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Immune-related effects from predation risk in Neotropical blue-black grassquits (*Volatinia jacarina*)

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ABSTRACT

Predation is a major force shaping natural history traits of birds because of their vulnerability during nesting and higher visibility during diurnal activities. For most birds in the Neotropics, predation is the major cause of nest failure due to the region's high diversity and abundance of predators. The blue-black grassquit (*Volatinia jacarina*), similarly to other small passerines in the savanna region of central Brazil, suffers extremely high rates of nest predation. Additionally, males may be particularly vulnerable to predators since they are very conspicuous when executing courtship displays. We assessed some of the non-lethal costs of predation risk on this species by comparing physiological and morphological parameters of birds exposed to predator vocalizations with that of control subjects exposed to non-predator vocalizations. Birds exposed to the predator vocalizations exhibited an immune-related reaction (changes in their *H*/*L* ratio), but no changes were observed in other biological parameters measured.

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1. Introduction

Functionally defined, predation involves the death of the prey during consumption, thus directly reducing prey populations. However, predators also produce non-lethal effects that induce changes in prey morphology, physiology and behavior (Werner and Peacor, 2003). These effects may negatively influence individual fitness due to decreased foraging, growth and breeding rates (Cresswell, 2008). In many cases, non-lethal predator effects are as critical to prey population demographics as death through consumption (Preisser et al., 2005).

The risk of predation is likely a strong selective pressure, although we know little about how organisms respond physiologically to such risk. Recent studies indicate that predation risk may induce adaptive physiological responses that may entail negative effects for the individual, including oxidative damage (McGraw et al., 2010; Janssens and Stoks, 2013). It is thus important to determine which circumstances involving the risk of predation may result in physiological responses (Wingfield et al., 1998). Similarly to other stressors, the risk of predation could increase production of glucocorticoids, resulting in a state of physiological and behavioral alertness (Johnson et al., 1992) that improves chances of immediate survival but involves a tradeoff leading to lower reproductive capacity and higher mortality (Wingfield and Sapolsky, 2003). Several studies have assessed different vertebrate taxa to determine possible physiological adaptations to predators or their cues. In Belding's ground squirrels (*Spermophilus beldingi*), for instance, populations living in habitats that differ in predation pressure not only exhibit specific behavioral adaptations but also have different cortisol profiles (Mateo, 2007). Cortisol metabolites are also regulated during development of the squirrels, peaking when young animals are learning anti-predator and survival strategies (Mateo, 2006). In an experimental study using male tree lizards (*Urosaurus ornatus*), the exogenous elevation of corticosterone enhanced antipredator behaviors (Thaker et al., 2009).

In studies with birds, physiological response to environmental stressors is often measured through corticosteroid levels in the bloodstream (Siegel, 1980; Gross and Siegel, 1983). However, corticosteroid levels change very rapidly (Clinchy et al., 2011) and can reflect the bird's response to being captured and handled. Other useful indicators of physiological responses to external disturbances involve changes in the leukocyte profile, as the latter is closely associated with circulating glucocorticoid levels (Maxwell, 1993). One positive aspect of using such measures is that these do not exhibit such rapid variations as seen with glucocorticoid levels. Measurements such as the total leukocyte count and the ratio of heterophils to lymphocytes in blood (*H*/*L*-ratio) are typically used to indicate the stress level of birds (Gross and Siegel, 1983; Ots

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et al., 1998; Elston et al., 2000; Hõrak et al., 2002; Owen and Moore, 2006). Studies show that social and environmental disturbances may affect the *H*/*L*-ratio (reviewed in Maxwell, 1993). Caution is needed in interpreting *H*/*L* ratios, however. Some mild disturbances, such as heat and road transportation, increase *H*/*L* ratio in poultry; more extreme and life-threatening situations, however, apparently do not lead to predictable increases in the *H*/*L* ratio (Maxwell, 1993). This author suggests that birds may exhibit a two-phase cellular reaction depending upon the type and duration of threat and fear they experience.

In this study we used a Neotropical passerine, the blue-black grassquit (*Volatinia jacarina*), to test the prediction that predation risk can generate physiological and morphological changes. The blue-black grassquit is an abundant passerine with a range extending from Mexico to Argentina (Sick, 2001), and is well-suited for studies that involve predation. Males molt into a blue-black nuptial plumage during the breeding season while females have cryptic brownish coloring throughout the year (Sick, 2001). The iridescent and UV-reflecting nature of the male plumage results from its structural organization, with a layer of keratin and an underlying layer of melanin granules; external disturbances during development of the plumage, for example, possibly alter color patterns (Maia et al., 2009). During the breeding season (October–March) males perform a notable display, which consists of repeated vertical flights with simultaneous vocalizations. The male display and conspicuous coloring could result in males being more vulnerable than females to visually oriented predators. Both sexes care for the offspring (Alderton, 1963), and nest predation is intense (Aguilar et al., 2008), often involving more than 80% of all nests (Diniz, pers. obs.). Additionally, a past study indicated that predators use the displaying male as a cue to find active nests (Dias et al., 2010).

We predicted that perception of predation risk in blue-black grassquits induces morphological and physiological changes in individuals, as detailed below. To test this prediction we exposed one group of individuals to the calls of regional avian predators and a control group to the calls of non-predatory birds. We anticipated an immune response reflected through changes in the *H*/*L*-ratio in the birds exposed to predator cues when compared to changes over the same time period in the birds exposed to non-predator cues. We expected that exposure to calls of predators would also negatively affect body mass and feather development (growth and color). Furthermore, we expected that males would suffer greater consequences from exposure to predator cues because of a possible greater vulnerability to predators when compared to females.

2. Materials and methods

2.1. Subjects and housing

We captured 132 adult blue-black grassquits in March of 2009 and April of 2010 at Fazenda Água Limpa (15°56′58.21″ S, 47°55′57.60″ W), a property of the University of Brasilia. We subsequently kept these birds within the university campus in two different outdoor aviaries, one in 2009 and another in 2010. All birds were provided with an *ad libitum* diet of seeds specific for small passerines, eliminating the need for other dietary supplements (Nutrópica®Passaros Brasileiros). Capture, aviary maintenance and banding procedures were authorized by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA licenses no. 22/2009 and 17765-1).

2.2. Experimental protocol

We conducted the experiments in November 2009 using 61 birds (32 females and 29 males) and in March of 2010, using 54 birds (27 females and 27 males). Before beginning the experiments, we conditioned groups of three randomly selected individuals of the same sex within visually (but not acoustically) isolated cages of 70 cm × 40 cm × 30 cm for a habituation period of 20 days. During this period, the birds received water and food *ad libitum*.

We randomly assigned the caged groups to two treatments: (1) “predatory”, composed of individuals submitted to vocalizations of 15 potential aerial predators; and (2) “control”, composed of individuals submitted to vocalizations of 15 non-predatory birds (Table 1). We chose potential predators and non-predators based on the local occurrence of these species (Braz, 2001), and upon the feeding characteristics of predator species (Sick, 2001). The experiment occurred in a laboratory at Universidade de Brasília with reduced acoustical influence of other birds occasionally singing outside the building, when compared to natural environments inhabited by grassquits. The predatory treatment group was composed of 15 females and 15 males in 2009, and 12 females and 13 males in 2010; and the control treatment was composed of 17 females and 14 males in 2009, and 15 females and 14 males in 2010. The sample sizes reported here include only individuals for which there are no missing data values. The same experimental protocol was used in both years, as detailed below.

In both predatory and control treatments, birds were placed in the experimental laboratory (2009: 7 m × 3 m; 2010: 8 m × 4 m) and then exposed to a playback for one hour per day during fifteen continuous days at one of three randomly selected

Table 1
Species for which vocalization playbacks were used in the experiment, divided between potentially predatory birds of blue-black grassquit adults and nests and non-predatory birds.

Predators		Non-predators	
Family	Species	Family	Species
Accipitridae	*Gampsonyx swainsonii*	Caprimulgidae	*Hydropsalis albicollis*
Accipitridae	*Accipiter striatus*	Tyrannidae	*Elaenia cristata*
Accipitridae	*Rupornis magnirostris*	Tyrannidae	*Tyrannus savana*
Accipitridae	*Buteo brachyurus*	Donacobiidae	*Donacobius atricapilla*
Falconidae	*Micrastur ruficollis*	Turdidae	*Turdus rufiventris*
Falconidae	*Falco rufigularis*	Turdidae	*Turdus amaurochalinus*
Falconidae	*Falco femoralis*	Mimidae	*Mimus saturninus*
Strigidae	*Tyto alba*	Coerebidae	*Coereba flaveola*
Strigidae	*Megascops choliba*	Thraupidae	*Saltator maximus*
Strigidae	*Glaucidium brasilianum*	Thraupidae	*Saltator similis*
Strigidae	*Athene cunicularia*	Thraupidae	*Saltatricula atricollis*
Strigidae	*Aegolius harrisii*	Thraupidae	*Neothraupis fasciata*
Strigidae	*Asio clamator*	Emberezidae	*Zonotrichia capensis*
Corvidae	*Cyanocorax cristatellus*	Emberezidae	*Sporophila nigricollis*
Corvidae	*Cyanocorax cyanopogon*	Icteridae	*Molothrus bonariensis*

times (800 h, 1200 h or 1600 h) to avoid habituation. We aligned cages along the walls of the laboratory with a space of 15 cm between them and placed visual barriers between the cages so the birds could not seen any of the neighboring individuals. The 15-day period of the experiment allowed the complete growth of new feathers induced by plucking (see details below). We produced playbacks using computer-based multimedia speakers (Troni 3D Plus Surround System PMPO 300W), after standardizing the amplitude for both treatments. We acquired the vocalizations from Xeno-Canto (Xeno-Canto-Foundation, 2009; http://www.xeno-canto.org/), which were then standardized for amplitude, screened and edited to remove excerpts of songs from other birds using Raven Lite 1.0 software (Charif et al., 2006). We reproduced playbacks using two computer-based multimedia speakers (Troni 3D Plus Surround System PMPO 300W), which faced the cages at a distance of 2 m.

To avoid habituation (Thompson, 2009) of the grassquits to the playbacks we used two approaches. First, for each predator or non-predator species we obtained three different vocalization recordings to build the playbacks. In each daily trial we exposed the grassquits to playback subsets containing the calls of three predatory species (predatory treatment) or three non-predatory species (control), but using the call variations obtained for each species to increase the overall variation. Second, we changed the subsets of playbacks each day, so that the focal birds were subjected to the cues of fifteen different species, whether predatory or non-predatory, along the experimental period.

2.3. Body mass, rectrix growth and feather color measurements

At the end of the habituation period and before starting the experiment, we measured body mass from a subsample of individuals (females: $n_{\text{control}} = 26$, $n_{\text{predatory}} = 19$; males: $n_{\text{control}} = 18$, $n_{\text{predatory}} = 24$) and induced feather growth (so that it would occur during the experimental trials) by plucking the first three feathers (rectrices) on the left side of the tail. After the experiment, we plucked the newly grown rectrices and measured their length with calipers (to nearest 0.1 mm), and measured each individual's body mass (to nearest 0.01 g) again. Females and males assigned to control or predatory treatments did not differ in body mass between treatments before the experiment (two sample t-test: $t = 1.06$, $P = 0.29$, $n = 45$; $t = -0.86$, $P = 0.39$, $n = 42$, respectively).

Feather growth was also induced before the experiment for covert feathers from the dorsal region of each bird, in an area of approximately 10 mm × 15 mm between the wings, to measure color properties of the newly grown feathers. We measured reflectance spectra with an Ocean Optics USB-4000 spectrometer attached to a pulsed xenon light source (PX-2, Ocean Optics, Dunedin, FL). A fiber optic, attached to a holder to minimize ambient light influence, was placed at a 45° angle from the feather surface to measure plumage reflectance using SpectraSuite software (Ocean Optics, Dunedin, FL) (Maia et al., 2009). Ten covert feathers were set on a surface of black velvet paper, overlaid as to simulate their natural pattern on the bird's body (Quesada and Senar, 2006). We took three measurements of the same sample of feathers by repositioning the fiber optic support on different parts of the sample. Each measurement was calculated relative to a WS-1-SS white standard and to the black velvet paper as a dark reference. The average of the three spectra, interpolated to a step width of 1 nm between 320 and 700 nm was used as a reference value for each individual. We processed the reflectance data using the R program (R Development Core Team, 2011) and the pavo package (see Maia et al., 2013). Spectral reflectance characteristics used in the analyses were brightness, contrast, and hue. Brightness was calculated as the average of percent reflectance from 320 to 700 nm; contrast, which indicated the saturation of the color, was measured as the difference between the maximum and minimum reflectance; and hue, was considered the wavelength at the peak reflectance (Montgomerie, 2006).

2.4. Immunological condition

Before and after the experiment in 2010, we assessed the immune condition of 21 birds (10 females and 11 males) from the predatory group and 17 birds (10 females and seven males) from the control group by measuring the heterophil to lymphocyte ratio (H/L; Gross and Siegel, 1983). We collected blood samples from the brachial vein of each individual in the afternoon period (1400–1800 h), and prepared blood smears that were fixed in absolute methanol. Hemograms and cell counts were conducted in a commercial laboratory (Santé Laboratório, Brasilia-DF), allowing us to calculate the H/L ratio.

2.5. Data analyses

We used multivariate analyses of variance (MANOVA) to test if length of the rectrices, body mass and color properties of males and females differed according to treatment (predatory or control). We additionally included "year" as a variable, since a possible physiological effect could have resulted from maintaining birds in different aviaries in different years. After running both MANOVAs the significant effects of the explanatory variables were evaluated using univariate analysis of variance (ANOVA) for each response variable including treatment and year as explanatory variables.

Variations in the immunological condition of the animals before and after the experiment were calculated as the difference between the final and initial H/L of each individual. To test whether birds of the predatory group differed from those of the control group in H/L degree of change during the experiment, we used ANOVA considering sex, treatment and the interaction between them as explanatory variables.

In all analyses the assumption of normality was evaluated using Shapiro Wilk tests and homoscedasticity using the Bartlett test (Quinn and Keough, 2002). We also tested for collinearity among dependent variables in MANOVA, by calculating Pearson's product moment correlations. As all correlations had r values lower than 0.5, all variables were included in the analyses.

3. Results

Individuals exposed to predator vocalizations did not differ in body mass, length of newly grown rectrices, nor in feather color properties when compared to those exposed to control vocalizations (females: Pillai's trace = 0.16, $F_{5,52} = 1.96$, $P = 0.10$; males: Pillai's trace = 0.18, $F_{5,49} = 2.16$, $P = 0.07$; Table 2). Females from both treatment groups differed between years 2009 and 2010 in these measurements when considered in the MANOVA (Pillai's trace = 0.22, $F_{5,52} = 2.90$, $P = 0.03$), whereas males did not (Pillai's trace = 0.14, $F_{5,49} = 1.62$, $P = 0.17$). ANOVAs of each response variable showed that average brightness and contrast of female covert feathers varied across years ($F_{1,56} = 10.61$, $P = 0.02$ and $F_{1,56} = 4.24$, $P = 0.04$, respectively), and both measurements showed greater values in 2009 (brightness: 22.2 ± 7.51% and contrast: 19.5 ± 6.3) in comparison to 2010 (brightness: 16.8 ± 4.4% and contrast: 16.7 ± 3.7). Females' body mass, rectrice length and feather hue did not change across years ($F_{1,56} = 0.98$, $P = 0.32$; $F_{1,56} = 2.15$, $P = 0.15$; $F_{1,56} = 0.004$, $P = 0.95$).

When evaluating leukocyte counts after the experiment, individuals of the predatory group differed significantly from those in the control group, with the former exhibiting negative H/L variation values and individuals from the control group showing positive H/L variation values ($F_{1,34} = 9.16$, $P = 0.005$; Fig. 1 and Table 3). The

Table 2
Mean values ± SD of body mass, rectrix length and feather color properties of male and female blue-black grassquits after exposure to vocalization playbacks of predators and non-predators (control).

Variables	Females		Males	
	Predatory ($n = 27$)	Control ($n = 32$)	Predatory ($n = 28$)	Control ($n = 28$)
Body mass (g)	10.9 ± 1.1	11.2 ± 1.0	10.9 ± 1.0	10.8 ± 1.3
Rectrix length (cm)	3.2 ± 0.3	3.0 ± 0.3	3.1 ± 0.2	3.3 ± 0.2
Brightness (%)	19.6 ± 6.0	19.9 ± 7.5	20.6 ± 6.8	19.8 ± 7.5
Contrast	17.5 ± 4.3	18.8 ± 6.2	15.1 ± 6.6	18.8 ± 7.2
Hue (nm)	699.6 ± 1.0	699.3 ± 1.5	327.7 ± 43.6	351.5 ± 54.8

Table 3
Mean values ± SD of leukocyte counts and heterophil/lymphocyte ratio (H/L) of female and male blue-black grassquits before and after exposure to vocalization playbacks of predators and non-predators (control).

Variables	Predatory		Control	
	Females ($n = 10$)	Males ($n = 11$)	Females ($n = 10$)	Males ($n = 7$)
Before treatment				
Heterophil	3.50 ± 2.59	4.55 ± 4.59	2.50 ± 2.12	1.29 ± 1.38
Lymphocyte	2.90 ± 1.85	4.00 ± 1.90	5.20 ± 2.25	5.14 ± 2.97
H/L	1.40 ± 1.15	1.12 ± 1.22	0.59 ± 0.51	0.44 ± 0.72
After treatment				
Heterophil	1.90 ± 1.52	1.18 ± 0.98	1.90 ± 1.29	1.57 ± 1.13
Lymphocyte	3.70 ± 2.71	4.09 ± 2.70	3.20 ± 2.04	2.86 ± 1.07
H/L	0.59 ± 0.39	0.35 ± 0.29	0.88 ± 0.90	0.67 ± 0.64

magnitude of the variation for the predatory group between the beginning and end of the experiment was almost fourfold that of the control group. This result was true for both males and females, as shown by the non-significance of the interaction term between sex and treatment group in ANOVA ($F_{1,34} = 0.02$, $P = 0.90$). Therefore, both males and females from the predatory group exhibited an immune response. Irrespective of treatment group, males did not differ from females in immunological condition ($F_{1,34} \sim 0.00$, $P = 0.99$).

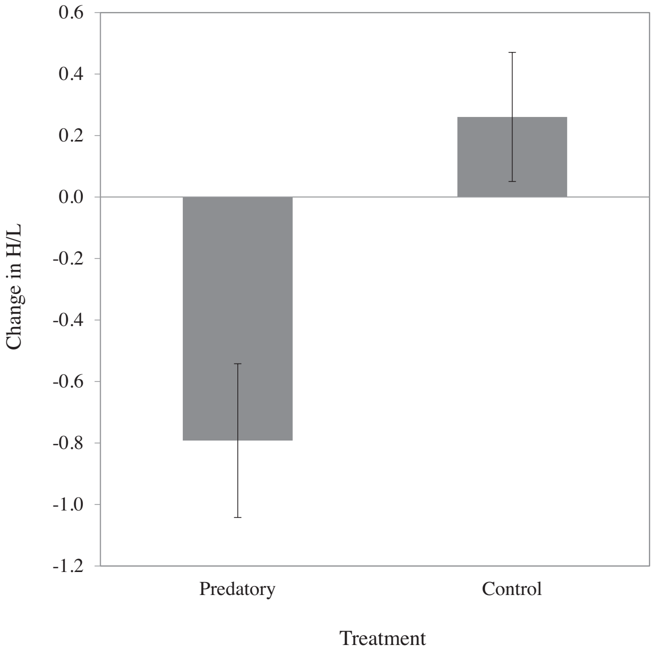

Fig. 1. Mean values (±SD) of the difference between the final and initial heterophil/lymphocyte ratio (H/L) for individuals (males and females, pooled) exposed to vocalizations of predators ("predatory") or non-predators ("control").

4. Discussion

Our study shows empirically that exposure of a passerine, the blue-black grassquit, to predator vocalizations resulted in a dramatic change in the H/L ratio for both males and females, indicating that this external stimulus affected the birds' immune systems. However, contrary to expectations, the exposure to potentially distressing predator vocalizations did not result in any deleterious morphological consequence concerning body mass, growth of new feathers or their spectral properties. In other words, it is clear that the birds exhibited a physiological response when exposed to the predator vocalizations, but the change in their leukocyte profile did not lower their capacity for growth of new feathers and maintenance of body mass.

Experimental manipulations such as the one conducted can identify specific environmental elements that provoke changes in an organism's physiology that possibly interfere with other biological functions. Both disease and physical injuries induce physiological changes in wild birds, shown through elevated H/L ratios. For example, house finches (*Carpodacus mexicanus*) infected with mycoplasmal conjunctivitis had elevated H/L ratios (Davis et al., 2004), as did penguins injured in fights (Vleck et al., 2000). Despite these confirmations of physiological responses to external physical and social stimuli, or in response to disease, there is less evidence indicating that perception of predation risk may also trigger immune responses (but see Thomson et al., 2010).

Few studies have used experimental approaches to assess how animals perceive and respond to the threat of predators. Such studies highlight the significance of acoustic cues, and show that birds exhibit behavioral as well as physiological responses to the possibility of predation. In one study, predator removal and subsequent decreased rates of predator vocalizations led to changes in the reproductive strategies of 12 bird prey species (Fontaine and Martin, 2006). In another study, recordings of predators produced near song sparrow *Melospizia melodia* nests reduced their reproductive success by approximately 40% (Zanette et al., 2011). In addition to changes in behavioral strategy, recent evidence suggests that certain acoustic stimuli can induce a physiological response in birds, reflected in changes in the H/L ratio and circulating corticosterone.

For example, pied flycatcher *Ficedula hypoleuca* nestlings exposed to nestling distress calls exhibited increased H/L ratios (Tilgar et al., 2010).

Our results show that the perception of predator risk as assessed by acoustic cues can modify the leukocyte profile of individuals, a key element that could trigger the suite of adaptive breeding behaviors shown in other studies. It is important to note that the continuous threat of predation simulated in our experiment yielded reduced H/L ratios, contrary to increased ratios seen in some studies. Given that increased H/L ratios are widely accepted as reliable indicators of physiological stress, how can our experimental results be interpreted? According to Maxwell (1993), mild to moderate stress in birds may induce a heterophilia, which raises the H/L ratio. Under more severe stress, a second phase occurs, apparently unique to birds, when a heteropenia associated with a basophilia becomes evident (Maxwell, 1993; Maxwell and Robertson, 1998). Our analyses revealed that a dramatic reduction in H/L ratios occurred only for the birds exposed to the predator acoustic stimulus. This suggests that the repeated exposure to predator calls in the confined situation of the caged birds was probably severe enough to result in the two-phase cellular reaction that may be unique to birds.

The significant change in leukocyte profile of blue-black grassquits induced by avian predator vocalizations indicates that individuals interpret such vocalizations as danger cues, irrespective of physical confirmation of the predator in the environment. Thus, predator vocalizations may represent a non-lethal indirect cost for avian prey species. The effects of predators on prey populations could be much greater than those attributed to direct death by predation given that anti-predation response costs may reduce breeding rates and increase mortality resulting from other factors (Lima, 1998; Creel and Christianson, 2008).

In our study, both sexes exhibited a similar decline in H/L values when exposed to predatory calls. Although females typically suffer higher rates of predation under natural conditions, especially during the nesting period (reviewed in Donald, 2007), males of some species may be more conspicuous due to courtship behaviors and acoustic signaling, which may attract predators (Magnhagen, 1991). The motor display of blue-black grassquits can certainly expose males to visually oriented predators, such as the birds whose calls we used in the experiment. Our expectation that male blue-black grassquits would exhibit a stronger physiological response to predator calls than females was not supported by the results, since both sexes exhibited similar reductions in the H/L ratio. Predators may target both sexes of this species at similar rates but in different contexts. Males may be more exposed during their displays while females may be more vulnerable during nesting, as they are the sole incubators.

Animals in threatening circumstances typically mobilize nutritional reserves to increase the availability of glucose and metabolic fuels so they can cope with an imminent threat; thus, energy storage in the form of fat generally decreases in such situations (Nelson, 2000). Additionally, nutritional and social instability have been shown to slow the growth rate of feathers and also lead to plumage irregularities (Jenkins et al., 2001; Bortolotti and Murza, 2002; Jovani and Blas, 2004; Talloen et al., 2008). Based upon these observations, we predicted that birds submitted to predator calls would exhibit reductions in body mass and feather growth, as well as changes in plumage spectral properties. Our data did not confirm these expectations, as birds exposed to predator calls did not differ from the control group relative to body mass, growth of rectrice feathers or in spectral properties of dorsal feathers. These results could be a possible effect of the *ad libitum* feeding. Contrary to birds exposed to predators in a natural environment, our captive population had continuous access to a rich food supply and did not expend energy fleeing from predators or foraging. Therefore, the *ad libitum* food provided may have allowed the birds to maintain their state of alertness without any deleterious effects. Future studies should consider nutritional limitation when testing for negative non-lethal effects of predators.

In conclusion, while many bird species recognize and respond behaviorally to vocalizations produced by predators (Miller, 1952; Hauser and Caffrey, 1994; Rainey et al., 2004; Reudink et al., 2007), our results emphasize the role of such acoustic cues in inducing altered leukocyte profiles. Physiological responses based on acoustic information may be a general phenomenon for susceptible prey species. Since blue-black grassquits used in the experiment were captured in their natural environment, their ability to recognize predator vocalizations can be attributed to former direct contact or social learning, an issue to be explored in the future. Furthermore, it is necessary to evaluate how the bird's immune system responds to the combination of hostile ecological scenarios and nutritional condition.

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