



AMBIENT TEMPERATURE, BUT NOT PATERNITY, IS ASSOCIATED WITH IMMUNE RESPONSE IN SAVANNAH SPARROWS (*PASSERCULUS SANDWICHENSIS*)

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ABSTRACT.—Females may engage in extrapair copulations to receive indirect benefits in the form of more immunocompetent young. Nestling quality is often assessed by examining immune function using the phytohemagglutinin (PHA) skin test, which can be a predictor of nestling recruitment. However, the PHA test can also be influenced by factors such as nest temperature. We tested the prediction that extrapair young mount greater immune responses to a PHA challenge and recruit at a greater rate than within-pair young in a wild population of Savannah Sparrows (*Passerculus sandwichensis*). We also investigated ambient temperature and its relationship with immune response. Extra- and within-pair young did not significantly differ with respect to PHA-induced immune response or with respect to recruitment. In contrast to other studies, we found that PHA-induced immune response was not associated with nestling recruitment rates. However, it was positively correlated with minimum ambient temperature for males hatched in 2002 and 2003, and for females in 2003 (with a similar, but marginally nonsignificant, trend in 2002). Immune response to PHA also was positively correlated with nestling mass for males hatched in 2002 and for females hatched in 2002 and 2003. We concluded that given the lack of relationship among immune responses, paternity, and recruitment rates in this population of Savannah Sparrows, nestling immune response to PHA injection should not be considered predictive of recruitment in populations where this relationship has not been explicitly explored, and environmental variables such as nest temperature may be an important covariate in immunoresponsiveness. Received 16 September 2008, accepted 17 January 2009.

Key words: extrapair paternity, *Passerculus sandwichensis*, phytohemagglutinin, recruitment, Savannah Sparrow, temperature.

La Temperatura del Ambiente, Pero No la Paternidad, Está Asociada con la Respuesta Inmune en *Passerculus sandwichensis*

RESUMEN.—Las hembras pueden involucrarse en cópulas extrapareja para recibir beneficios indirectos en forma de crías con mayor inmunocompetencia. La calidad de los pichones a menudo es evaluada examinando su función inmune usando la prueba cutánea de fitohemaglutinina (PHA, por sus siglas en inglés), la cual puede predecir el reclutamiento de los pichones. Sin embargo, la prueba de PHA también puede ser influenciada por factores como la temperatura de los nidos. Pusimos a prueba la predicción de que los pichones extrapareja presentan respuestas inmunes mayores ante el desafío de PHA y reclutan a una tasa mayor que los pichones intrapareja en una población silvestre de *Passerculus sandwichensis*. También investigamos la temperatura del ambiente y su relación con la respuesta inmune. Los pichones extrapareja e intrapareja no presentaron diferencias significativas con respecto a la respuesta inducida por la prueba de PHA ni con respecto al reclutamiento. En contraste con otros estudios, encontramos que la respuesta inmune inducida por PHA no se asoció con la tasa de reclutamiento de los pichones. Sin embargo, dicha respuesta estuvo correlacionada positivamente con la temperatura ambiental mínima para machos que eclosionaron en 2002 y 2003 y para hembras que eclosionaron en 2003 (existió una tendencia similar, marginalmente no significativa, en 2002). La respuesta inmune ante la PHA también se correlacionó positivamente con la masa de los pichones para los machos eclosionados en 2002 y para las hembras que lo hicieron en 2002 y 2003. Concluimos que dada la ausencia de relación entre las respuestas inmunes, la paternidad y las tasas de reclutamiento en esta población de *P. sandwichensis*, la respuesta inmune de los pichones a la inyección de PHA no debería ser considerada como una variable que predice el reclutamiento en las poblaciones en donde esta relación no ha sido explorada de forma explícita, y que condiciones ambientales como la temperatura de los nidos pueden ser covariables importantes de la respuesta inmune.

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MANY STUDIES HAVE investigated the “good genes” benefits of extrapair mating in terms of an improved nestling immune system (Johnsen et al. 2000, Kleven and Lifjeld 2004, Garvin et al. 2006, Kleven et al. 2006, Edler and Friedl 2008, Forsman et al. 2008, Fossøy et al. 2008, Wilk et al. 2008), with mixed results. Results of some studies have shown that a higher degree of nestling immunity is correlated with an increased nestling recruitment rate (Cichoń and Dubiec 2005, Moreno et al. 2005). However, it is possible that the benefits of extrapair mating are context-dependent (Welch 2003, Schmolle et al. 2005). Environmental factors such as temperature, precipitation, seasonality, parasite abundance, and food availability may affect the costs of pursuing extrapair copulations and the rearing environment of the offspring, as well as directly affecting nestling immune responses (Lifjeld et al. 2002, O’Brien and Dawson 2007). Garvin et al. (2006) suggested that the benefits of extrapair mating may be more evident in unfavorable conditions, as supported by their study showing that immune-response differences between within- and extrapair young were evident only during colder ambient temperatures. However, O’Brien and Dawson (2007) suggested the opposite: genetic benefits were more apparent under favorable conditions, given that extrapair young had longer flight feathers at fledging than within-pair young when parasite abundance was low. These and other discrepant findings suggest that selection of genetic benefits may not be consistently strong under either favorable or unfavorable conditions and that each form of benefit may have its own specific context-dependence. Thus, it is important to consider the specific environmental context in which the particular genetic benefit of interest may occur.

We hypothesized that females engage in extrapair copulations to improve the quality, specifically the immune function, of their offspring and that these increases in immune function vary in magnitude, depending on the environmental conditions (e.g., temperature) present during the nestling period (*sensu* Ardia 2005). Specifically, we predicted that females have extrapair young (EPY) that display greater immune responses than within-pair young (WPY). However, we also predicted an interaction between temperature and paternity, with differences in immune responses between EPY and WPY not becoming manifest until temperatures were low enough to reveal the benefits conveyed by extrapair paternity, according to Garvin et al. (2006), who investigated the same form of genetic benefit within a similar context. Finally, we predicted that young with greater immune functionality would be more likely to survive to the next breeding season (recruit) and to produce more fledglings over the individual’s lifetime, thereby providing evidence that increased nestling immune function is indeed an indirect benefit to the female in the form of increased fitness.

We studied a population of Savannah Sparrows (*Passerculus sandwichensis*) nesting on Kent Island, New Brunswick, during the breeding seasons of 2002 and 2003. About 10–40% of male Savannah Sparrows are polygynous (Wheelwright et al. 1992), and a longer-term study investigating extrapair paternity and sexual selection in this population found that rates of extrapair paternity were 56% and 37.4% in 2002 and 2003, respectively, with 73.4% of females producing at least one extrapair young in 2002, and 61.4% in 2003 (Freeman-Gallant et al. 2005). The population on Kent Island is highly philopatric, with an average natal dispersal

distance of 228 m (Wheelwright and Mauck 1998). Environmental conditions can be highly variable on Kent Island. For example, daytime highs ranged from 9.4 to 24.4°C during June, July, and August 2002. Variation in nest temperature may affect immune responsiveness, specifically the phytohemagglutinin (PHA) skin test that we employed (see below), which is positively correlated with nest and ambient temperatures (Ardia 2005, Garvin et al. 2006). Thus, this is an appropriate system in which to study the associations among environmental conditions, extrapair paternity, offspring immune function, and offspring recruitment.

To examine immune function, we used a PHA skin test (see Smits et al. 1999). Phytohemagglutinin is a benign mitogen that induces an immune response that can be measured by the amount of resultant swelling at the site of injection, with larger swellings associated with an increased chance of same-season fledgling recapture (Suter et al. 2007) and of recruitment the following year (Cichoń and Dubiec 2005, Moreno et al. 2005). In the literature, the swelling response to PHA injection has been cited as an indication of the T-cell-mediated immune response (e.g., Roberts et al. 2007, Roulin et al. 2007). However, Martin et al. (2006) experimentally demonstrated that the immune response to PHA injection recruits many different immune cell types to the injection site and more accurately models both innate and adaptive immune capability.

METHODS

Study population.—All research was conducted from May through July in 2002 and 2003 on Kent Island, an 80-ha island located at the southern end of the Grand Manan Archipelago in New Brunswick (44°35′N, 66°46′W). Savannah Sparrows are migratory, arriving to breed between April and May, and generally initiate clutches between late May and late June (Wheelwright and Mauck 1998). Beginning in mid-May, we used mist nets to capture all adults. We banded each adult with a U.S. Fish and Wildlife Service (USFWS) aluminum numbered band and a random, unique combination of three plastic color bands for individual identification. We also took a 50- μ L blood sample from the brachial vein for genetic analysis (see below).

We conducted daily censuses of the study area from late May until late July and identified social pairs (Wheelwright and Mauck 1998). We located nests using behavioral observations and then checked the nests every other day until hatching (day 1). We banded nestlings with a USFWS aluminum band and a single colored leg band six days after hatching (day 7). At this time we took a blood sample and began immune assessment (see below). Nestling recruitment was noted in all individuals captured or observed in island-wide surveys the following year. Because of the high degree of natal philopatry recorded in this population, and breeding dispersal distances typically <50 m (Wheelwright and Mauck 1998, Wheelwright et al. 2006), we are confident that this is a reasonable method to assess recruitment. Additionally, we recorded breeding data (e.g., total number of fledglings over the individual’s lifetime) through the summer of 2008 for all birds that returned. Maximum and minimum daily temperature and precipitation (cm) were recorded on Kent Island daily throughout the duration of the study by a weather station located on the study site.

Nestling sampling.—We removed all nestlings from the nest (79 nests in 2002, 60 in 2003; mean brood size = 3.2 in 2002 and 2003) on the morning of day 7. We recorded the mass (to 0.1 g) and wing length (to 1 mm) of each nestling and then removed 50 μ L of whole blood by puncturing the brachial vein and collecting blood in 70- μ L capillary tubes. The blood was transferred from the capillary tubes to a micro tube filled with lysis buffer (Seutin et al. 1991), which preserves DNA. We then drew a black dot on the patagium (wing-web) of each nestling and measured the thickness with a digital micrometer (Mitutoyo model 293-369). Measurements were taken twice to obtain repeatability statistics ($r = 0.97$ and 0.98 for days 7 and 8, respectively) and, because of the high repeatability, means were used in subsequent analyses. Directly under the black dot, we injected 0.15 mg of PHA suspended in 20 μ L of phosphate-buffered saline (Sigma L-8754). Each nestling was then returned to the nest. Twenty-four hours later (± 1 h), we returned to the nest site and measured the thickness of the patagium at the black dot. We used the difference in thicknesses as our measure of immune response (Smits et al. 1999). Within years, all measurements were performed by a single observer. However, because observers differed between years, we performed all analyses regarding the swelling response only within each year to eliminate potential year and observer bias.

Paternity and sex assignment.—The paternity data we used were derived from a larger, three-year study of extrapair paternity and sexual selection in Savannah Sparrows (Freeman-Gallant et al. 2005, 2006). Details of primer sequences, annealing temperatures, reaction amounts, and paternity assignment protocol can be found in Freeman-Gallant et al. (2005), and information on heterozygosities and locus characteristics can be found in Freeman-Gallant et al. (2006).

We determined the sex of nestlings by amplifying an intron in the chromohelicase-DNA binding gene that differs in size between the W and Z sex chromosomes (Kahn et al. 1998). Our 25- μ L reactions contained 20 mM Tris-HCl, 50 mM KCl, 4.2 mM MgCl₂, 0.2 mM dNTPs, 16 pmol of each primer, and 0.5 U Taq. Reactions were run in a Hybaid Omn-E polymerase-chain-reaction (PCR) machine over 30 cycles; cycling conditions followed Kahn et al. (1998). In Savannah Sparrows, PCR yields a single 238-base-pair (bp) fragment in males (ZZ) and an additional 286-bp fragment in females (WZ; Freeman-Gallant et al. 2001). Therefore, the sex of all 411 offspring in the present study is known without ambiguity.

Statistical analysis.—Data were analyzed using SAS 9.1 (SAS Institute, Cary, North Carolina). We used mixed-model analysis of variance (ANOVA) to examine the prediction that extrapair young have greater immune responses than within-pair young. We ran separate models for males and females because sex and mass were confounded (males are larger than females; $F = 34.22$, $df = 1$ and 430 , $P < 0.001$). Also, because minimum temperature and precipitation were significantly correlated ($r = 0.8$, $P < 0.001$), we included only minimum temperature, but not precipitation, in our model. We chose temperature because previous research has demonstrated the relevance of temperature to the nestling immune response (Ardia 2005, Garvin et al. 2006). Within each sex, we analyzed our results by year to control for between-year differences in PHA-test observer bias. We examined PHA-induced immunity and used paternity as a fixed effect, natal nest as a random effect, and nestling mass and minimum temperature during

the day of PHA injection as covariates. Interactions were excluded from the final model if $P > 0.05$ in the initial model.

We examined indirect benefits in three ways. First, we used a chi-square analysis to see whether there were gross patterns of recruitment attributable to paternity. Second, we ran a generalized linear mixed model (GLMM), which permits random factors and non-normally distributed dependent data. We ran separate analyses by year with recruitment as the dependent variable, PHA-induced immunity and mass as covariates, paternity and nestling sex as fixed effects, and natal nest as a random effect. We also included nestling thermal environment as a covariate, calculated as the mean minimum temperature for the first seven days after hatch. To explicitly test whether any effect of paternity depended on environmental conditions, we included the paternity*nestling-thermal-environment interaction in our model. Using only the data from birds that recruited, we ran a mixed model with number of lifetime fledglings as the dependent variable (data which were normally distributed) and the same independent variables as in the GLMM. Because there was a single nest from which multiple birds recruited in 2002, we ran two ANOVAs for that year (each with a single sibling removed) with the above variables, with the removal of natal nest as a random effect.

RESULTS

Mixed models examining immune response.—Paternity had no effect on immune response of males in either year (Table 1A). Immune response increased with mass in 2002 but not in 2003 (Fig. 1A), whereas immune response increased with minimum temperature in both 2002 and 2003 (Fig. 2A). However, there was a significant interaction of mass and minimum temperature in 2002, with lighter birds (lowest tercile) showing a positive relationship between immune response and minimum temperature, heavier birds (highest tercile) showing a negative relationship, and medium birds (middle tercile) displaying no relationship (Fig. 3). All other terms and interactions were nonsignificant (all $P > 0.05$).

Paternity had no effect on female immune response in either year (Table 1B). Larger females had greater immune responses in both 2002 (Fig. 1B) and 2003 (Fig. 1C). In 2002, there was a trend toward greater immune responses at higher minimum temperatures (Fig. 2B), and the same relationship was significant in 2003 (Fig. 2C). One interaction term remained in 2002 but was not significant, and none remained in 2003. All other terms and interactions were nonsignificant (all $P > 0.05$).

Recruitment and lifetime fledgling production.—For birds hatched in 2002, we found that recruitment status was not associated with immune response ($F = 0.00$, $df = 1$ and 154 , $P = 0.956$), nestling sex ($F = 0.42$, $df = 1$ and 154 , $P = 0.518$), paternity ($F = 0.32$, $df = 1$ and 154 , $P = 0.574$), rearing environment ($F = 0.32$, $df = 1$ and 154 , $P = 0.573$), or a paternity*environment interaction ($F = 0.52$, $df = 1$ and 154 , $P = 0.474$), but heavier birds were more likely to recruit ($F = 6.45$, $df = 1$ and 154 , $P = 0.012$). For nestlings hatched in 2003, recruitment status was not associated with immune response, nestling sex, paternity, mass, rearing environment, or the paternity*environment interaction (all $P > 0.05$), yielding two years in which recruitment was not associated with immune response. Overall, EPY did not recruit more often than WPY ($\chi^2 = 0.285$, $df = 1$, $P = 0.594$).

TABLE 1. Mixed-model ANOVA tables for three mixed models with PHA-induced immunity as the dependent variable, with (A) only male nestlings as subjects and (B) only female nestlings as subjects.

Variable	2002			2003		
	df	F	P	df	F	P
A. Male nestlings						
Paternity	89.8	0.11	0.737	80	0.18	0.676
Minimum temperature	101	12.53	0.001	80	4.49	0.037
Mass	101	16.33	0.001	80	2.45	0.122
Minimum temperature*mass	101	13.08	0.001			
B. Female nestlings						
Paternity	112	0.02	0.889	88.6	1.85	0.177
Minimum temperature	119	2.97	0.087	38.2	4.49	0.041
Mass	123	4.17	0.043	87.4	5.94	0.017
Minimum temperature*mass	119	2.50	0.116			

Notes: Paternity = WPY vs. EPY; significant *P* values (<0.05) are in bold.

We could not use a mixed model with natal nest as a random effect because there was only one nest from which more than one sibling recruited in 2002. Therefore, we ran two ANOVAs, each with one sibling removed. Immune response, nestling sex, rearing environment, and the paternity*environment interaction were not significantly related to lifetime number of fledglings in either model (all $P > 0.05$). However, nestling weight was positively associated with lifetime number of fledglings in both models (both $P < 0.05$), and EPY fledged fewer lifetime young than WPY in one model ($F = 7.41$, $df = 1$ and 5 , $P = 0.0416$), with a similar, nonsignificant trend in the other ($F = 5.19$, $df = 1$ and 5 , $P = 0.0717$). In 2003, number of fledglings was not significantly related to immune response, nestling sex, nestling weight, paternity, rearing environment, or the paternity*environment interaction (all $P > 0.05$).

DISCUSSION

Extrapair young were not more immunocompetent, more likely to recruit, or more likely to fledge more lifetime offspring than within-pair young in either year. Furthermore, immune response to PHA was never an indication of likelihood to recruit. Although there seems to be a correlational relationship in some study systems—for example, Blue Tit (*Parus caeruleus*; Cichoń and Dubiec 2005) and Pied Flycatcher (*Ficedula hypoleuca*; Moreno et al. 2005)—we found no such evidence in Savannah Sparrows on Kent Island. Therefore, although females in this population that raise young with greater PHA-induced responses may still receive indirect benefits (e.g., longer life span), neither increased recruitment rates nor greater lifetime reproductive success appears to be among them, and any advantage that EPY have over WPY is yet to be determined. On the basis of this finding, which included data from >400 nestlings, the assumption that nestling PHA-induced immune response is a good predictor of recruitment does not appear to be warranted, at least in this system.

Although female Savannah Sparrows are more likely to produce EPY when socially paired to males that are genetically similar to themselves (Freeman-Gallant et al. 2003, 2006), this infidelity does not result in increased levels of immune response to PHA injection among offspring. Additionally, because EPY did not recruit more frequently than WPY overall, we were unable to demonstrate

any fitness benefit to females engaging in extrapair copulations using our available data. In fact, in one model, EPY recruits had a lower lifetime reproductive success than WPY. However, given the lack of a paternity effect in the other 2002 model and the lack of a paternity effect in 2003, the conservative conclusion is that there is not a difference between EPY and WPY lifetime reproductive success. Thus, our predictions that EPY have an immune advantage over WPY, that paternity is predictive of recruitment, and that there is some other indirect benefit to females that raise EPY were not supported. Furthermore, the temperature*paternity interaction was not significant for either sex in 2002 or 2003 with regard to PHA-induced swelling, and there was no recruitment advantage based on a temperature*paternity interaction. Therefore, our prediction that differences in immune function or recruitment between EPY and WPY would manifest with different ambient temperatures was not supported.

Although there were exceptions, for the most part, immune response increased with both minimum temperature and mass, which is consistent with previous correlational studies (Liffield et al. 2002, Ardia 2005, Moreno et al. 2005, Garvin et al. 2006). This correlation between immune response and minimum temperature was seen in lightweight males in 2002 and all males in 2003, and in females during 2003, with a trend in the same direction in 2002. We concluded that Savannah Sparrows can more effectively mount an immune response to a PHA challenge when minimum temperatures are higher. One potential explanation for this finding is that at colder temperatures, nestlings need to allocate more resources to thermoregulation, at the expense of immune activation (Svensson et al. 1998). Parents brood young only for 7 min h⁻¹ by the time the young are seven days old (Wheelwright and Rising 1993). We did not examine energetic tradeoffs explicitly; however, PHA-induced immune response has been shown to be temperature-dependent in other correlational avian studies (Liffield et al. 2002, Garvin et al. 2006, Ardia 2007, Pitala et al. 2007). However, in an experimental study in which the temperature of nest cavities was manipulated, temperature had no effect on nestling immune response to PHA (Dawson et al. 2005). Thus, temperature may affect the immune response indirectly by influencing factors such as food availability (Liffield et al. 2002; but see Ardia 2007) or parasite prevalence (Walker et al. 2003). We recommend that future

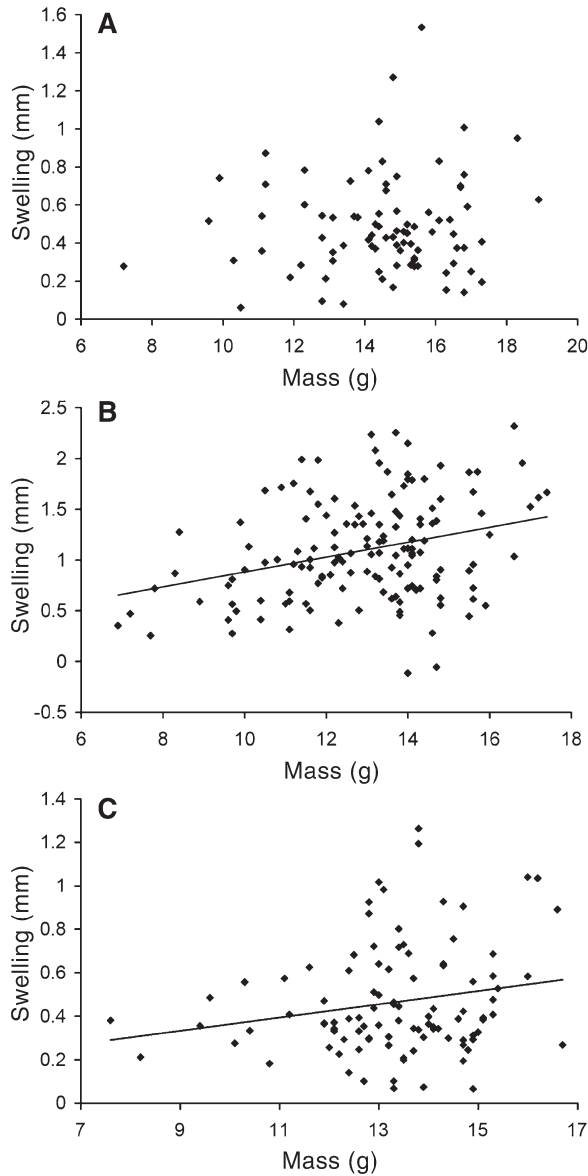


FIG. 1. PHA-induced swelling as a function of mass in nestling (A) males in 2003, (B) females in 2002, and (C) females in 2003. Significant relationships are indicated by a regression line. Males from 2002 are not shown, because there was an interaction of mass and temperature.

researchers manipulate these various factors while performing PHA challenges, to tease out the direct and indirect effects of temperature on immune response.

The interaction of mass and minimum temperature on immune response of males in 2002 is somewhat puzzling. This relationship was not found in females in 2002, and it did not exist for either sex in 2003. In the 2002 males, however, lighter males

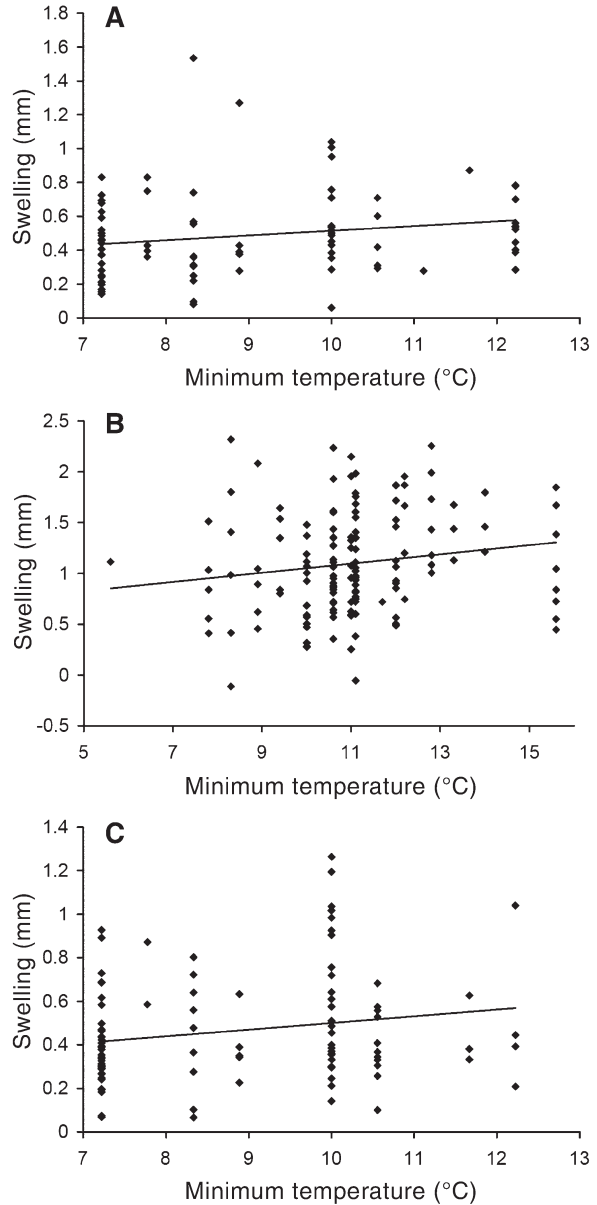


FIG. 2. PHA-induced swelling as a function of minimum temperature in nestling (A) males in 2003, (B) females in 2002, and (C) females in 2003. Significant relationships are indicated by a regression line in A and C, and the trend ($P = 0.087$) is indicated by a regression line in B. Males from 2002 are not shown, because there was an interaction of mass and temperature.

had greater immune responses at higher temperatures, whereas heavier birds showed the opposite relationship. It is possible that lighter birds are more subject to the energetic constraints of thermoregulation (Svensson et al. 1998), but this does not

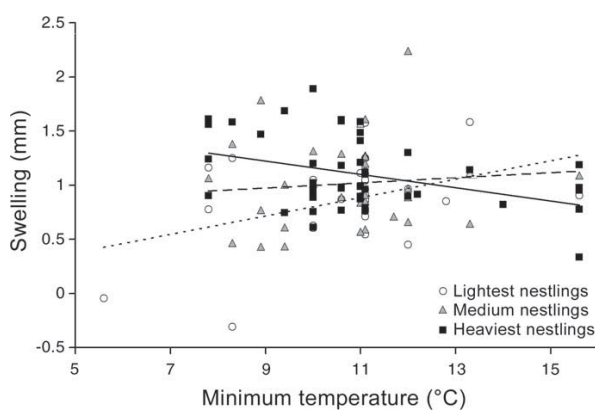


FIG. 3. PHA-induced swelling as a function of minimum temperature in male nestlings in 2002. Because of the interaction of minimum temperature and mass, we present regression lines for the lightest nestlings ($F = 4.94$, $df = 1$ and 25 , slope = 0.09 , $P = 0.035$; open circles, dotted line), medium nestlings ($F = 0.28$, $df = 1$ and 31 , slope = 0.02 , $P = 0.60$; gray triangles, dashed line), and heaviest nestlings ($F = 7.58$, $df = 1$ and 44 , slope = -0.06 , $P = 0.009$; black squares, solid line).

explain why heavier birds showed a negative relationship or why this interaction was found only in 2002 males. It is possible that the heavier males in 2002 invested more resources into growth than in immune defenses, a tradeoff shown in some studies (Brommer 2004, Soler et al. 2007) that is not always consistent across years (Garvin et al. 2006). Although food availability may mediate mass-dependent effects on immune response (Brzęk and Konarzewski 2007), and temperature can greatly influence food availability in insectivorous species (Liffield et al. 2002), this is an incomplete explanation for the patterns observed here. Future studies could further examine this interaction between ambient temperature and body mass on PHA-induced immunity in a more controlled environment where manipulation of specific variables, such as nest temperature and food availability, is possible.

We found that both mass and minimum temperature are important factors in assessing a PHA-induced immune response. Individual immune responses to PHA can be affected by conditions immediately prior to testing (Ewenson et al. 2003), which indicates that the response is somewhat plastic. Some researchers do not use temperature or mass as covariates in their statistical models, even though there is evidence that these factors can contribute significantly to variation (Liffield et al. 2002, Ardia 2005, Martínez-Padilla 2006), whether directly or indirectly. Therefore, we urge other researchers to investigate these covariates and other relevant variables such as food availability and parasite prevalence when measuring PHA responses, and also not to assume that these responses predict nestling recruitment.

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