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Communications

Phytohaemagglutinin injection has a long-lasting effect on immune cells

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Measurement of phytohaemagglutinin (PHA)-induced skin swelling is the most popular assay of immune function in avian studies. The mechanisms causing swelling have been relatively well studied; however, very little is known about the potential long term physiological effects of PHA. Here we show that injection of PHA into patagium of captive greenfinches *Carduelis chloris* increases the concentration of heterophils (phagocytic cells of the innate immune response) in the peripheral blood for at least 30 days. Such long-term consequences should be taken into account when using PHA skin test in studies monitoring changes in individual physiological condition and/or immune status.

Immunoecology deals with trade-offs between immune function and other vital functions. Understanding why and how these trade-offs emerge is crucial for explaining the evolution of parasite virulence, resistance and tolerance, and mechanisms responsible for life-history trade-offs and honesty of signal traits. To study these issues, ecologists need to assess immune function of organisms. The most popular method to assess immune function in avian studies is the phytohaemagglutinin (PHA) skin test (Smits et al. 1999, Kennedy and Nager 2006, Martin et al. 2006).

PHA is a plant lectin (carbohydrate-binding protein), which functions in plants as antimicrobial and anti-herbivore toxin. Subcutaneous injection of PHA induces T-cell mitogenesis and produces a localized swelling response involving local infiltration of tissue by most types of immune cells. The magnitude of this swelling can reflect both acquired T-cell-mediated immunocompetence (Tella et al. 2008) and non-specific basophile-mediated inflammation (Martin et al. 2006).

PHA assay has also been applied for assessment of the physiological costs of mounting an immune response. This approach stems from the life-history theory assuming that immune defence can only be maximised at the expense of some other important physiological function. PHA injections elevate metabolic rate in some species (Martin et al. 2003, Nilsson et al. 2007), but decrease it in others (Lee et al. 2005). PHA injection can change the levels of reactive oxygen metabolites, antioxidant capacity, carotenoids and nitric oxide metabolites in plasma (Costantini and Dell'Omo 2006, Hõrak et al. 2007, Perez-Rodriguez et al. 2008, Sild and Hõrak 2009).

Most studies have assessed relatively short-term effects (24 to 72 h) of PHA injection. Two studies on birds, however, hint that the inflammatory response elicited by PHA may have a long-lasting (7–14 d) effect on individual immune status (Adler et al. 2001, Hõrak et al. 2000). These findings, together with evidence from mammalian studies about systemic effects of dietary lectins (Vasconcelos and Oliveira 2004) suggest that PHA injection may induce a long-lasting physiological effect. Here, we test for this possibility in a study of captive greenfinches by measuring leukocyte concentrations in blood, 30 days after PHA injection.

Methods

We captured male greenfinches in mist-nets in the Sõrve Bird Observatory on the island Saaremaa (57° 55′N, 22° 03′E) on 25–27 Jan. 2007. Birds were transported to Tartu and housed indoors in individual cages (27 × 51 × 55 cm). In the aviary, temperature and humidity averaged 15.9 ± 1.7 (SD) °C and 53.2 ± 2.6%, respectively. The birds experienced natural day length (artificial lighting) during our experiment, and were supplied with sunflower seeds and water *ad libitum*.

Blood samples were collected on 21 and 27 Feb. and 26 Mar. in the morning before the lights turned on. In the evening of 24 Feb. (on the 4th d after 1st blood sampling and three d before second blood sampling), six birds were injected intradermally in the wing web with 0.2 mg of PHA (Sigma, St. Louis, MO., L-8754) in 0.04 ml of sterile isotonic saline. At the same time, seven birds were injected with saline. For counting leukocytes, a drop of blood was smeared on microscope slide, air-dried, fixed in absolute methanol, and stained with azure-eosin (Romanowsky stain). We estimated the proportion of different types of leukocytes by examining 100 leukocytes

under 1000 × magnification under oil immersion. Estimates of the total white blood cell count (WBC) were obtained by counting the number of leukocytes per approximately 10,000 erythrocytes. Differential leukocyte counts were obtained by multiplying their proportions with WBC. Repeatabilities (Lessells and Boag 1987) of leukocyte counts, obtained from a larger sample of smears counted twice were: WBC; $r = 0.82$, $F = 10.0$, $df = 19,20$, $P < 0.001$; heterophil concentration: $r = 0.79$, $F = 8.4$, $df = 19,20$, $P < 0.001$; lymphocyte concentration: $r = 0.82$, $F = 10.1$, $df = 19,20$, $P < 0.001$; H/L ratio: $r = 0.79$, $F = 8.5$, $df = 19,20$, $P < 0.001$. For all calculations, leukocyte count values were ln-transformed. Effects of treatments on leukocyte concentrations were analysed by repeated measures ANOVA's.

Results

Three days after injections, heterophil concentrations and H/L ratios increased among both groups of greenfinches, but more so among PHA-injected birds (Fig. 1 A, D). Thirty days after injections, heterophil counts of PHA-injected birds were still 29% higher than those of saline-injected controls (7.4 ± 4.8 (SD) vs. 2.1 ± 4.8 (SD) heterophils per 10^4 erythrocytes). We found a 37% difference in H/L ratios (0.34 ± 0.09 (SD) vs. 0.13 ± 0.08 (SD)). Post-injection values of total leukocyte count or lymphocyte hemoconcentration did not differ between treatment groups (Fig. 1 B, C).

Discussion

PHA injection caused a long-lasting increase in heterophil concentration in greenfinches. This effect was evidently also responsible for increased H/L ratios, since lymphocyte concentrations were not affected by the treatment. Our results are thus consistent with two previous studies (Hõrak et al. 2000, Adler et al. 2001), showing a long-term effect of PHA injection.

How does the long-lasting effect of PHA injection on immune cells emerge? The increase in the number of circulating heterophils is generally considered as a symptom of inflammatory response (e.g., Campbell and Ellis 2007). Its main mediators are pro-inflammatory cytokines released by activated macrophages; these cytokines mediate changes in glucocorticoids, acute-phase proteins, and recruitment of monocytes and heterophils from the bone marrow (Leshchinsky and Klasing 2001). In mammals, PHA-elicited immune response induces a complex gene activation cascade where some genes are up-regulated 14 d after injection (Crabtree 1989). In case of dietary administration, the pro-inflammatory effect of PHA has been attributed also to its ability to increase the translocation of gut-derived bacteria to the periphery (Cordain et al. 2000); however, it is not known whether such mechanism might also function in case of intra-dermal injection. In addition to stimulating acute phase response and T cells, a protein that contaminates PHA also activates B cells (K. Klasing pers. comm.). It is thus also possible that germinal center formation for antibody maturation

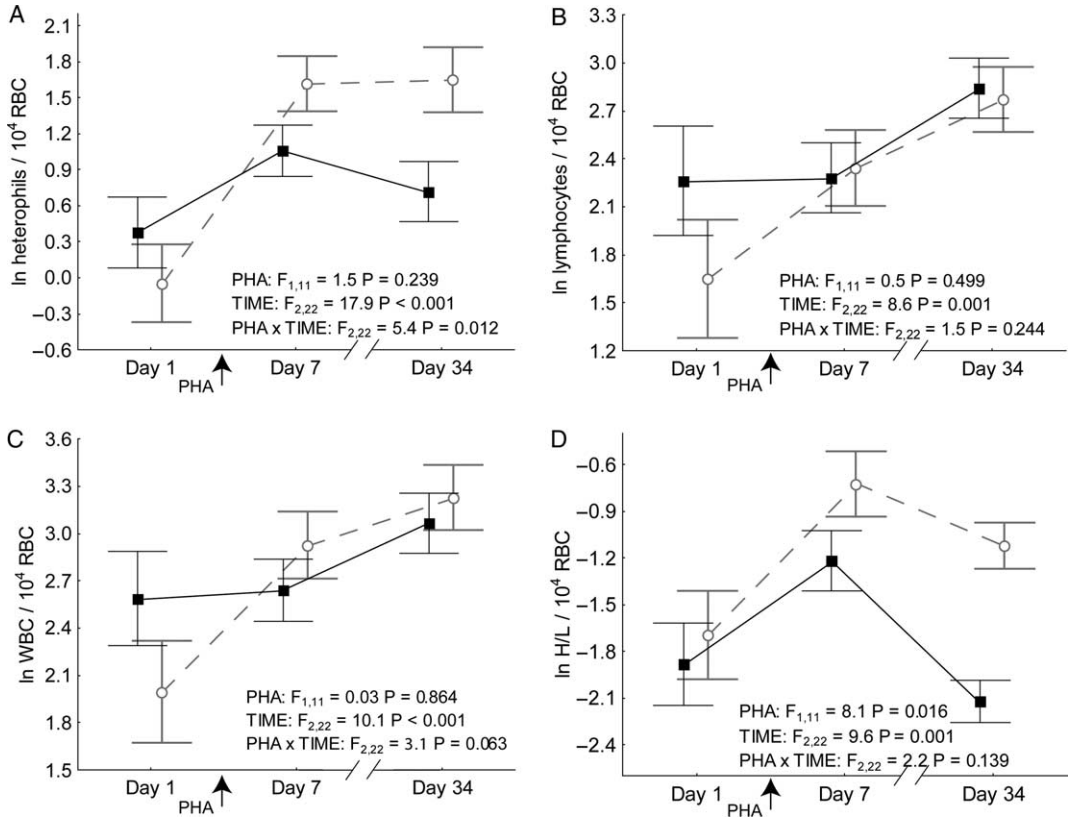

Figure 1. Effects of PHA injection on dynamics of leukocyte counts and differentials in greenfinches. Open symbols – PHA injection ($n = 6$), filled symbols – saline injection ($n = 7$). Arrow indicates time of injection. Statistics from repeated measures ANOVA.

triggered the heterophilia. This would be consistent with a study of mice (Hoshi et al. 1984), showing that as long as 21 d after PHA injection into footpad, lymph follicles contained prominent germinal centres.

What consequences do elevated heterophil concentrations have for birds? Heterophils play an important role in innate immune response. In poultry, these phagocytes are the first cells to migrate to the site of infection where they engage in phagocytosis and killing of pathogens by producing toxic reactive oxygen species and releasing bactericidal substances and proteolytic enzymes in the process of oxidative burst and degranulation (He et al. 2008). Under certain conditions, such responses can also damage host tissues, even more than infectious agents (e.g., Harmon 1998, Bojesen et al. 2004). However, marked heterophilia is not necessarily protective against infection (Campbell and Ellis 2007) and in some instances, peripheral heterophilia can co-occur with suppression of the functional activity of heterophils (Kogut et al. 1999). Therefore we cannot confirm that the observed change in leukocytic parameters implies serious long term health impact. However, it would be important to consider the possible long-term impact of PHA injections to immune system when PHA skin test is used in studies monitoring changes in individual physiological condition.

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