TECHNICAL COMMENT

Sex-Specific Effects of Handling Time on an Index of Immune Activity in Zebra Finches

Lisha L. Berzins
Elinor Tilman-Schindel
Gary Burness*
Department of Biology, Trent University, Peterborough, Ontario K9J 7B8, Canada

Accepted 10/5/2007; Electronically Published 2/15/2008

ABSTRACT

Recently, there has been considerable interest in the role of the immune system in shaping life-history evolution, sexual selection strategies, and indexes of individual quality. The most frequently used assay of immune function, particularly in avian field studies, is the phytohemagglunitin (PHA) skin test. PHA is injected subcutaneously into the wing web, and the magnitude of the resultant swelling has traditionally been interpreted as an index of an individual's cell-mediated immunocompetence. The test follows one of two protocols: the traditional two-wing injection protocol, with one wing web injected with PHA and the other with phosphate-buffered saline (PBS), or the simplified one-wing protocol that omits the PBS injection. In this technical comment, we alert researchers to the importance of considering handling time when performing the PHA test. We show that zebra finches (*Taeniopygia guttata*) subjected to the two-wing protocol had a lower wing-web swelling than individuals injected in one wing. In males, handling time explained over 50% of the variation in an individual's skin swelling response; females were relatively unaffected by handling time. We suggest that caution should be exercised when comparing the magnitude of wing-web swelling across studies in which the alternate protocol was followed. In addition, the recording of handling time, and its inclusion in subsequent statistical analyses, may aid in the detection of subtle differences across treatments.

* Corresponding author; e-mail: garyburness@trentu.ca.

Physiological and Biochemical Zoology 81(3):383–387. 2008. 1522-2152/2008/8103-70782$15.00
DOI: 10.1086/529461

Introduction

Recently, there has been considerable interest in the role that the immune system plays in shaping the evolution of life-history traits (e.g., Tella et al. 2002), sexual selection strategies, and indexes of individual quality (see Martin et al. 2006 for a recent review of studies). The most popular assay of immune function, particularly in avian field studies, is the phytohemagglutinin (PHA) skin test (Kennedy and Nager 2006). PHA is a carbohydrate-binding protein, or lectin, isolated from red kidney beans, that when injected subcutaneously into an animal produces a localized swelling response. PHA activates T lymphocytes to undergo mitosis, and the magnitude of this resultant swelling has traditionally been considered an index of the cell-mediated immunocompetence of the injected individual (e.g., Lochmiller et al. 1993). Recent work, however, suggests that PHA also activates components of the innate immune system, with multiple immune cell types infiltrating the injection site at different times postexposure (Martin et al. 2006). As such, the complexity of the response to PHA led Martin et al. (2006) to urge caution when interpreting greater swelling as representing greater cell-mediated immunocompetence.

Despite the complexity surrounding interpretation of an individual's immunological response to PHA (e.g., Kennedy and Nager 2006; Martin et al. 2006), the PHA test is widely used in ecological immunology. In birds, the PHA skin test has been conducted using either a two-wing protocol (e.g., Lochmiller et al. 1993) or a simplified one-wing protocol (Smits et al. 1999). In the two-wing protocol, one wing web is injected with PHA dissolved in phosphate-buffered saline (PBS), and the other wing web is injected with PBS, to act as a control. The injection sites are measured preinjection and 24 h postinjection, and the immune response to PHA is indexed as the difference in swelling between the two wings (Lochmiller et al. 1993). Smits et al. (1999) demonstrated that within an individual, the injection of PBS as the vehicle induced no measurable wing-web swelling response, and therefore contributed little/if anything to the overall PHA skin test. As such, they advocated omitting the "control" injection of PBS and proposed injecting a single wing only. The one-wing protocol thus estimates the immune response as the difference between the pre- and post-injections to PHA only (Smits et al. 1999). Although the one-wing technique has not been without criticisms (Siva-Jothy and Ryder 2001), it has gained much popularity in studies of ecological immunity.

Part of the appeal of the one-wing protocol is that it decreases handling time by approximately half and reduces the physiological stress experienced by the bird (Smits et al. 1999). When experiencing stress, birds release the glucocorticoid corticosterone (CORT), which when elevated chronically has been shown in some species to blunt the PHA response (Martin et al. 2005). Although there are no data of which we are aware on the effect of acute elevations of CORT on the PHA response in birds, acute handling stress has been shown to reduce indexes of innate immunity (Matson et al. 2006). If handling stress impacts the PHA response, then the decreased handling time associated with the one-wing protocol may result in a greater swelling response (interpreted typically as greater "immunocompetence") than if the same individual had been subjected to a two-wing protocol. If this were the case, then immunological comparisons among studies, or between treatments within the same study in which the alternate protocol had been used (e.g., Moreno et al. 2005), may become challenging. In this technical comment we suggest this has gone largely unrecognized in studies of ecological immunology.

We used captive zebra finches (*Taeniopygia guttata*) as a model to test two questions: (1) what is the relationship between handling time and the magnitude of an individual's PHA-induced swelling response, and (2) does the swelling response differ among individuals subjected to a one-wing protocol versus a two-wing protocol?

Material and Methods

Study Species

Adult male ($n = 23$) and female ($n = 24$) zebra finches were obtained from a local breeder and group-housed by sex at the Trent University Animal Care Facility for a minimum of 2 mo before beginning experiments. Individuals were randomly assigned to one of two treatment groups: the one-wing or the two-wing protocol. These birds were then moved to individual cages (35 cm [L] × 41 cm [W] × 36 cm [H]) for 24 h before the experimental protocol was performed. By moving birds to individual cages, our aim was to minimize capture time, which has been shown previously to influence the PHA swelling response (Ewenson et al. 2003). All birds were held at ca. 25°C with a 14L : 10D cycle. Commercial birdseed (Topcrop finch/budgie seed in 50 : 50 mix) and water were supplied ad lib.

Immune Challenge

The immune response was elicited by subcutaneous injection of crystalline phytohemagglutinin (PHA-P, Sigma L8754) dissolved in sterile 0.01 M PBS (pH 7.4). Birds were injected in the wing web with either a 20-μL PHA solution (0.1 mg in 20 μL PBS) or the equivalent volume of PBS. Dosages of PHA injected into the wing web vary widely in the literature. The dose we used was based on Martin et al. (2006), although we reduced the volume injected. Finches assigned to the two-wing injection protocol were injected with PHA in the right wing web followed by injection of PBS into the left wing web, and those assigned to a one-wing injection protocol were injected with PHA in the right wing web only. For each individual, the handling time was recorded, beginning when the cage door was opened and ending once the bird had been placed back into the cage. Although we recognize that this measure of handling time includes the capture of the bird in its cage, the duration of this capture/chase was less than 25 s and was minimal compared with the overall time the bird was held in the hand.

Before injection, the thickness of the injection site was measured six times to the nearest 0.001 mm using a digital pressure-sensitive micrometer (Mitutoyo 227-221). The site was then measured again 24 h postinjection. Individuals were weighed (± 1 g) upon recapture using a Pesola spring scale. The same individual performed all injections and measurements and held the birds (L.L.B.).

The PHA swelling response for the two-wing treatment was calculated as $(PHA_{postinject} - PHA_{preinject}) - (PBS_{postinject} - PBS_{preinject})$, following Lochmiller et al. (1993). The swelling for the one-wing treatment was calculated as the thickness of the wing web pre- and postinjection of PHA, $PHA_{postinject} - PHA_{preinject}$, following Smits et al. (1999). The repeatability (Lessels and Boag 1987) of the six preinjection measurements was 0.90 ($F_{46,235} = 53.95$, $P < 0.0001$) and of the six postinjection measurements on the PHA wing was 0.95 ($F_{46,235} = 107.49$, $P < 0.0001$).

Statistical Analyses

Data for PHA response, handling time, body mass, and tarsus length were $\log_{10}$ transformed to improve normality and homogeneity of variance. We calculated body condition as residuals from $\log_{10}$ body mass on $\log_{10}$ tarsus length. We first used a two-way ANOVA to test for an effect of protocol (one- or two-wing injection) and sex on wing-web swelling in response to PHA injection. To explore further the mechanistic basis for variation in wing-web swelling, we subsequently performed an ANCOVA with sex, protocol, body condition, and handling time as independent variables. In each analysis, the model initially contained all two-way interactions between sex and the other variables; nonsignificant interaction terms ($P > 0.05$) were subsequently removed. For clarity, figures are presented using untransformed data. All values are presented as mean ± 1 SE, and all analyses used JMP IN (ver. 5.0) statistical software (Sall et al. 2005). Comparisons were considered statistically significant at $P \leq 0.05$.

Results

Individuals injected with PHA using the one-wing protocol had a significantly greater wing-web swelling than those injected using the two-wing protocol (ANOVA: $F_{1,44} = 4.05$, $P = 0.05$; Fig. 1). Sex had no effect on the wing-web swelling response (ANOVA: $F_{1,44} = 1.67$, $P = 0.20$). The average handling time (± 1 SE) for individuals injected in one wing was 200.0 ± 11.0 s, while the handling time for those injected in both wings

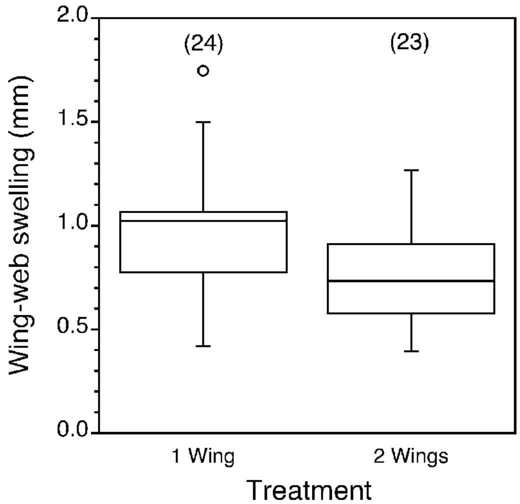

Figure 1. Wing-web swelling (mm) in zebra finches injected in either one wing with PHA or in two wings (PHA in right wing and saline in left wing). Box plot shows horizontal lines at fifth, twenty-fifth, fiftieth (median), seventy-fifth, and ninety-fifth percentiles. Points falling outside this range are indicated with a circle. Sexes are pooled. Sample sizes are in parentheses. $P = 0.05$.

was more than double at 430.0 ± 22.1 s (t-test: $t_{45} = 10.25$, $P < 0.001$). When handling time was included in the analysis, protocol (one- or two-wing injection) no longer affected wing-web swelling (ANCOVA: $P = 0.66$). Interestingly, the impact of handling time on wing-web swelling differed between males and females (ANCOVA, handling time × sex: $F_{1,41} = 4.18$, $P = 0.047$). Handling time was negatively related to the wing-web swelling in males (ANCOVA, male: $F_{1,20} = 14.20$, $P = 0.001$; Fig. 2*A*), while females showed no relationship ($P = 0.20$; Fig. 2*B*). Strikingly, handling time alone explained >50% of the variation in a male's wing-web swelling ($r^2 = 0.53$, $F_{1,21} = 24.08$, $P < 0.001$; Fig. 2*A*). When handling time, protocol, and sex were controlled for, body condition was also related to wing-web swelling, with individuals in better condition showing greater swelling responses (ANCOVA, $F_{1,41} = 6.23$, $P = 0.017$).

Discussion

Zebra finches experiencing the two-wing PHA protocol had less wing-web swelling than did individuals experiencing the one-wing PHA protocol (Fig. 1). We suggest it was not the actual protocol per se that resulted in the blunted response in the two-wing birds but rather the length of time each individual was held. The importance of handling time as a factor influencing an individual's PHA response has, to our knowledge, not been reported previously. Interestingly, males and females responded differently with respect to handling time. While handling had a negative impact on a male's wing-web swelling, females showed no clear effect.

Previous work has shown that chasing birds before capture blunts the swelling response to PHA (Ewenson et al. 2003). Our results suggest that restraint during injection of PHA likely poses an additional stressor, particularly in males. Although our measure of handling time included a small chase component (the time to capture an individual in its cage), this was a relatively small fraction of the total time the bird was held. It would be interesting in future studies to record and analyze these two stressors separately to further isolate sources of variation in the PHA response.

Detecting an effect of handling time on the PHA response is perhaps not surprising given that restraint is used to elicit an elevation of the stress hormone CORT in a variety of avian species (e.g., Wingfield et al. 1992), including zebra finches (Salvante and Williams 2003). Chronic CORT elevation most typically depresses indexes of immunity. For example, artificial CORT elevation results in a blunted PHA swelling response in some populations of house sparrows (*Passer domesticus*; Martin

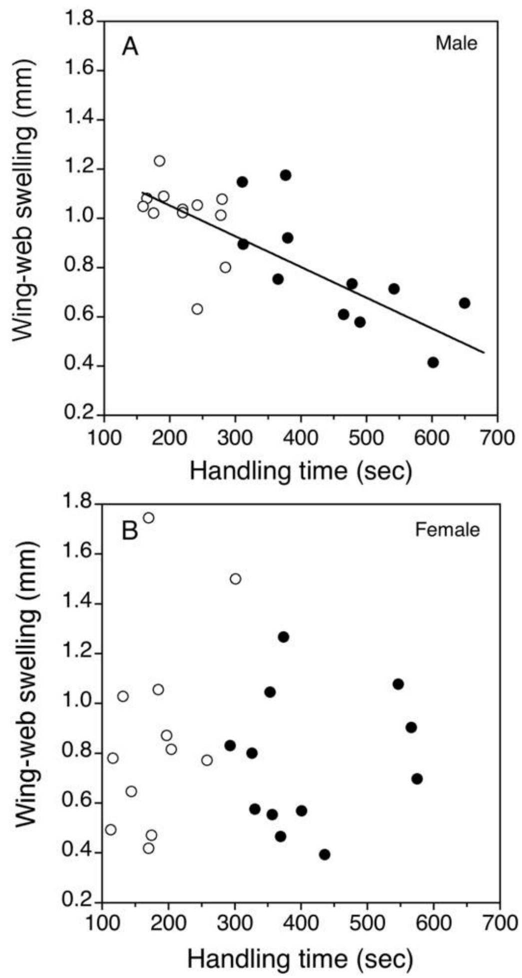

Figure 2. Influence of handling time on the wing-web swelling of (*A*) male and (*B*) female zebra finches following injection of phytohemagglutinin (PHA). *Unfilled circles*, birds injected in one wing with PHA; *filled circles*, birds injected in one wing with PHA and one wing with saline. Equation of regression line in *A*: wing-web swelling (mm) = 1.29 − 0.0012 × handling time (s), $r^2 = 0.57$, $F_{1,21} = 27.79$, $P < 0.0001$.

et al. 2005) and in captive chickens (*Gallus gallus domesticus*; El-Lethey et al. 2005). In the field, chronic stress, such as that faced by adults rearing artificially enlarged broods (e.g., Moreno et al. 1999) or by nestlings in an enlarged brood (Ilmonen et al. 2003), can also have suppressive effects on the PHA response.

Despite the number of studies detailing the effects of chronic stress, the impact of acute stress on the immune system has been less studied. In contrast to our results, acute stress in male mice has been shown to enhance the immunization or sensitization phase of cell-mediated immunity when compared with mice that had not been stressed (Viswanathan et al. 2005). However, these results were obtained using a 2.5-h restraint stress, a duration that far exceeded the handling time faced by the zebra finches in our study. Whether the blunted response we detected in zebra finches is taxon specific or is perhaps a transient phenomenon that after a longer period of restraint may result in enhancement is unknown but would be worth exploring in future studies.

In contrast to our results that showed a blunted PHA response in birds subjected to the two-wing protocol (Fig. 1), previous studies have generally reported an insignificant effect of the type of wing-web treatment on PHA response (e.g., Smits et al. 1999; Moreno et al. 2005). In these previous studies, the authors calculated the swelling response of individuals injected in two wings using the two-wing calculation (Lochmiller et al. 1993) and then recalculated the swelling response in those same individuals using the one-wing calculation (whereby the thickness of the PHA response either included or excluded the swelling in the control wing due to PBS injection). The PHA swelling response was highly associated between the injection protocols, supporting the exclusion of a control injection (Smits et al. 1999). The effect of differences in handling time between the two treatments and the potential impact of such a difference on an individual's PHA response was not considered.

In our study, only males showed a negative effect of handling time on the PHA response; females did not. Interestingly, females were also more variable in their PHA response than were males (Levene's test on residuals from regression of wing-web swelling on handling time: $F_{1,45} = 6.26$, $P = 0.016$). The mechanistic basis for these differences is not clear, although sex-specific differences in immune function have been reported previously in other species. For example, artificially increasing brood size resulted in a more blunted wing-web swelling response in male than in female blue tit nestlings (*Parus caeruleus*; Dubiec et al. 2006). Similarly, only male European starling nestlings (*Sturnus vulgaris*) are negatively affected by increasing natural brood size, particularly in years of low food availability (Chin et al. 2005). As far as we are aware, ours is the first study to suggest that an acute stressor may also impact the PHA response in a sex-specific manner.

How much does handling time blunt an individual zebra finch's wing-web swelling? We calculated that for males subjected to the two-wing injection protocol (regression using untransformed data: wing web [mm] $= 1.48 - 0.0016 \times$ handling time [s]; $r^2 = 0.57$, $F_{1,9} = 11.96$, $P = 0.007$), individuals held for 300 s (5 min) had an average swelling response of 1.00 mm, while those held for 600 s (10 min) had a swelling of only 0.52 mm. This difference of 0.48 mm is similar to or exceeds that of significant treatment effects reported previously for this species (e.g., Blount et al. 2003; McGraw and Ardia 2003). Given the magnitude of the response, handling time may play a contributing role in studies that have failed to reject null hypotheses, as suggested previously by Ewenson et al. (2003). Nevertheless, in comparative studies others have reported significant within-species repeatability despite differences in dose of PHA, protocol, sex, and age of individuals (Tella et al. 2002).

In conclusion, zebra finches experiencing a two-wing PHA injection protocol had a lower swelling response than individuals experiencing the one-wing protocol, due largely to differences in handling time. Whether these patterns can be extended to other species or to field studies is not known. However, until handling time can be discounted as a contributing factor to the PHA wing-web swelling, we suggest that researchers record the length of time an individual is held and consider including this value in their subsequent statistical analyses. In addition, our results suggest that unless handling time is reported, caution may be warranted when comparing the degree of wing-web swelling across data sets in which different PHA injection protocols were followed.

Acknowledgments

We thank the Trent University Animal Care staff for help with animal husbandry. C. Brunetti and J. Tella made useful comments on the pilot study that prompted us to pursue this current one. We also thank two anonymous reviewers for many useful comments on an earlier version of this article. Funding was provided by a Natural Sciences and Engineering Research Council Discovery Grant to G.B. and by the Canadian Foundation for Innovation. E.T.-S. was supported by an Ontario Graduate Scholarship in Science and Technology. All animals were cared for in accordance with the Canadian Council on Animal Care, and all research was approved by the Trent University Animal Care Committee.

Literature Cited

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

Chin E.H., O.P. Love, A.M. Clark, and T.D. Williams. 2005. Brood size and environmental conditions sex-specifically affect nestling immune response in the European starling *Sturnus vulgaris*. J Avian Biol 36:549–554.

Dubiec A., M. Cichon, and K. Deptuch. 2006. Sex-specific development of cell-mediated immunity under experimentally altered rearing conditions in blue tit nestlings. Proc R Soc B 273:1759–1764.

El-Lethey H., B. Huber-Eicher, and T.W. Jungi. 2005. Exploration of stress-induced immunosuppression in chickens reveals both stress-resistant and stress-susceptible antigen responses. Vet Immunol Immunopathol 95:91–101.

Ewenson E., R. Zann, and G. Flannery. 2003. PHA immune response assay in captive zebra finches is modulated by activity prior to testing. Anim Behav 66:797–800.

Ilmonen P., D. Hasselquist, Å. Langefors, and J. Wiehn. 2003. Stress, immunocompetence and leukocyte profiles of pied flycatchers in relation to brood size manipulation. Oecologia 136:148–154.

Kennedy M.W. and R.G. Nager. 2006. The perils and prospects of using phytohaemagglutinin in evolutionary ecology. Trends Ecol Evol 21:653–655.

Lessels C.M. and P.T. Boag. 1987. Unrepeatable repeatabilities: a common mistake. Auk 104:116–121.

Lochmiller R.L., M.R. Vestey, and J.C. Boren. 1993. Relationship between protein nutritional status and immunocompetence in northern bobwhite chicks. Auk 110:503–510.

Martin L.B., II, J. Gilliam, P. Han, K.A. Lee, and M. Wikelski. 2005. Corticosterone suppresses immune function in temperate but not tropical house sparrows (*Passer domesticus*). Gen Comp Endocrinol 140:126–135.

Martin L.B., II, P. Han, J. Lewittes, J.R. Kuhlman, K.C. Klasing, and M. Wikelski. 2006. Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. Funct Ecol 20:290–299.

Matson K.D., B.I. Tieleman, and K.C. Klasing. 2006. Capture stress and the bactericidal competence of blood and plasma in five species of tropical birds. Physiol Biochem Zool 79: 556–564.

McGraw K.J. and D.R. Ardia. 2003. Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. Am Nat 162:704–712.

Moreno J., S. Merino, J. J. Sanz, E. Arriero, J. Morales, and G. Tomás. 2005. Nestling cell-mediated immune response, body mass and hatching date as predictors of local recruitment in the pied flycatcher *Ficedula hypoleuca.* J Avian Biol 36:251–260.

Moreno J., J.J. Sanz, and E. Arriero. 1999. Reproductive effort and T-lymphocyte cell-mediated immunocompetence in the female pied flycatchers *Ficedula hypoleuca.* Proc R Soc B 266: 1105–1109.

Sall J., L. Creighton, and A. Lehman. 2005. JMP Start Statistics. 3rd ed. Thomson Brooks/Cole, Pacific Grove, CA.

Salvante K.G. and T.D. Williams. 2003. Effects of corticosterone on the proportion of breeding females, reproductive output and yolk precursor levels. Gen Comp Endocrinol 130:205–214.

Siva-Jothy M.T. and J.J. Ryder. 2001. Assaying PHA-induced mitosis: out of control? Funct Ecol 15:812–817.

Smits J.E., G.R. Bortolotti, and J.L. Tella. 1999. Simplifying the phytohemagglutinin skin-testing technique in studies of avian immunocompetence. Funct Ecol 13:567–572.

Tella J.L., A. Scheuerlein, and R.E. Ricklefs. 2002. Is cell-mediated immunity related to the evolution of life-history strategies in birds? Proc R Soc B 269:1059–1066.

Viswanathan K., C. Daugherty, and F.S. Dhabar. 2005. Stress as an endogenous adjuvant: augmentation of the immunization phase of cell mediated immunity. Int Immunol 17: 1059–1069.

Wingfield J.C., C.M. Vleck, and M.C. Moore. 1992. Seasonal changes of the adrenocortical response to stress in birds of the Sonoran Desert. J Exp Zool 264:419–428.