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Does immune suppression after moderate stressors occur to free resources in the house sparrow (Passer domesticus)?

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by

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Abstract:

Stressors affect immune functions, but exactly why has yet to be discerned entirely. Two non-exclusive hypotheses have been proposed to explain this phenomenon: (i) immunosuppression abates the autoimmune response to self-antigens exposed by the rigors of a stressor, and (ii) immunosuppression allows allocation of resources to functions more valuable than protection against infection during a stressful event, (e.g., physical performance augments predator evasion). In this experiment, the second hypothesis was tested in wild house sparrows (*Passer domesticus*) by comparing rates of change in vertical flight performance versus antibacterial capacity of blood over a six week period of captivity. I predicted that if the latter hypothesis was true, immune function would decline over time, while physical capability would remain relatively stable, or even increase due to the reallocation of resources. Body mass, vertical hover duration, and antibacterial blood capacity all decreased over time in captivity. However, immune function did not decline more rapidly than physical performance, lending no support to the idea that resources are scavenged from immune function for physical activities. However, the possibility that demands other than vertical flight are traded-off against immune function cannot be dismissed.

Introduction:

Stress in the following study was defined as a behavioral and physiological state implemented during intense, yet transient, aversive conditions; this state allows the organism to avoid, endure, or even recover from possibly detrimental stimuli (Martin 2009; Apanius 1998). The release of hormones from the hypothalamic-pituitary-adrenal (HPA) axis during a stressful event can augment immunological responses if the exposure to a stressor is for a brief duration (i.e., seconds-minutes), but chronic exposure to a stressor can lead to immune dysregulation (i.e., days-months; Glaser and Kiecolt-Glaser 2005). All organisms have limited energy supplies with which to maintain homeostasis, reproduce, and grow. When energy is allocated to one process, the amount available for other functions is reduced (Sheldon & Verhulst 1996; Molles 2010), resulting in trade-offs among various physiological functions.

Energy reallocation among functions is the framework for one of the two hypotheses that attempt to explain why immunity is suppressed during stress. The first hypothesis is that suppression of the immune system occurs during a stress response because pathogen defense is less critical to immediate survival than physical ability (Sapolsky et al. 2000). For example, the resources available are distributed to muscles and tissues in preparation for predator evasion, territorial defense, or other aggressive encounters, rather than the mobilization and activation of immune cells and proteins. The second hypothesis proposes that a decrease in immune function allows an animal to compensate for novel self-antigens released during stress, which cause antigen processing centers to initiate an autoimmune response (Råberg et al. 1998). An autoimmune response could be very costly in terms of tissue degradation and release of stored energy from those tissues (e.g. protein, glycogen, etc.).

Here, I tested the first hypothesis using the house sparrow (*Passer domesticus*), a wild passerine. Wild animals in their natural setting experience different challenges and stressors than wild animals in captivity: predators, pollution, weather changes, and habitat destruction versus artificial lighting, and smells, sounds, and surroundings unlike the natural habitat (Morgan and Tromborg 2007). The environment of a captive animal is highly constrained, altering natural behavior patterns such as foraging and ranging. For example, flicker frequency of fluorescent lighting alters mate choice in wild, captive female European starlings (*Sturnus vulgaris*; Evans et. al 2006). The use of disinfectants in enclosure cleaning removes meaningful olfactory information, which can elicit stress, and can pose health risks in the form of self-anointing, or licking at the source of a disinfectant scent and spreading the scent on itself (Clark and King 2008; Wells 2009). In caged carnivores, the greater the home range size, the more likely the animal is to provide poor parental care and to exhibit abnormal behavior, such as pacing or rocking (Clubb and Mason 2003). Findings such as these indicate potential adverse effects of captivity on wild animals, emphasizing the need to use caution when extrapolating data from captive individuals to those in their natural habitat or vice versa.

Research on wild, captive, and domesticated species, as well as humans, has found that exposure to a stressor causes a cascade of effects in the brain that exerts many downstream effects on immunological function (de Kloet et. al 2005; Minton 1994; Moberg and Mench 2000). The increase in neural activity induced by a stressor causes the hypothalamus to secrete corticotrophin releasing hormone (CRH; Yadav 2008). CRH acts on the anterior pituitary, leading to the release of adrenocorticotropic hormone, which in turn acts upon the adrenal glands to stimulate the secretion of adrenal hormones, such as glucocorticoids (GCs); in birds, the adrenal glands release the GC corticosterone (CORT; Anderson 2006). These GCs feed back

onto the pituitary and hypothalamus to decrease their activity and, hence, to modulate the body's hormonal response to the stressor (Fig. 1; Squires 2003). Significantly elevated GCs over an extended period of time (i.e chronic stress) can cause the feedback process to cease, leading to hyperactivity of the HPA axis, which translates into further increases in GC and CRH levels that, in extreme cases, lead to hippocampal atrophy and destruction (Magiakou and Chrousos 2005; McEwen 1998; Romero 2004). The hippocampus has high levels of adrenal steroid receptors—mineralocorticoid (Type I; MR) and glucocorticoid (Type II; GR)—that help modulate the adrenal stress response (McEwen 1998). MRs are linked to the appraisal process and onset of the response; GRs are important in mobilizing the energy resources to terminate the stress response process, as well as aiding in recovery (de Kloet et. al 2005). Repeated stress gives rise to a reduction in GR, which is correlated to reduced sensitivity to the GCs, and thus a dissipated ability to signal the hypothalamus to stop producing stress induced levels of CRH (Reul et al. 1990).

High GC levels have been cited as a direct byproduct of interaction with a stressor and thus are used to identify the degree of stress during experimentation (Carsia and Harvey 2000). Previous work indicates that captivity is a stressor for wild animals, including avian species (Dickens and Romero 2009; Dickens et al. 2009). Wild-caught chukars (*Alectoris chukar*) exhibited "temporary chronic stress" in response to captivity over a period of 10 days, demonstrated by weight loss, changes in hematocrit levels, and an inability to shut off CORT secretion, i.e. interrupted negative feedback of the HPA axis, by day 5 (Dickens et al. 2009). Wild European starlings (*Sturnus vulgaris*) increased baseline heart rate for 2 days post initial introduction to captivity. The decreased heart rate variability that accompanied the increased

heart rate during the first few days of captivity indicated excessive, sustained sympathetic nervous system activity (Dickens and Romero 2009).

Chronic stress induced activation of the HPA axis, and the resultant increase in GC levels, can suppress phagocytosis and decrease the redeployment of blood leukocytes (mechanism unknown; Dhabhar and McEwen 1997), actions which are key in the function of the innate immune system (Koutsos and Klasing 2008). The innate immune system is the first line of defense against infection, including physical barriers (e.g. epithelial surfaces), whole-body defense mechanisms (sweat, mechanical action of mucus and tears, etc.), the complement system (plasma proteins which induce the inflammatory response, enhance B and T lymphocyte response of the adaptive immune system, etc.), and a particular group of leukocytes (Sell and Max 2001; Juul-Madsen et. al 2008). Some of these innate immune system leukocytes, such as mast cells and dendritic cells, are found in tissue, while others, such as monocytes, neutrophils, and eosinophils, are found in circulation (Sell and Max 2001). It is the complement system and these particular leukocytes that eliminate certain pathogens and signal to the specialized leukocytes of the adaptive response (B and T lymphocytes) for help in invader elimination (Juul-Madsen et. al 2008). The innate system leukocytes in circulation allow us to determine the ability of an individual sparrow to control bacteria *in vitro* (Liebl and Martin 2009); Gram-negative bacteria (such as *Escherichia coli*) are extinguished by the complement system (Taylor 1983), and Gram-positive bacteria (such as *Staphylococcus aureus*) are killed by phagocytic cells (Rubin et al. 2008).

Here, I tested the resource reallocation hypothesis by exposing house sparrows to a chronic stressor (captivity) and measuring antibacterial blood capacity and physical performance to determine trade-offs in investment. If pathogen resistance is less critical than physical ability

during times of stress, antibacterial response should decrease more rapidly than physical performance over time in captivity (the stressor), presumably due to reallocation of resources. Physical robustness was determined using a vertical flight chamber, because vertical flight/hover is the most challenging form of locomotion that a bird can perform (Møller 2010). During a hover, the mechanical power output and fiber shortening of the pectoralis muscle are maximal (Dial et al. 1997). In order to hover, wings are beat more or less horizontally to generate enough lift to exactly compensate for gravity (Podulka et. al 2004). Most birds, other than the hummingbird and kingfisher, are unable to maintain this energetically expensive mode of flight for any extended period of time, if at all.

Methods:

Capture, blood samples, and captivity

A group (n=9) of house sparrows were captured in Brandon, Florida over a period of 3 months in Fall 2009 using mist nets. Birds were removed from the net in less than 3 minutes (before circulating CORT increases in response to restraint) to take a 50 μL blood sample from the brachial vein using a 26 gauge needle and heparinized capillary tube after first sterilizing the wing with alcohol. As soon as possible (within 5 minutes), each blood sample was processed for antibacterial killing activity (see "Antibacterial assay" below). Birds were massed to the nearest 0.1 g, and tarsus and wing chord were measured to 0.1 cm and 0.1 mm, respectively. Sex and age were determined as accurately as possible; birds were in molt, so classification of males was difficult, and is not used as a variable in statistical analysis. The house sparrows were restrained in a cotton cloth bag until transport to captive housing at USF, where they were housed in traditional songbird cages with access to mixed seeds and water, *ad libitum*, in a room with

steady ambient climate conditions. This room was isolated from human disturbance, other than a daily ~15 minute period when food and water were refreshed. One day following capture, all specimens were subjected to a flight performance test as described below. Birds were kept for 6 weeks in captivity, with a weekly blood sample (within 3 minutes of entering the room) and one day later, a weekly flight test. After completion of the final flight test, birds were released at the site of capture. All procedures were approved by the USF IACUC (W3202).

Antibacterial assay

Following Liebl and Martin, 2009, a 1.5 μL aliquot of each blood sample was added to 34.5 μL of CO_2-independent media plus 4 mM L-glutamine in seven separate tubes: one blank tube (media, but no bacteria), then 3 tubes each for *Escherichia coli* (Gram-negative bacteria) and *Staphylococcus aureus* (Gram-positive bacteria). All blood samples and aliquots were kept on ice until arrival in lab. In a laminar flow hood, 12.5 μL of 10^5 bacteria/mL solution (bacteria from lyophilized pellets (Microbiologics, St. Cloud, MN) reconstituted in sterile phosphate-buffered saline) was added to each corresponding sample tube and 12.5 μL sterile PBS was added to each blank tube. Tubes were then vortexed, incubated at 37°C for one hour, and vortexed again. A control blank tube was filled with 48.5 μL of sterile PBS and 3 control tubes each for *E. coli* and *S. aureus* were filled with 48.5 μL 10^5 bacteria/mL. 250 μL of tryptic soy broth (TSB) was added to all blank, control, control blank, and sample tubes, which were then incubated for 12 hours at 37°C. After the 12 hour incubation, absorbance was evaluated using a spectrophotometer (Nanodrop 1000) with a 300 nm filter. Absorbance shows how much bacteria is present in the control tubes versus the sample tubes, which translates into antibacterial activity of the blood. Each tube was vortexed immediately before the 2 μL subsample was withdrawn

from the center of the tube. The spectrophotometer was blanked using the corresponding media blanks for each sample prior to measurement. All samples were measured relative to the positive control.

Flight performance test

Approximately 24 hours after each weekly blood sample, each bird was subjected to a vertical flight test in a Plexiglas chamber (46 in x 8 in x 8 in) in a room free of other birds. A video camera was placed in the room prior to the trial at the same distance from the chamber each time. A single bird was placed in the box and given 45 seconds to acclimate to the room and box without a human present. I burst in through the door stomping my feet after the 45 second interval. After the bird's initial reaction, I left the room for an additional 45 seconds and then burst through the door for a second time. The bird was then removed from the box and placed back in its cage. Video recordings for each bird were assessed to determine 4 flight parameters: height and duration of the first vertical flight in response to each of the two experimenter intrusions.

Data Analysis

Variable variances were homoscedastic and distributions were not significantly non-normal. Repeated measures general linear models (GLM) were used to determine whether there were changes over the course of captivity in antibacterial activity, body mass, and physical fitness (i.e., vertical flight performance). Bonferroni post-hoc comparisons were used to identify pair-wise differences, and body mass at capture and percent mass loss over the first week in captivity were used as covariates. To determine the relationship between the immune and physical

parameters, raw data were converted to z-scores (over all time intervals, but for each variable separately), and another repeated measures GLM (with trait as a between-subject factor and mass loss as a covariate) was conducted. Z-scores were used to convert the data sets with different raw units to comparable distributions with known characteristics and averages set to equal. Only the duration of the hover flight in response to the second experimenter intrusion was used in the analysis because 1) repeated hovering flight would be the most demanding activity the flight chamber could measure, and 2) the vertical flight variables were highly correlated in individuals within and between the two experimenter intrusions. Slopes of time by immune and performance trait values were determined, and Spearman rank correlation analysis were used, to further investigate the results with the expectation that birds with the smallest reduction in physical performance would be those that had sacrificed antibacterial capability most rapidly. All data for captive week 2 were dropped because the TSB used in the antibacterial assay was contaminated. All analyses were performed with SPSS v18 and significance attributed at $\alpha \leq 0.05$.

Results:

Antibacterial assay

Over the six week period of captivity, *E. coli* killing ability of the blood decreased ($F_{5, 40} = 2.4$, $P = 0.05$; Fig. 2). *Staphylococcus aureus* antibacterial capability changed over time ($F_{5, 35} = 3.3$, $P = 0.02$; Fig. 2), but the pattern was more variable, indicating an obvious decline only at the last measured point.

Flight performance test

Hover flight duration changed over captivity ($F_{5, 40} = 5.8$, $P < 0.001$; Fig. 3), showing a sharp increase in week 1 followed by a steady decrease through the final measurement. In addition, body mass declined over time ($F_{5, 40} = 2.55$, $P = 0.05$; Fig. 4A), with the most mass lost (nearly 5%) over the first week of captivity. It is interesting to note that individuals who lost the most mass during the experiment (i.e. during week 1) also tended to be the birds that exhibited the greatest decrease in hovering capacity (Spearman $r = 0.66$; $P = 0.06$; Fig. 4B).

Relationship between immunity and physical function

All three traits—*E. coli* and *S. aureus* antibacterial activity and hover flight duration as z-scores—declined over the six week captive period ($F_{5, 120} = 4.8$, $P < 0.001$; Fig. 5), but rates of trait change did not differ significantly. Also, Spearman rank analysis indicated that the rates of decline of antibacterial activity were unrelated to the rate of decline of hover duration ($P = 0.55$) i.e. there was no significant relationship between the variables.

Discussion:

Captivity acts as a chronic stressor on wild animals, including passerines, as indicated by elevated GC levels and decreased GR and MR in the house sparrow (unpublished, Andrea Liebl, Martin Lab), and 2-3 times higher CORT levels in 35 day captive white-throated and white-crowned sparrows than in free-living individuals (*Zonotrichia albicollis* and *Zonotrichia leucophrys*; Marra et. al 1995). Such sustained levels of elevated CORT tend to have a negative effect on the immune system (Dhabhar and McEwen 1997; Evans et. al 2000; Saino et. al 2003). Accordingly, over six weeks in captivity, blood antibacterial capacity decreased in the house

sparrow, with *E. coli* showing an obvious downward trend over time (Fig. 2). The pattern of *S. aureus* killing was more complicated, ultimately showing the lowest activity during the last week of captivity (Fig. 2). Immune function thus declined over time in response to the chronic stress of captivity.

In addition to a decline in immune function, house sparrow vertical hover capacity decreased during weeks 2-6 after an initial increase during the first week of captivity (Fig. 3). Concurrently, the birds experienced a decline in body mass, with the majority of this loss during the first week of captivity (5% of body mass; Fig. 4A). These results are similar to the approximate 4% loss in wild-caught chukar during a 10 day period in captivity (Dickens et. al 2009), and the rate of weight loss in European starlings around day 6 when subjected to chronic stress for 18 days (Cyr et. al 2007).

Birds that lost the most mass over the first week of captivity were also the birds that showed the greatest decline in hover duration during the experiment (Fig. 4B). Both of these changes could be due to the increased levels of CORT present during exposure to a stressor, which would lead to muscle wasting as well as the observed down-regulation of the immune system (Bradshaw 2003). Gluconeogenesis, especially from the breakdown of muscle protein, is known to occur from the increased level of circulating glucocorticoids (Wingfield et. al 1997). In house sparrows with implants of CORT, it is the pectoralis flight muscle that loses a great deal of mass (Wingfield et. al 1997). CORT treatment of wild song sparrows (*Melospiza melodia*), pied flycatchers (*Ficedula hypoleuca*), and dark-eyed juncos (*Junco hyemalis*) results in protein loss from flight muscles, but no change in body mass due to increased fat depots (Wingfield and Silverin 1986; Silverin 1986; Gray et al. 1990). It is possible that the group of birds in this experiment lost mass from flight muscles, but did not experience an increase in fat depots,

because the animals were in a stressful environment (Wingfield and Silverin 1986). To further evaluate the relationship between weight loss and flight performance, future experiments should incorporate the methods of Wingfield and Silverin (1986) wherein fat depots near the abdomen and furculum are evaluated by an arbitrary scale of 1 to 5.

Vertical/hover flight was chosen as the physical characteristic to measure because it is a very energetically costly form of locomotion (Dial et. al 1997; Møller 2010). If there were some change in resource allocation, it should be easily discernible in this function as hover flight can be scored repeatedly and accurately. Rather than muscle wasting, perhaps the decline in hover duration in all birds over time in captivity was due to acclimation to the flight box and general startle procedure. Many birds only flinched upon human reentry to the isolated room; those birds gave no jumping or true startle response as generated during the earlier flight tests. Other birds have shown acclimation to experimental procedures. Mallard ducks (*Anas platyrhynchos*) habituated to daily treadmill exercise after 7 days (Rees et. al 1983). Common eiders (*Somateria mollisima*) became accustomed to periods of gentle handling after 10 days (Cabanac and Guillemette 2001). Western screech-owls (*Otus kennicottii*) may become accustomed to handling and learn that they will be returned to their cage (Dufty Jr. and Belthoff 1997).

If the resource reallocation hypothesis, and that of this experiment, was correct, chronic stress would lead to energy transfer from immune function to other physiological functions, such as flight or, in nature, territorial defense. The decline in physical performance over the experimental period contrasted with the expected results—stable or increased physical ability—lending little support to my original hypothesis. When the immune parameters and physical parameter were compared, there was no significant relationship between the rates of decline (Fig. 5). In other words, one function did not decline faster than the other, failing to support the

hypothesis that resources are allocated from immune function to other physical functions more important to survival.

Despite my equivocal results, future experimentation on stress-induced immunosuppression is still warranted. Experiments could be designed to determine whether muscle wasting actually occurs (from the chronic stress of captivity) and thus decreases the ability of the birds to initiate or maintain vertical flight. This could be accomplished with a combination of the fat store method (Wingfield and Silverin 1986) and the use of ultrasound technology to measure thickness of the pectoral muscle (Lindström et. al 2000). Alternatively, a new version of the flight box could be used with some sort of mechanism to ensure that the birds do not become accustomed to the procedure. The second option has already been considered by other Martin lab members. A box that emits varying "white noise" ensures the animal does not become accustomed to a particular sound, and a trap door ensures that the animal must initiate vertical flight. Regardless of any further experimentation, data from this project lends support to the idea that chronic stress negatively impacts immune function.

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Figures

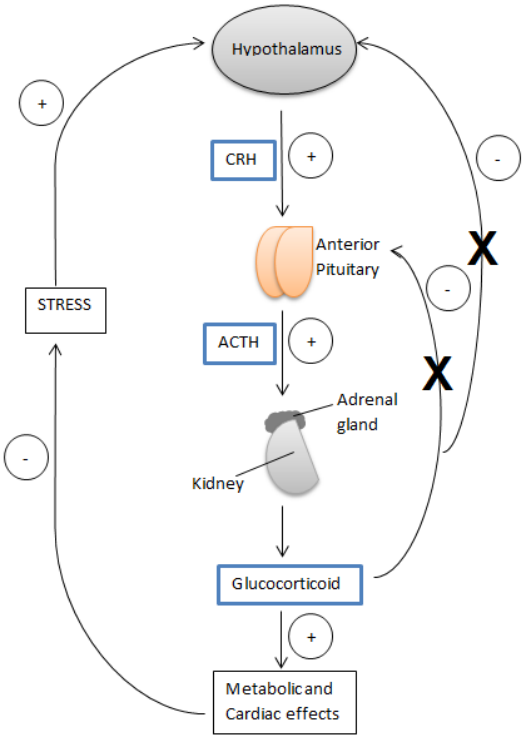

Figure 1. Schematic of the basic stress response of the hypothalamic-pituitary-adrenal axis in mammals. - indicates downregulation, while + indicates upregulation; CRH refers to corticotropic releasing hormone; ACTH refers to adrenocorticotropic hormone. (based on Squires 2003)

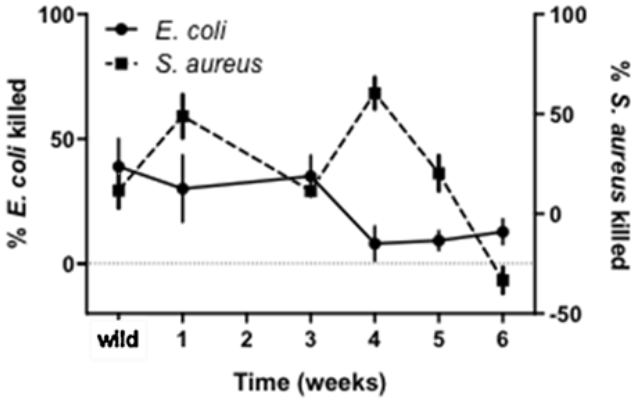

Figure 2. Capacity of whole blood to control *E. coli* (solid line; P= 0.05) and *S. aureus* (dashed line; P= 0.02) declined over time in captivity.

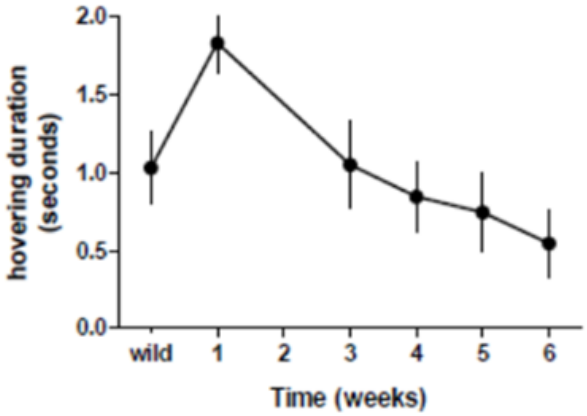

Figure 3. Hover duration of house sparrows declined over time in captivity after an initial increase during the first week (P<0.001).

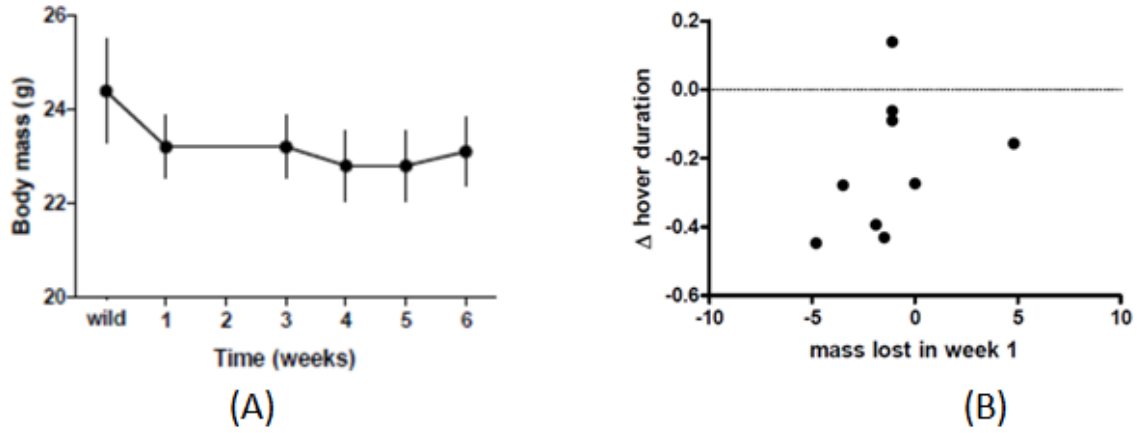

Figure 4. (A) Bird body mass declined over time in captivity, with a notably large decrease during the first week (P=0.05). (B) Birds that lost the most mass during the first week of captivity, also tended to show the greatest decline in hover duration (P=0.06).

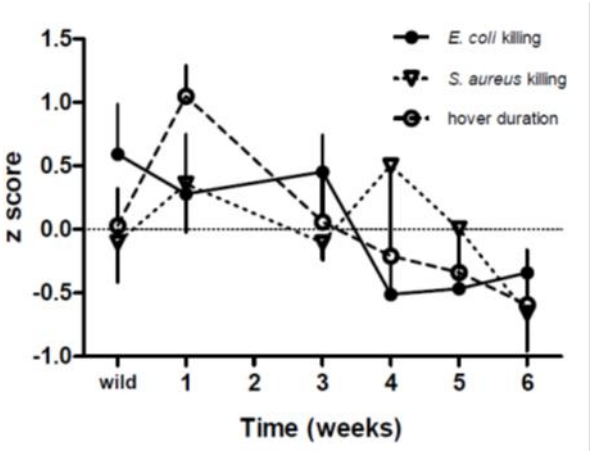

Figure 5. Although all three traits declined (P < 0.001), there was no relationship between the rates of decline in antibacterial activity and the rate of decline in hover duration (P = 0.55).