 2006), but more knowledge is still required (Buehler et al. 2007; Vallender et al. 2007a, Vallender et al. 2009).

Among the drivers of species extinctions, infectious diseases of wildlife seem to be increasing in importance (Smith et al. 2009; Ishak et al. 2008). It is also suggested that individuals with greater heterozygosity may have increased resistance to disease-causing agents and that this trait is inherited to the future generations (Penn et al. 2002; Hawley et al. 2005). This heterozygote advantage hypothesis is particularly relevant to small or rapidly declining populations that experience a loss of heterozygosity. Lowered levels of heterozygosity were associated with decreased immune functions, such as natural antibody levels (Whiteman et al. 2006) and induced humoral and cell-mediated immunity (Reid et al. 2007). In addition, when

two species co-occur and one experiences more severe effects of shared parasites than the other, displacement by the less-affected species is shown to be possible (Tompkins et al. 2001).

Heterozygosity may also have important implications for reproductive success of an individual. In Blue Tits (*Parus caeruleus*), females frequently engage in extra-pair mating (mating outside the pair bond), and males that are genetically dissimilar were found to be more likely to be chosen as extra-pair partners (Foerster et al. 2003). Females thereby increased heterozygosity in the extra-pair young, and the more heterozygous offspring had greater chance of survival and reproduction (Foerster et al. 2003).

Further, it has been hypothesized that immune quality may be related to reproductive success. Hamilton and Zuk (1982) provided the first evidence that immunity could affect mate choice by suggesting that females choose males that would provide offspring with genes that would contribute to immunity against blood parasites. Immune functions are important in the defense against infectious agents such as blood parasites (Ots and Hõrak 1998; Sol et al. 2003), and birds seem to time their peak functions in some branches of immunity during the breeding season when parasite transmission is highest (Møller et al. 2003). Still, immune functions are considered energetically costly (Norris and Evans 2000; Bonneaud et al. 2003). Thus, under limited resources, birds could face an energetic tradeoff between immunity and breeding activity (Nordling et al 1998). For example, males may experience a tradeoff between immunity and development of secondary sexual characteristics (e.g. bright plumage) or vigor in territorial behavior (Hamilton and Zuk 1982). Hence, achieving both could indicate superior quality in males (Folstad and Karter 1992).

The suggested connections between heterozygosity, immunity, and mate choice may be important in understanding the GWWA X BWWA hybridization system and the decline of the GWWA. It is known that the hybrids are fertile and readily backcross with either of the parent

species, resulting in genetic introgression (Confer 2006; Vallender et al. 2007a). Thus, in the hybridization zone, females have the options of mating with males of the same species, the other species, or the hybrids. At least in GWWA, extra-pair mating appears common and could be playing a key role in the ongoing hybridization (Vallender et al. 2007b). Additionally, because these two species prefer similar shrubland habitat with extensive territory overlaps (Frech and Confer 1987; Fowlds 2010), they could share similar infectious agents such as parasites. If differences in immune quality exist among GWWA, BWWA, and their hybrids, it may have significant implications for the displacement mechanism and introgression. However, relatively little attention has been paid to disease and immunity in the GWWA X BWWA hybridization system. In the present study, I aimed to investigate the role of infectious diseases and immunity in the decline of GWWA.

To the best of my knowledge, there has been only one study that focused on immune functions in GWWA and BWWA. The study suggested that GWWA may have lower standardized heterozygosity and slightly greater prevalence of blood parasites as compared to BWWA (Vallender-Fraser 2006). However, the study did not test specific immune functions directly or include hybrids in the analysis. The performance of hybrids is of great interest (Confer, 2006; Buehler et al. 2007; Reed et al. 2007), yet there are disagreements in the literature. Some indicate that hybrids are selected against in breeding and thus reproduce poorly (Confer 2006; Kubel 2005), but others found no disadvantage in their breeding performance as compared to GWWA (Reed et al. 2007; Vallender et al. 2007b; Neville et al. 2008). If hybrid fitness is comparable to the parent species, hybridization is likely to continue, and the number of pure GWWA is predicted to decline further as the two species come into contact (Reed et al. 2007). To advance knowledge on this hybridization system, it is important to study immune functions in GWWA, BWWA and their hybrids; however, there is currently a lack of publication on this topic.

In the present study, I compared two measures of immune functions (natural antibodies and white blood cell counts), tick and blood parasite prevalence, and hematocrit in GWWA, BWWA, and their hybrids. If differential resistance to infectious diseases is indeed involved in the displacement of GWWA by BWWA, as suggested by Vallender-Fraser (2006), GWWA is expected to show lower levels of natural antibodies and white blood cell counts, and higher prevalence of parasites than BWWA, indicating relative disadvantage in disease resistance (Ishak et al. 2008; Nordling et al. 1998; Kilgas et al. 2006). Immune performance in hybrids is difficult to predict: mixed results are reported depending on the parental species and infectious agents (Mouliia 1999). Yet, at least one study on avian hybridization showed hybrid having increased strength in immune functions as compared to the endangered parent species (Tompkins et al. 2006). The present study performed the first immunological tests along with blood parasite prevalence in the GWWA X BWWA hybridization system, and thereby investigated the possibility of disease-mediated displacement of GWWA by BWWA and their hybrids.

Methods

All procedures were permitted by the USGS Patuxent Wildlife Research Center (permit #22522) and under the University of Wisconsin-Madison Animal Care and Use Committee (protocol #A01431-0-04-10).

Bird capture and sampling

GWWA, BWWA, and their hybrids were captured using mist nets in their shrubland habitat from May 25 to July 19, 2010. The period of capture was intended to span the entire breeding season. Sampling was done in Central Wisconsin (generalized location: 44.3° N, 90.2°W), which was in the middle of the hybridization zone where the two species co-occurred,

with relative abundance of GWWA (Fowlds 2010). Playback calls (recordings of mate attraction songs of male birds) were used to selectively capture the target species. The playback call has been known to be very effective for territorial males during the breeding season and commonly used for these species (e.g. Vallender et al. 2007b). Consequently, sampling was terminated when the birds disintegrate their territory and stop responding to the playback call.

Upon capture, the phenotype of the bird (Figure 1) was recorded. Phenotypic hybrids were identified as individuals showing intermediate characteristics of the two parental species (Figure 1C and 1D), and later confirmed from photographs (see Parkes 1951 for general phenotypic characteristics of hybrids). The “Brewster’s Warbler” phenotype indicates very recent hybridization event in its ancestry (Parkes 1951). As for the introgressed GWWA, I specifically focused on the relatively strong yellowness on the breast and undersides (Figure 1C) or a band of yellow below the black throat patch as indicators of the bird’s hybrid status. Caution was taken because second-year birds (first-time breeders) could have yellow hints on the breast or undersides from the juvenile plumage (Rachel Vallender, personal communication). Captured birds were banded with USGS aluminum bands with a unique number to avoid re-sampling.

Standard measurements were taken from each bird to compare structural size and condition. Bird mass was measured with a Pesola spring scale to the nearest 0.25g. Tarsus (length from the ankle to the base of digits) was measured to the nearest 0.01mm using digital calipers. Wing chord (length from the wrist to the longest feather tip of the wing, without applying pressure on the wing) and tail were measured with a ruler to the nearest mm. Birds were sexed and aged (hatch-year, second-year, or after second-year) using plumage characteristics based on Pyle (1997).

The presence of ticks (Acari: Ixodidae) was recorded for each bird. Ticks were commonly found on the facial region of birds, particularly around the eyes. Infestation by Ixodid

ticks could lead to reduced hematocrit (packed cell volume) in birds, suggesting its negative impact on the host's health (Heylen and Matthysen 2008).

Birds were bled (<75 μ L) by puncturing the brachial vein, and blood was collected directly into heparinized capillary tubes. A single blood smear was prepared from a drop of blood on a glass slide for leukocyte counts. The slides were air-dried and fixed in absolute methanol. Whole blood (~10 μ L) was transferred into lysis buffer and later separated for two analyses: mitochondrial DNA analysis for cryptic hybrid identification and blood parasite screening. The remaining blood was centrifuged in the field. Hematocrit, or packed cell volume, was measured as the percent of cell volume (mostly red blood cells) in the total blood column after centrifugation (Campbell and Ellis 2007). Hematocrit indicates efficiency of oxygen uptake and transport, and low hematocrit is associated with reduced body conditions in birds (Ots et al. 1998). Plasma samples for natural antibody assays were frozen in liquid nitrogen in the field and later transferred into a -20°C freezer.

Identification of cryptic hybrids

Not all GWWA X BWWA hybrids show phenotypic characteristics that are intermediate of the two parental species: they can be identical in morphology to the parent species (Vallender et al. 2007a). To identify some of these cryptic hybrids, I submitted the whole blood samples stored in lysis buffer to the Cornell Laboratory of Ornithology (Laura Stenzler) for mitochondrial DNA (mtDNA) genotype analysis as described by Vallender et al (2007a). Briefly, the technique amplified regions of the mtDNA that are uniquely fixed in the parental species using polymerase chain reaction (PCR). Thus, any individual with a phenotype-genotype mismatch would be identified as a cryptic hybrid. For example, a bird with GWWA phenotype but with BWWA mtDNA genotype would be identified as a cryptic GWWA hybrid. Yet, because mitochondria are

only inherited through the maternal line, the method was incapable of identifying all cryptic hybrids. Thus, I cannot conclude with certainty that a phenotypically pure bird without the phenotype-genotype mismatch was in fact genetically pure.

Natural antibodies

Natural antibodies (NAbs) are considered to be one of the most important innate immune components (Matson et al. 2005, Mendes et al. 2006). Mostly immunoglobulins M and G, the major roles of NAbs seem to be early resistance against infections and initiating specific immune responses of B and T cells by enhancing antigen presentation (Ochsenbein and Zinkernagel 2000). Because NAbs are known to react to a wide range of antigens including foreign red blood cells, species-specific reagents are not required (Matson et al. 2005).

The levels of NAbs in the plasma were indexed using the hemolysis-hemagglutination assay described by Matson et al. (2005). This technique characterizes the ability of NAbs to activate complement and to agglutinate foreign red blood cells. Because of the small sample volume I could obtain from many of the birds, I used this assay with minor modifications. I first replicated the original assay (described with 25 μ L of reagents) with decreased reagent volumes (10 μ L reagents). After confirming that the results with reduced volumes mirror those of the original, I performed all assays with 10 μ L of reagents. Further, it was observed that the birds had very low hemolysis activity (none showed any lysis activity at 1:2 plasma dilutions). Thus, I decided to focus on agglutination reactions only.

Specifically, 10 μ L of plasma samples were pipetted into column 2 of 96 well plates (Corning Coaster #3795, Corning, NY). Then 10 μ L of 0.01 M Phosphate buffered saline (Sigma, #P3744, St. Louis, MO) was pipetted into columns 1-12. Then, samples from column 2 were serially diluted (1:2) through column 11 using a multi-channel pipetter. Columns 1 and 12 served

as a negative control. Then 10 μ L of a 1% rabbit red blood cell suspension was then added to all wells. The plates were gently shaken, covered with an acetate plate sealer (Thermo #3501, Milford, MA), and then incubated in a 37 °C water bath for 90 min. Plates were then removed from the water bath and tilted at a 45 degree angle for 20 minutes at room temperature to enhance the visualization of agglutination. Plates were then scanned at 200 dpi using the positive transparency setting of a flatbed scanner (HP Scanjet 3970). Because of the subjective nature of the assay, all plates were scored blindly by a single scorer. For each sample, the score was obtained as the last well (highest dilution) that exhibited agglutination.

Leukocyte count (in progress)

Differential leukocyte (white blood cells) counts from blood smears, based on Clark et al. (2009), will be another measure of innate immunity. Higher relative abundance of leukocytes is thought to indicate greater innate immune capacity (Zuk and Johnson 1998), and in a study on hybridizing parakeet populations, the hybrids showed the greatest leukocyte counts (Tompkins et al. 2006). Thus, the leukocyte counts from GWWA X BWWA hybrids relative to the parent species were of great interest.

In short, the slides prepared in the field will be stained in Wright's solution (Clark et al. 2009) and studied under a light microscope. Under the assumption that 5 microscope views of a monolayer of blood cells with $\times 1000$ magnification (oil-emersion) correspond to approximately 1000 red blood cells (Campbell and Ellis 2007), the number of white blood cells per 10,000 red blood cells will be approximated. From the count, heterophil/lymphocyte will be determined to index stress, which is an important measure of hematological health status in birds (Ardia et al. 2008), but also could confound total leukocyte counts (Tompkins et al. 2006).

Blood parasite prevalence (in progress)

Prevalence data of blood parasites could be used to infer population-level immune quality (Ishak et al. 2008; Nordling et al. 1998; Marzal et al. 2005). Blood parasites of the genera *Haemoproteus* and *Plasmodium*, transmitted by blood-sucking insects, have a cosmopolitan distribution and are commonly found in the peripheral blood in a wide variety of avian hosts (Atkinson and Van Riper 1991), including GWWA and BWWA (Vallender-Fraser 2006). These parasites, collectively known as avian malaria, can have negative effects on host performance during stressful periods such as the breeding season (Nordling et al. 1998; Marzal et al. 2005; Atkinson and Van Riper 1991; Ots and Hõrak 1998). Studies have shown that infections decrease both reproductive success (Marzal et al. 2005) and survival (Sol et al. 2003; Marzal et al. 2008) in birds, a characteristic of important infectious agents that have a population impact (Wobeser 2006).

Previous work (Vallender-Fraser 2006) from three allopatric GWWA and BWWA populations (Manitoba and Ontario GWWA, and Kentucky BWWA) indicated that GWWA suffer higher blood parasite prevalence as compared to BWWA. I aim to replicate this study including hybrid birds in a sympatric (Central Wisconsin), hybridizing population.

For the analysis, whole blood samples stored in lysis buffer will be sent to the Canadian Museum of Nature (Rachel Vallender) for PCR-based screening of blood parasites (Ricklefs and Fallon 2002). Essentially, the technique amplifies the cytochrome-*b* region of the mtDNA of *Haemoproteus* and *Plasmodium*. The method is much more sensitive to the traditional blood smear-based detection and preferred in determining prevalence (Ricklefs and Fallon 2002).

Data Analyses

I excluded the females from the comparison. This is because the vast majority of captures were males (inevitable consequence of capture method exploiting territorial behavior) and differences in immune functions between the sexes were possible (Ots and Hōrak 1998; Ardia et al. 2008). Further, immune functions and health statuses of males were of greater interest, given the possibility of mate choice by females being the mechanism driving hybridization and displacement (Vallender et al. 2007b, Vallender-Fraser, 2006).

Because most of the hybrids in my total capture were of GWWA forms (phenotypically distinguishable: $n=9$; cryptic: $n=8$), I excluded the two BWWA hybrid forms (phenotypically distinguishable, $n=1$; cryptic, $n=1$) in the sample. Moreover, the performance of the GWWA hybrids relative to the pure GWWA has been the focus of debate (e.g. Reed et al. 2007, Vallender et al. 2007b). Due to the small sample size of hybrids, the phenotypically distinct “Brewster’s Warbler” forms ($n=2$) were combined with the GWWA forms, as in Vallender et al. (2007b). Hereon, hybrids in the analyses refer to individuals from the distinct “Brewster’s” form to the cryptic GWWA hybrids in the GWWA X BWWA hybridization spectrum.

Thus far, all analyses were performed using Program R (version 2.9.2, The R Foundation for Statistical Computing) and Rcmdr. For NAb and hematocrit, I used the analysis of covariance (ANCOVA) to compare GWWA, BWWA, and hybrids with season (capture date in days post May 24) as a covariate. Season was included because the capture period extended from the early breeding season to the end, and birds could have different energy allocations (breeding versus self-maintenance) during these periods (Nordling et al. 1998). In the analysis of hematocrit, tick presence (infested or uninfested) was added as a second covariate given the possible impact shown in previous work (Heylens and Matthysen 2008). Physiological studies often include “body condition” calculated from mass and tarsus length (e.g. residuals from a

regression of mass and tarsus³, Møller et al. 2003), based on the assumption that birds with greater mass with respect to an index of skeletal size are in better condition. However, I did not include “body condition” in the models for NAb and hematocrit because differences between species were observed in mass but not in tarsus (see *Body measurements* in the Results section). All ANCOVA models initially contained interactions, which were subsequently removed from the model when non-significant ($p>0.05$). Covariates were also removed when non-significant ($p>0.05$). Tick prevalence was compared among GWWA, BWWA, and hybrids using the χ^2 contingency test. Measurements of mass, wing chord, tail, and tarsus among GWWA, BWWA, and hybrids were compared using the analysis of variance (ANOVA). *Post hoc* Tukey tests were performed when significant differences were observed among groups.

Results

I still do not have the results from differential leukocyte counts and blood parasite prevalence. The following are the results I have thus far.

Natural Antibodies

I obtained NAb hemagglutination titers from 54 GWWA, 19 BWWA, and 14 hybrids (2 “Brewster’s” phenotype; 7 phenotypically distinguishable GWWA hybrids; 6 cryptic GWWA hybrids). The interaction between species and season was negligible. In all groups, NAb showed a significant linear increase as the season progressed (ANCOVA, $F_{1,83}=14.90$, $p<0.001$; Figure 2). However, no significant differences were observed among BWWA, GWWA, and their hybrids (ANCOVA, $F_{2,83}=0.301$, $p=0.74$; Figure 2).

Hematocrit and effect of ticks

I obtained hematocrit results from 75 GWWA, 25 BWWA, and 18 hybrids (2 “Brewster’s” phenotype; 8 phenotypically distinguishable GWWA hybrids; 8 cryptic GWWA hybrids). The interactions among species, season, and tick presence were all non-significant. Tick effect on the hematocrit was also non-significant and thus dropped ($F_{1, 112}=0.124, p=0.73$). Hematocrit significantly declined as the season progressed (ANCOVA, $F_{1, 113}=12.98, p=0.007$; Figure 3), yet no significant differences were observed among BWWA, GWWA, and their hybrids (ANCOVA, $F_{2, 113}=0.985, p=0.37$; Figure 3).

Tick prevalence

Presence or absence of Ixodid ticks was recorded from 27 BWWA, 90 GWWA, and 19 hybrids (2 “Brewster’s” phenotype; 9 phenotypically distinguishable GWWA hybrids; 8 cryptic GWWA hybrids) throughout the breeding season. Prevalence of ticks was significantly higher in BWWA had significantly higher tick prevalence (33.3%) as compared to GWWA (13.3%) and hybrids (10.5%) (χ^2 contingency test, $\chi^2=6.55, df=2, p=0.0386$; Figure 4).

Body measurements

Measurements of GWWA, BWWA, and their hybrids are summarized in Table 1. Statistically significant differences in mass (ANOVA, $F_{2, 113}=5.09, p=0.008$) and wing chord (ANOVA, $F_{2, 111}=8.86, p<0.001$) were observed. *Post hoc* Tukey tests revealed that GWWA and hybrids had greater mass and wing chord as compared to BWWA. Tail and tarsus lengths did not show significant differences (ANOVA, $F_{2, 113}=1.2, p=0.31$; $F_{2, 116}=1.02, p=0.36$, respectively).

Discussion

I tested the hypothesis that there might be differences in immune functions and hematological health statuses in the GWWA X BWWA hybridization system. This was based on the previous suggestions that BWWA may have greater heterozygosity levels and suffers less fitness consequences from infectious agents such as blood parasites as compared to GWWA (Vallender-Fraser 2006). However, the results I have thus far failed to identify any advantage in BWWA or hybrids. The observed patterns and the biological relevance are discussed further.

Natural antibodies

I did not observe any significant differences in levels of NABs among GWWA, BWWA, and their hybrids. NABs levels are not influenced by current infections or specific antibody response (Baumgarth et al. 1999), and suggested to be relatively stable with respect to the short-term health status of individuals (Matson et al. 2005). Further, because NABs are directly encoded in the germ-line genome, its expression could reflect the individual's genetic background (Matson et al. 2005). In fact, studies in chickens indicate that the levels of NABs respond to selection and may even reflect different genetic potential for acquired (adaptive) immune responses (Parmentier et al. 2004; Ardia and Schat 2008). Moreover, NAB levels have been shown to differ significantly, although not predictably, among naturally inbred populations, suggesting possible negative impact of low heterozygosity on this immune function (Whiteman et al. 2006). Thus, the lack of differences in GWWA and BWWA indicates that NAB levels are unaffected by the differences in heterozygosity suggested by Vallender-Fraser (2006). Moreover, it seems that the GWWA X BWWA hybrids do not suffer genetically-based disturbances, nor do they enjoy enhanced functions from increased heterozygosity levels in this immune component.

The general increase in NABs toward the end of the breeding season could be attributed to changes in energy allocation within individuals. Although birds may have higher NAb levels during the breeding season than during the non-breeding season, as suggested in the Red Knot (shorebird, *Calidris canutus*, Buehler et al. 2008), considering the energetic demand of breeding, immune functions could be limited by resources (Norris and Evans 2000, Nordling 1998). During the peak breeding season, males of these birds are known to defend territories and contribute to raising young along with the female (Vallender et al. 2007; Reed et al. 2007). Toward the end of the breeding season (July), it was observed that birds were becoming less responsive to the playback call, suggesting a decline in breeding activity. Because parasite transmission is considered highest during breeding (Møller et al. 2003), GWWA, BWWA, and their hybrids may have up-regulated their NAb levels toward the end as energy became available for immune functions.

Hematocrit

No significant differences were detected in hematocrit among GWWA, BWWA, and their hybrids. Variations in hematocrit in natural population can be difficult to interpret because a number of different factors are known to affect hematocrit. Low hematocrit values are associated with bacterial or parasite infections, but could also reflect starvation or deficiencies of specific nutrients (Coles 1997). Considering the correlation between low hematocrit and decreased body condition (Ots et al. 1998), however, it still seems to serve as a general indicator of hematological health status. Thus, at the population-level, GWWA, BWWA, and their hybrids seem to be in comparable health with respect to hematocrit values.

The seasonal trend observed among these species could be related to the level of physical exercise. Increase in hematocrit is associated with heightened activity and hence

increased demand for oxygen uptake and transport (Hörak et al. 1998). The intense physical exercise of male birds during breeding could be attributed to the high hematocrit values earlier in the season, which subsequently declined as males became less active.

Tick prevalence and effects on hematocrit

BWWA had much higher prevalence to ticks as compared to GWWA or their hybrids, which was unexpected. The reason behind this pattern is uncertain. Host density, often a major factor in parasite transmission (Wobeser 2006), does not seem to explain this pattern. BWWA occurred in much lower density as GWWA in the study area (Fowlds 2010), and these two species could occupy extensively overlapping territories (Frech and Confer 1987; Fowlds 2010). Instead, higher tick frequency in BWWA may be due to slight differences in habitat preference between the two species. BWWA appears to be a relative habitat generalist as compared to GWWA (Confer 1981). In Central Wisconsin, BWWA were shown to inhabit not only the shrubby, optimal GWWA habitat but also drier, more open areas with less stem density and older, more mature stands (Fowlds 2010). It could be that inhabiting dryer areas where ticks could be more abundant (Stafford et al. 1995) contributed to the higher frequency of tick infestation in BWWA.

Yet, the infested birds do not seem to suffer the hematological health effect (decrease in hematocrit) as was documented by Heylen and Matthysen (2008) in the Great Tit (*Parus major*). Thus, tick infestation and prevalence may be unimportant with respect to individual or population health in the GWWA X BWWA system. It would be interesting to observe whether there are any effects on the leukocyte counts, an effect not observed in the Great Tits (Heylen and Matthysen 2008). In any regards, it is unreasonable to believe that its higher ectoparasite prevalence favors BWWA.

Differences in body measurements

The differences of mass and wing chord between GWWA and BWWA were slight but highly significant when tested statistically. It is uncertain whether the major source of variations in these measurements was from differences in “condition” (nutritional status) or of genetic origin. However, considering that most of the hybrids in my data were of GWWA forms, the similarities of hybrids to GWWA in structural dimensions seem to be due to genetic similarities. If so, the observed differences in mass and wing chord between GWWA and BWWA should have resulted from species-specific genetic differences.

I recorded body measurements considering that there could be some physical attributes that may be used as a fitness indicator by females, favoring BWWA or hybrid males over GWWA. Particularly, longer wings and tails may indicate greater access to limited resources and hence better quality (Møller and Ninni 1998). In House Wrens (*Troglodytes aedon*), females seem to choose extra-pair partners based on size rather than immunocompetence (Forsman et al. 2008). However, a study on paternity and extra-pair mating in GWWA suggested a general lack of preference in females with respect to particular physical traits in male partners (Vallender et al. 2007b). Their results partially explain the pattern in the Wisconsin population, that although GWWA have larger physical attributes, BWWA seem to have been displacing GWWA since their initial contact (Confer 2006). It would be unreasonable that larger size confers an advantage to GWWA in mate choice as compared to BWWA, because if that were the case, it would be BWWA that becomes displaced. In short, the analysis of body measurements do not support the idea that BWWA have some physical traits that may makes them more attractive to females.

Immunity, heterozygosity, and GWWA X BWWA hybridization

It is important to note that the results I have thus far include only a single, constitutive innate immune branch (NAb). Multiple measures of immunity are desirable to draw concrete conclusions on immunocompetence (Ardia and Schat 2008, Mendes et al. 2006). In fact, it is thought that different branches of immune functions have varying energetic costs, and therefore tradeoffs could occur not only between immunity and other physiological functions, but also among the different branches of immunity (Norris and Evans 2000). Further, a study on hybridizing parakeets suggested that different branches of immunity could respond differently to hybridization, indicating that they are under different mechanisms (Tompkins et al. 2006). Thus, it is possible that other immune functions such as leukocyte abundance could show differences among GWWA, BWWA, and their hybrids.

Moreover, the measures of inducible immunity to novel antigens could be more relevant to heterozygosity. Initiating an immune response against disease-causing agents relies on the major histocompatibility complex (MHC) on the surface of cells (Goldsby et al. 2003). MHC is involved in initiating an immune response by binding to antigens and presenting them to T-lymphocytes (Goldsby et al. 2003; Westerdahl et al. 2005). Each MHC molecule can bind to only a limited number of antigens, and thus diversity in the MHC alleles is considered important (Westerdahl et al. 2005, Penn et al. 2002). Indeed, it is suggested that individuals with a large number of MHC alleles are protected against lethal effects of blood parasite infections in birds (Westerdahl et al. 2005). Therefore, individuals and populations with greater levels of heterozygosity at the MHC loci are thought to be able to respond better to a wider range of pathogens (Penn et al. 2002). Unfortunately, however, my methods were incapable of quantifying activities of specific, inducible immune responses. This is due to procedural difficulties of injections of novel antigens and potential harm on the individuals of a declining species.

Nevertheless, blood parasite prevalence (i.e. the population-level suppression of a potentially harmful parasite) and the possible increase in leukocyte count in infected birds may shed some light onto the relative strength of immune responses in general.

The mechanism behind the rapid displacement of GWWA by BWWA with hybridization in areas of sympatry remains unknown (Vallender et al. 2009). The present study aimed to test the hypothesis that differences in immunity and health status, factors that could affect relative fitness (Tompkins et al. 2001; Hamilton and Zuk 1982) may be disfavoring GWWA. I thus far only have one measure of immunity (NAbs), hematological health status (hematocrit), and parasitism (Ixodid tick prevalence) each. Based on the limited data, however, it seems unlikely that the immune function and health conditions I investigated are driving the displacement of GWWA. On the other hand, the comparable immune performance and health status of GWWA X BWWA hybrids support the pessimistic scenario for the pure GWWA populations, as indicated by Reed et al. (2007), Vallender et al. (2007b) and Neville et al. (2008). Their results showed no disadvantage in realized breeding success in the hybrids of both sexes. Indeed, without a physiological barrier, hybridization seems likely to continue, forecasting a future where the pure GWWA may become extremely rare.

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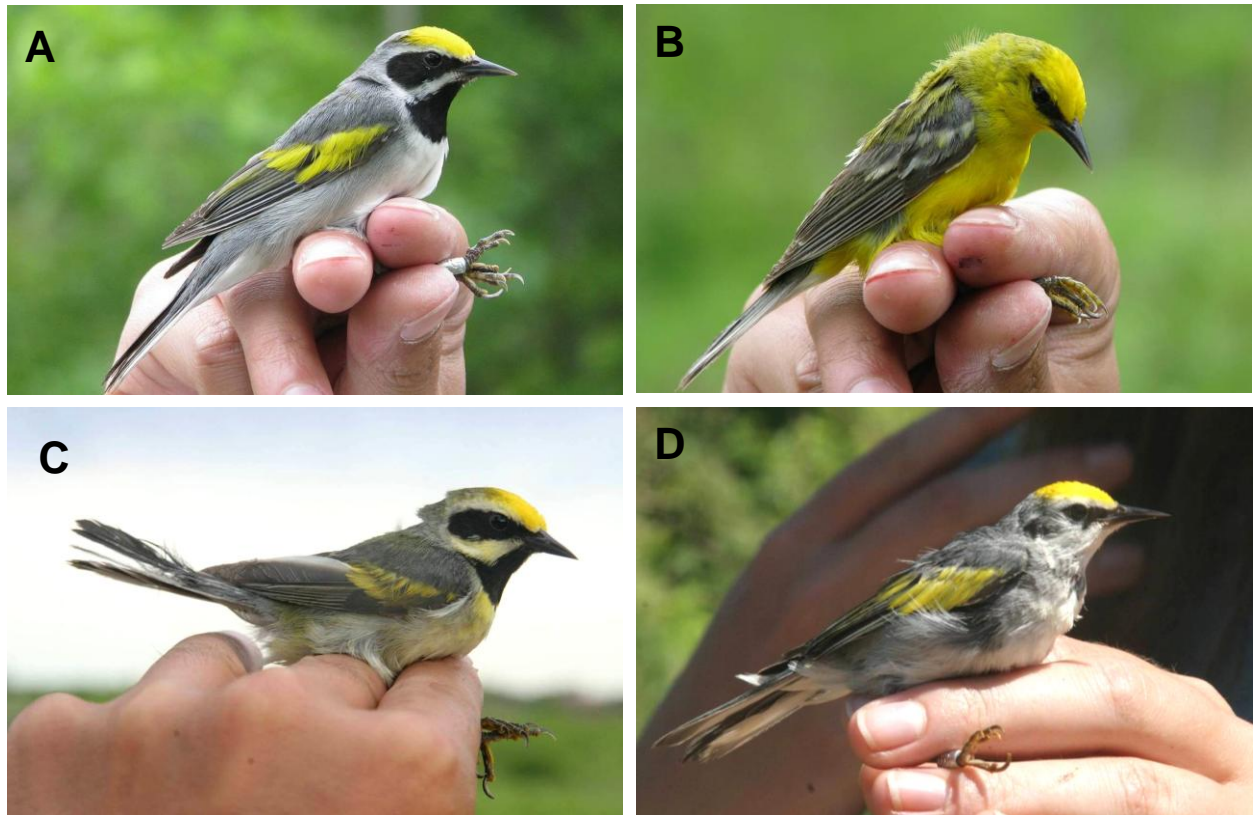
Figures and Table

Figure 1. Male warbler phenotypes. A: Phenotypically pure Golden-winged Warbler. B: Phenotypically pure Blue-winged Warbler. C: Phenotypically distinguishable Golden-winged Warbler hybrid, showing extensive yellow on the breast. D: Distinct hybrid of the “Brewster’s Warbler” phenotype. Photographs A and B: courtesy of R. Monica Fowlds.

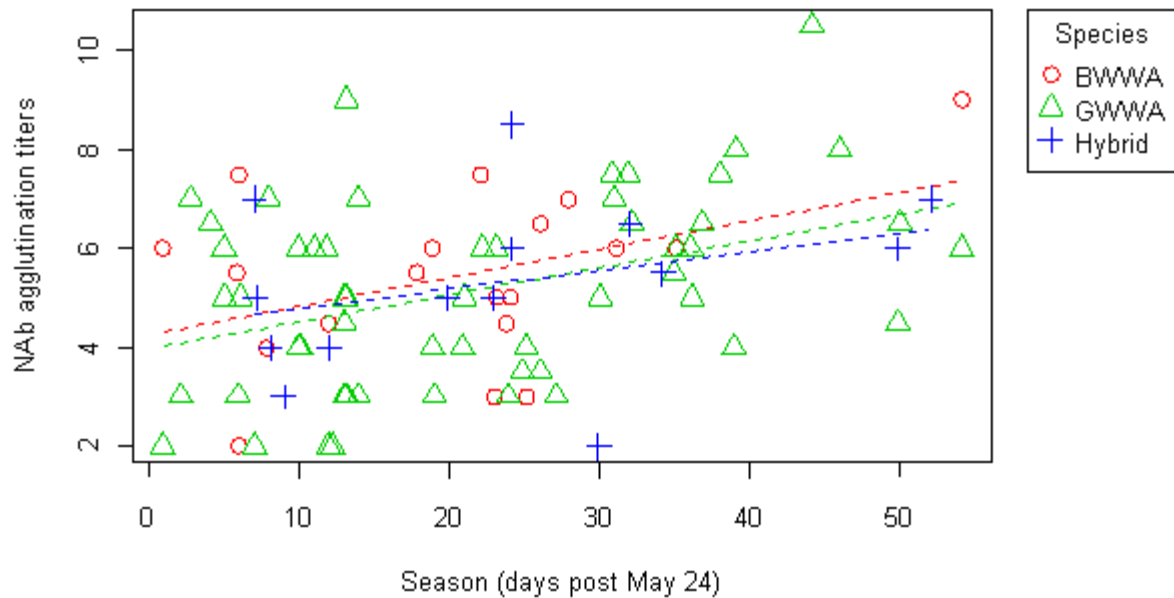


Figure 2. Natural antibody (NAb) titers in Blue-winged Warblers (BWWA, $n=19$), Golden-winged Warblers (GWWA, $n=54$), and their hybrids ($n=14$) from Central Wisconsin. Hybrids include birds of the “Brewster’s Warbler” phenotype ($n=2$), phenotypically distinct GWWA hybrids ($n=7$), and cryptic GWWA hybrids that were identified by mitochondrial DNA analysis ($n=6$). NAb titers increased as the season progressed (ANCOVA, $F_{1,83}=14.90$, $p<0.001$), yet no significant differences were observed among BWWA, GWWA, and their hybrids (ANCOVA, $F_{2,83}=0.301$, $p=0.74$).

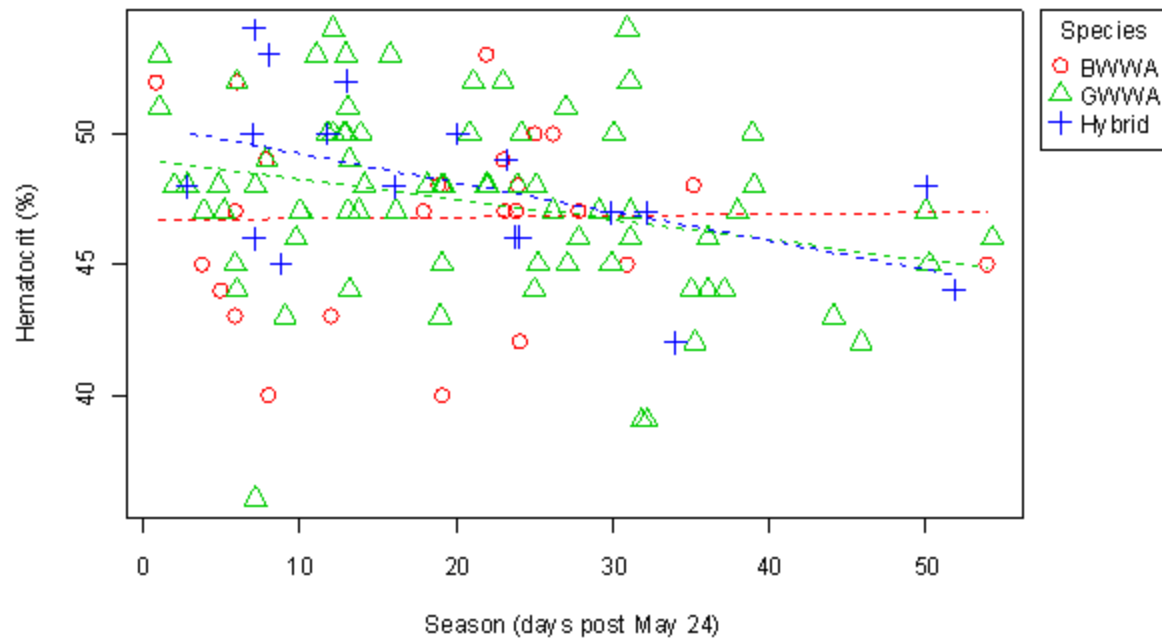


Figure 3. Hematocrit in breeding male Blue-winged Warblers (BWWA, $n=25$), Golden-winged Warblers (GWWA, $n=75$), and their hybrids ($n=18$) from Central Wisconsin. Hybrids include birds of the “Brewster’s Warbler” phenotype ($n=2$), phenotypically distinct GWWA hybrids ($n=8$), and cryptic GWWA hybrids that were identified by mitochondrial DNA analysis ($n=8$). Hematocrit declined as the season progressed (ANCOVA, $F_{1,113} = 12.98$, $p=0.007$), yet no significant differences were observed among BWWA, GWWA, and their hybrids (ANCOVA, $F_{2,113}=0.985$, $p=0.37$).

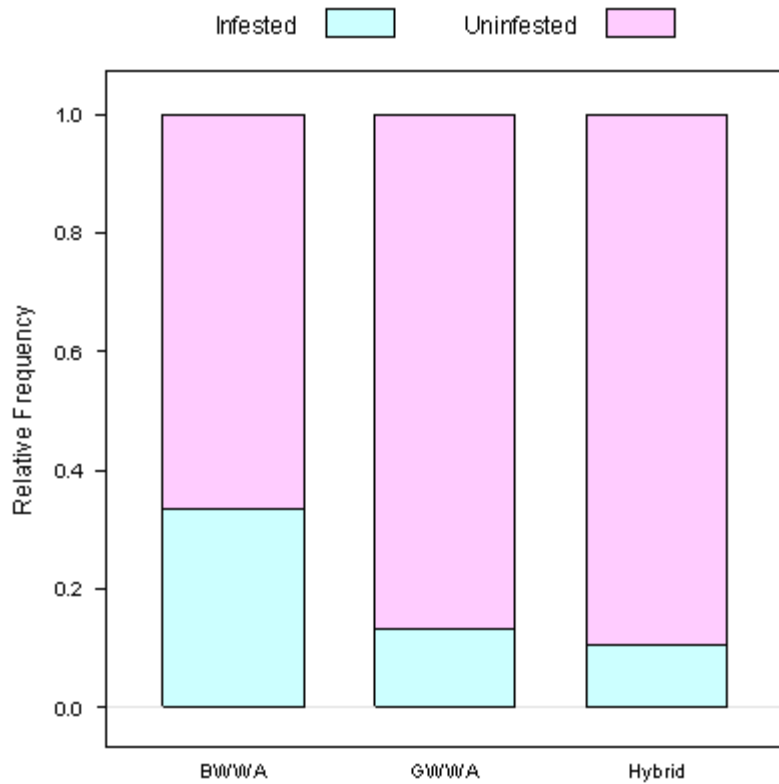


Figure 4. Frequency of tick infestation among breeding male Blue-winged Warblers (BWWA, $n=27$), Golden-winged Warblers (GWWA, $n=90$), and their hybrids ($n=19$) from Central Wisconsin. Hybrids include birds of the “Brewster’s Warbler” phenotype ($n=2$), phenotypically distinct GWWA hybrids ($n=8$), and cryptic GWWA hybrids that were identified by mitochondrial DNA analysis ($n=9$). BWWA had significantly higher tick prevalence as compared to GWWA and hybrids (χ^2 contingency test, $\chi^2=6.55$, $df=2$, $p=0.0386$).

Variable	BWWA (<i>n</i>)	GWWA (<i>n</i>)	Hybrid (<i>n</i>)	<i>F</i>	<i>p</i>
Mass (g)	8.8±0.1 (23)	9.2±0.1 (80)	9.3±0.1 (13)	5.09	0.0076*
Wing chord (mm)	60.0±0.4 (23)	61.6±0.2 (78)	61.4±0.4 (13)	8.86	<0.001*
Tail (mm)	47.1±0.3 (23)	47.6±0.2 (80)	46.8±0.8 (13)	1.20	0.31
Tarsus (mm)	17.40±0.07 (23)	17.45±0.04 (81)	17.31±0.08 (15)	1.02	0.36

Table 1. Measurements of breeding male warblers (mean±SE). BWWA = Blue-winged Warblers, GWWA = Golden-winged Warblers, and Hybrid = GWWA X BWWA hybrids. The larger means, identified by the *post hoc* Tukey test following ANOVA, are shown in bold. Asterisks (*) are given to $p < 0.05$. GWWA and GWWA X BWWA hybrids had significantly greater mass and wing chord length than BWWA, while measures of tail and tarsus showed no significant differences.