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Metabolic cost of inflammatory response of ruby-throated hummingbirds (*Archilochus colubris*)

L Gerardo Herrera M,[1] Nadia Bayram[2] and Kenneth C Welch Jr[2]

Abstract

Animals with a slow pace of life and high mass-specific metabolic rates are expected to invest less in innate immune responses. We measured skin inflammation and the resting metabolic rate (RMR) of ruby-throated hummingbirds (*Archilochus colubris*) after their immune system was challenged with phytohemagglutinin (PHA) and compared with the response of birds injected a saline solution. The PHA test measures the inflammatory process, a component of the innate response. Ruby-throated hummingbirds belong to a group that is under-represented in avian immunological studies characterized by a slow pace of life and fast metabolic rate. Hummingbirds developed an inflammatory response that lasted <28 h. PHA injection produced a significant increment of RMR (up to ~13%) with respect to RMR values after the injection of the saline solution indicating that immune response involved a metabolic cost for hummingbirds. This increment lies within the range of values previously reported for birds injected PHA (5%–29%).

Keywords

Hummingbirds, inflammatory response, immune system, metabolic rate, phytohemagglutinin

Introduction

Investment in immunological functions during the lifetime of organisms has long being examined within the framework of the pace of life theory.[1] Accordingly, animals with a fast pace of life (high reproductive rate, short developmental time and short adult life spans) should invest more in innate immune response whereas investment in adaptive response should be favored in animals with a slow pace of life. An extension to this theoretical framework incorporates metabolism, predicting that the energetic investment in adaptive immunity should be favored over innate immunity in animals with a slow pace of life and a high mass-specific metabolic rate.[2]

Hummingbirds are characterized by extreme lifespan for their mass and the highest mass-specific metabolic rates of all living birds.[3,4] In spite of their unique natural history, the immune system of hummingbirds has been very little studied.[5–7] We measured skin inflammation and the energetic response of ruby-throated hummingbirds (*Archilochus colubris*) after their immune system was challenged with phytohemagglutinin (PHA). Ruby-throated hummingbirds are small birds (~2–5 g) that can live up to 9 years (https://genomics.senescence.info/species).

PHA injection is widely used to study avian immunoecology and recent work shows that this test measures the inflammatory process, a component of the innate immune response.[8–10] Energetic response of birds to a PHA challenge has been tested in a handful of species representing several orders, feeding habits, body sizes and life spans.[11–16] Our study explores this response in a member of a group that is under-represented in avian immunological studies characterized by a slow pace of life and fast metabolic rate.[2]

Methods

Animal capture and husbandry

Experiments were conducted on male ruby-throated hummingbirds captured on the campus of the University of Toronto Scarborough with approval of the Laboratory Animal Care Committee. Birds were captured in July 2014 and housed individually in metal cages (91.5 cm W × 53.7 cm H × 50.8 cm D; Corners Limited) and offered a diet of 18% (w/v) Nektar Plus solution (Guenter Enderle). The hummingbirds' daylight schedule approximated the light-dark cycle they would naturally encounter. Accordingly,

[1]Estación de Biología Chamela, Instituto de Biología, Universidad Nacional Autónoma de México, San Patricio, Jalisco, México
[2]Department of Biological Sciences, University of Toronto Scarborough, Toronto, Canada

Corresponding author:
L Gerardo Herrera Montalvo, Estación de Biología Chamela, Instituto de Biología, Universidad Nacional Autónoma de México, Circuito Exterior s/n, San Patricio, Jalisco 04510, México.
Email: gherrera@ib.unam.mx

light intensity was gradually raised (over 60 min) from 0 to 100%, or decreased from 100% to 0%, beginning at 06:30 and ending at 16:00 h, respectively.

Experimental design

Data were collected between December 2014 and January 2015, corresponding to the nonbreeding period of ruby-throated hummingbirds.[17] At 16:00, sets of three hummingbirds were placed in individual, 500-ml metabolic chambers for 4 h each day 1 week before starting data collection to acclimate them to the experimental setup. After this period, birds were randomly assigned to receive an injection of PHA ($N = 6$) or of phosphate-buffered saline (PBS; $N = 6$) and they were tested during three consecutive days. Metabolic chambers were housed inside an environmental chamber with the lights off at an ambient temperature of 24.22 ± 0.05°C (mean ± S.E. here and thereafter) during the 10 days during which birds were tested.

Immune challenge

Birds received an injection of 20 µl of a PHA solution (1 mg PHA/1 ml PBS; L8754, Sigma-Aldrich) or 20 µl of PBS at ~15:50 h on the second day of the data collection period. The PHA dose was 1.34 ± 0.10 mg PHA kg^{-1} and it is within the range of doses previously used in birds (0.49–5.88 mg PHA kg^{-1}). The injection process lasted ~5 min and was conducted by the same person (KCW) with a Hamilton precision syringe on the right wing-web placed under a stereoscope (Carl Zeiss AG). The thickness of the wing web of each bird was measured by the same person (LGHM) with a micrometer (Mitutoyo; ± 0.01 mm) under a stereoscope within ~3 min before the injection (Th_0) and ~6 (Th_6) and ~28 (Th_{28}) hours after the injection of PHA or PBS. Wing web thickness was measured with no previous knowledge of injection treatment. All birds were measured three times on each occasion, and we used the mean of these measures for statistical comparisons. We measured the intensity of inflammatory response for each individual 6 ($I_{R6} = Th_6–Th_0$) and 28 ($I_{R28} = Th_{28}–Th_0$) hours after the injection of PHA or PBS. Birds were placed in individual metabolic chambers immediately after this processing at ~16:00 h.

Respirometry

Rates of oxygen consumption were measured via open flow respirometry simultaneously in three individuals placed in separate chambers during three consecutive days. The total length of recording varied for each respirometry period: 4 h the day previous to the injection, 6 h the day of the injection, and 4 h 1 day after the injection. Ambient air flow through the metabolic chamber containing the hummingbirds and an empty reference chamber was modulated with a Flowbar-8 mass flow controller (Sable Systems International) and maintained at 400 to 500 ml/min at all times. Excurrent air from the chambers was subsampled at a steady flow rate of 200 to 250 ml/min using a RM-8 flow multiplexor (Sable Systems International). Subsampled air was passed through a water vapor pressure meter, a drying column (Indicating Drierite, W.A. Hammond Drierite), and finally an oxygen analyzer (Turbofox-5, Sable Systems International). The oxygen analyzer was regularly calibrated according to manufacturer instructions.

Respirometry data were recorded at a frequency of 1 Hz (Expedata v. 1.84, Sable Systems). Data were recorded for 3 min while sampling from the empty reference chamber, followed by two 7.5-min recordings from the chamber holding the bird, separated by a 2-min recording period from the reference chamber. Subsampling was continued from the chamber containing the bird, continuing for 19-min periods, each separated by 2-min reference chamber recordings. A final 3-min sampling of the reference chamber was started, after which the bird was removed from its chamber and returned to the vivarium. Body mass of hummingbirds was measured with an analytical balance (Ohaus; ± 0.01 g) at the beginning and end of each respirometry period.

Data analysis

Raw gas measurements were drift and lag-corrected and rate of oxygen consumption ($\dot{V}_{O_2}$ in ml/min) was calculated using equation 10.6 from[18] and assuming a respiratory exchange ratio ($RER = \dot{V}_{CO_2} / \dot{V}_{O_2}$) of 0.71.[19] $\dot{V}_{O_2}$ for each hour during each respirometry period was calculated as the lowest 5-min mean value of instantaneous oxygen consumption. Metabolic rates were expressed as ml O^2 h^{-1}.

Statistical analyses

We used *t*-tests to compare I_{R6} and I_{R28} between birds injected with PHA or PBS. We compared body mass at the beginning (B_{Mi}) and end (B_{Mf}) of each respirometry period between and within injection treatments with separate repeated measure analyses of variance (RM-ANOVA). Similarly, we compared hourly values of $\dot{V}_{O_2}$ of birds assigned to the PHA or PBS treatments with separate RM-ANOVA for each respirometry period. Time, treatment, and their interaction (treatment × time) were included as fixed factors, while individual was included as a random factor. The mean of B_{Mi} and B_{Mf} was included as a covariate. $\dot{V}_{O_2}$ decreased significantly over the 4 h of respirometry trials each day (see below). This was not unexpected, as the stress of handling and placement in the respirometry chamber likely led to elevated $\dot{V}_{O_2}$ values early in the observation period. To account for this temporal effect, we normalized each birds' post-injection $\dot{V}_{O_2}$ values for hours 1 to 4 of that day to the same individuals' same-hour pre-injection value. The proportionate $\dot{V}_{O_2}$ (PV) was calculated as follows:

$$PV = \frac{Post-injection\,hour(y)\dot{V}_{O_2}}{Pre-injection\,hour(x)\dot{V}_{O_2}}$$

where, for example, $hour(y) = 1$ or 25, and $hour(x) = -23$, relative to injection.

Table 1. Body mass (B_M), wing web thickness (W_T) and inflammatory response (I_R) of hummingbirds with respect to time of injection of phosphate-buffered saline (PBS; $N = 6$) or of phytohemagglutinin (PHA; $N = 6$). Values are mean ± S.E.

	Treatment	Time with respect to injection (hr)					
		−24	−20	0	6	24	28
BM (g)	PBS	4.63 ± 0.36	4.40 ± 0.35	4.17 ± 0.29	3.91 ± 0.31	3.93 ± 0.30	3.60 ± 0.32
	PHA	4.70 ± 0.36	4.47 ± 0.35	4.63 ± 0.29	4.30 ± 0.31	4.39 ± 0.30	3.87 ± 0.32
WT (mm)	PBS			1.43 ± 0.16	1.37 ± 0.08		1.42 ± 0.07
	PHA			1.35 ± 0.08	2.00 ± 0.05		1.56 ± 0.07
IR (mm)	PBS				0.08 ± 0.04		0.09 ± 0.03
	PHA				0.65 ± 0.12		0.24 ± 0.07

We compared hourly PV values on post-injection days 0 and 1 and examined variation in these values via RM-ANOVA. Because data were normalized by individual, ID was dropped as a random factor. As with $\dot{V}_{O_2}$, models included the same fixed effects (time, treatment, treatment × time) and mass as a covariate. All analyses were performed in R (version 3.6.2)[20] using a level of significance $p < 0.05$.

Results and discussion

Inflammatory response

Ruby-throated hummingbirds developed a significantly higher inflammatory response 6 h after being challenged with PHA than with PBS ($t_{10} = 4.34$, $p = 0.001$; Table 1), but no difference between treatments was found 28 h after injections ($t_{10} = 1.64$, $p = 0.13$; Table 1). Thickness of the wing web of hummingbirds increased to the same extent (1.4 times the pre-injection thickness) but lasted for a shorter period than in other bird species administered a similar PHA dose.[14,15] Inflammatory responses to PHA injection varies with reproductive activity in male birds, with lower values in the breeding season.[21] Because we challenged non-breeding males, our findings are not generalizable to females or males in other reproductive conditions.

Body mass

Hummingbirds lost body mass over the course of the pre-injection respirometry period (Table 1; time: $F_{1, 10} = 60.87$, $p < 0.001$), but this loss was not related to the treatment to which they were assigned (Table 1; treatment: $F_{1, 17.5} = 0.03$, $p = 0.866$, treatment × time: $F_{1, 10} = 0.29$, $p = 0.605$). We found the same pattern the day of the injection (Table 1; time: $F_{1, 10} = 40.29$, $p < 0.001$, treatment: $F_{1, 10.2} = 1.18$, $p = 0.301$, treatment × time: $F_{1, 10} = 0.62$, $p = 0.448$), and 1 day after the injection (Table 1; time: $F_{1, 10} = 11.89$, $p = 0.006$, treatment: $F_{1, 11.4} = 0.97$, $p = 0.345$, treatment × time: $F_{1, 10} = 0.63$, $p = 0.447$) of PHA and PBS. Birds had no access to food during each measurement period, which most likely explains body mass losses. However, birds lost 18% to 22% of body mass from the first to the last day of the respirometry period probably due to manipulation stress as birds were fed freely each time that they were returned to the vivarium.

Resting metabolic rate

$\dot{V}_{O_2}$ decreased significantly over the 4 h of respirometry data collection on the pre-injection day (Figure 1; time: $F_{1, 34} = 22.50$, $p < 0.001$), but this decrease was not related to the treatment (Figure 1; treatment: $F_{1, 37.7} = 0.09$, $p = 0.769$, treatment × time: $F_{1, 34} = 0.15$, $p = 0.698$; mass: $F_{1, 9} = 0.30$, $p = 0.530$). We found the same pattern during the 6 h measured the day of injection (Figure 1; time: $F_{1, 58} = 22.90$, $p = <0.0001$; treatment: $F_{1, 12.8} = 0.28$, $p = 0.60$, treatment×time: $F_{1, 58} = 0.78$, $p = 0.38$, mass: $F_{1, 9} = 9.35$, $p = 0.013$) and over the 4 h measured the following day (Figure 1; time: $F_{1, 34} = 13.60$, $p < 0.001$; treatment: $F_{1,38.1} = 0.98$, $p = 0.328$, treatment×time: $F_{1, 34} = 0.79$, $p = 0.380$, mass: $F_{1, 9} = 2.16$, $p = 0.176$).

PV was significantly elevated in PHA-injected birds on the day of injection (Figure 2; treatment: $F_{1, 43} = 6.61$, $p = 0.018$, time: $F_{1, 43} = 0.29$, $p = 0.499$; treatment×time: $F_{1, 43} = 0.18$, $p = 0.674$; mass: $F_{1, 43} = 3.10$, $p = 0.085$). The PV of birds injected with PHA increased 13.2 ± 0.04% over pre-injection day values (estimated marginal means; emmeans package). This increment lies within the range of values previously reported for birds injected PHA (5–29%).[11,13,15,16] In contrast, the RMR of saline-injected birds decreased by 2.2 ± 0.04% relative to the pre-injection day period. The day after the injection, PV values averaged slightly higher in PHA-injected birds but this difference was not significant (Figure 1; treatment: $F_{1, 43} = 3.61$, $p = 0.064$, time: $F_{1, 43} = 0.17$, $p = 0.679$; treatment × time: $F_{1, 43} = 0.11$, $p = 0.743$; mass: $F_{1, 43} = 0.07$, $p = 0.788$).

Conclusion

Investment in innate immunity should be less favored for vertebrates with relatively high mass-specific metabolic rates and a slow pace of life.[1,2] Nevertheless, energetic investment in the immune response triggered by PHA appears to be independent of metabolic rate and life history as indicated by our findings with hummingbirds and those with other species of birds, mammals, and reptiles.[14–16,22,23] Comparing the responses to PHA injection of birds with different life histories might be limited by the use of different mass-specific doses of the antigen. However, the strength of the energetic response of birds to PHA injection appears to be dose-independent as demonstrated in controlled experiments, and by previous studies using a variety

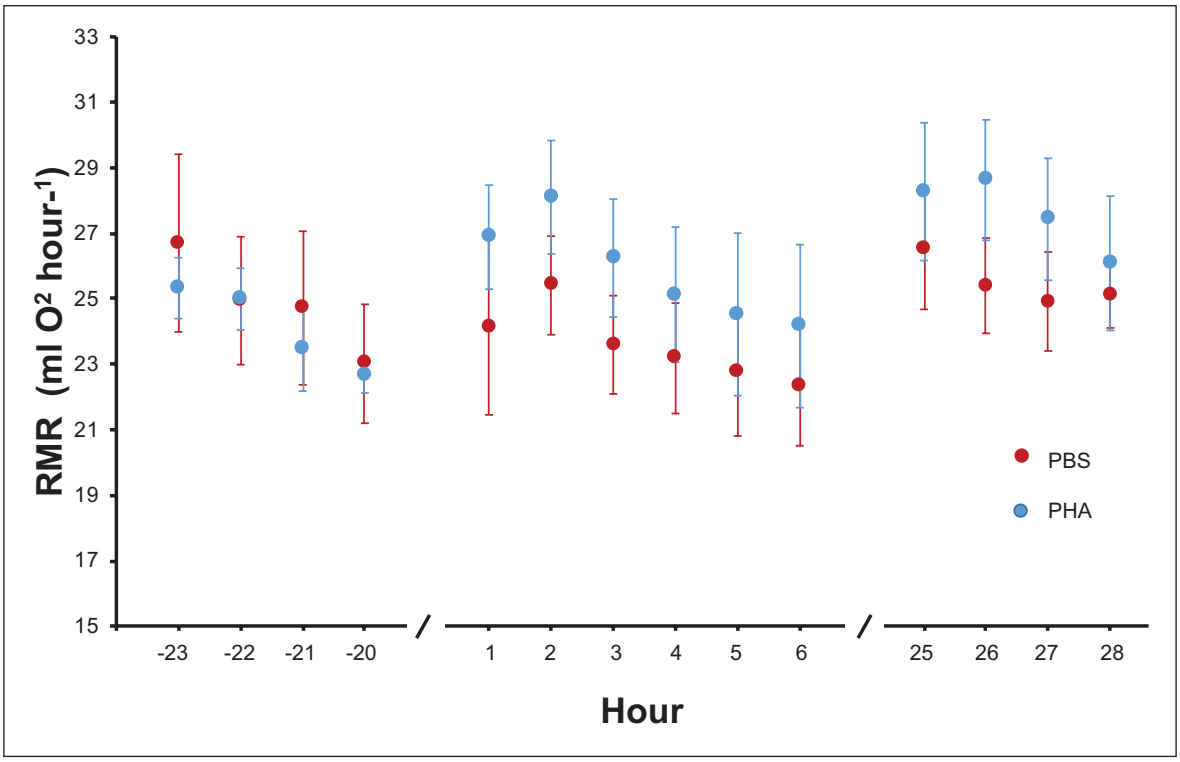

Figure 1. Resting metabolic rate (RMR; mean ± S.E.) of ruby-throated hummingbirds (*Archilochus colubris*) measured with respect to time of injection (hour 0) of phytohemagglutinin (PHA; $N = 6$) or of phosphate-buffered saline (PBS; $N = 6$). The total period of recording was 4 h the day prior to injection, 6 h the day of injection, and 4 h 1 day after injection. Individual rates of oxygen consumption were calculated as the lowest 5-min mean value of oxygen consumption during each hour for each respirometry period, and then expressed as ml O^2 h^{-1}.

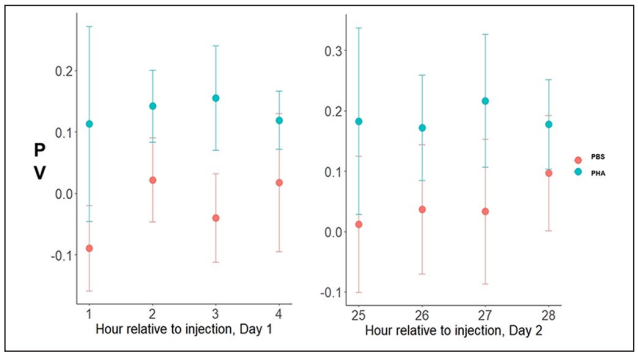

Figure 2. Proportionate $\dot{V}_{O_2}$ (PV; post-injection $\dot{V}_{O_2}$ values divided by time-matched pre-injection values; mean ± S.E.) of ruby-throated hummingbirds (*Archilochus colubris*) measured with respect to time of injection (hour 0) of phytohemagglutinin (PHA; $N = 6$) or of phosphate-buffered saline (PBS; $N = 6$).

of mass-specific doses that produced no increment of RMR (0.49–3.70 mg kg^{-1}) or that increased RMR significantly (1.17–5.88 mg kg^{-1}).[11–16]

Declaration of conflicting interests

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ORCID iD

L Gerardo Herrera M https://orcid.org/0000-0001-9348-5040

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