

## RESEARCH ARTICLE

# Common measures of immune function vary with time of day and sampling protocol in five passerine species

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## ABSTRACT

Ecological immunology is a rapidly growing field of study that focuses on understanding variation in immune systems across species and how this relates to species ecology and evolution. Newly developed field methods aimed at studying variation in immune function in a field setting have yielded many insights. Nonetheless, there continues to be much debate regarding the interpretation of field measures of immune function. There is substantial evidence to suggest that handling stress could introduce variation into measures of immune function, yet no study has examined the impacts of incremental changes in handling times under 30 min on immune measures. Nor has any study examined variation in immune function with time of day, though other physiological measures, including glucocorticoids known to impact immune function, vary with time of day. Here, I used observational field data to test the hypothesis that innate immune function varies with handling stress. Furthermore, I tested the hypothesis that innate immune function changes over the course of the day. I show that measures of innate immune function vary with (1) handling stress over short time periods typical of sample collection in the field, and (2) the time of day that an individual is sampled. I discuss these findings from an ecological perspective and suggest that the observed variation is not random, but is likely to have important adaptive functions. I end with a summary of the practical implications of these findings for field studies of ecological immunology.

**KEY WORDS:** Innate immune function, Ecological immunology, Natural antibodies, Complement, Lysozyme acute phase protein, PIT54 acute phase protein, Passerines

## INTRODUCTION

The burgeoning field of ecoimmunology focuses on understanding variation in immune systems and how this relates to ecological and evolutionary differences within and between species (Lee and Klasing, 2004; Norris and Evans, 2000; Ricklefs and Wikelski, 2002). To this end, scientists have developed a variety of methods to measure variation in immune function in a field setting (Boughton et al., 2011; Matson et al., 2006; Millet et al., 2007). While measuring immune function in an ecological context has yielded many insights, scientists continue to struggle with understanding what particular immune measurements indicate about the overall immune function of an individual (Boughton et al., 2011). One basic obstacle to interpreting field immune data is separating variation in immune function that is related to factors of biological interest from measurement artifacts, such as variation associated

with handling stress (Buehler et al., 2008). This issue is compounded by the fact that field-collected blood samples are typically obtained opportunistically over the course of the day as individuals are captured, and that some amount of time elapses between capture of an individual and taking of a blood sample (handling time).

Indeed, there is substantial evidence to suggest that handling stress could introduce variation into measures of immune function. Chronic stress is generally considered immunosuppressive (Råberg et al., 1998), and increased physiological demands associated with breeding, fluctuations in environmental conditions, and the stress of captivity can all reduce immune function, though the magnitude of these effects varies by species (Ardia et al., 2003; Hangalapura et al., 2005, 2003; Matson et al., 2006). However, different stressors alter immune function in different ways, with some stressors actually increasing certain aspects of immune function in the short term; in particular, innate and inflammatory responses tend to increase rapidly in the minutes and hours after exposure to a stressor (Martin, 2009). This rapid change in response to acute stressors poses a potential problem for field studies of ecoimmunology, which often focus on measures of innate immune function as a result of the constraints of field sampling (e.g. that immune function be measured using blood samples or benign biopsies so as to avoid terminal sampling, that assays require only small volumes of blood given the often limited size of samples that can be taken, and that animals not be held in captivity to obtain samples) (Millet et al., 2007).

In the case of the stress-response hormone corticosterone, a baseline sample must be obtained within 3 min to avoid the impact of handling stress on hormone levels (Romero and Reed, 2005). However, despite the rapid induction of the stress response system and the rapid impact of stress on some aspects of immune function, only a few studies have examined the impact of handling time on measures of immune function in wild birds; furthermore, they have done so at a coarse level, examining the impact of relatively long periods of handling stress on immune function (i.e. 30 min, 60 min or even 2.5 h after capture). Several of these studies reported a discernible difference in immune function between individuals sampled within 3 min of capture versus those sampled 30 min or more after capture, especially in the case of bactericidal activity (Buehler et al., 2008; Davis, 2005; Fratto et al., 2014; Matson et al., 2006; Millet et al., 2007). It remains unknown whether measures of innate immune function in wild birds vary with capture stress over shorter time intervals; however, it is variation over these shorter time intervals that is likely to be the most relevant for field studies using mist nets or walk-in traps, given that welfare considerations dictate individuals be processed expeditiously post-capture, with the result that blood samples are more likely to be collected within minutes rather than hours of capture. In addition, previous studies have generally focused on two aspects of immune function, leukocyte counts and bactericidal activity [although Buehler and colleagues

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**List of abbreviations**

GFC	Galápagos fly catcher, <i>Myiarchus magnirostris</i>
MGF	medium ground finch, <i>Geospiza fortis</i>
SGF	small ground finch, <i>Geospiza fuliginosa</i>
STF	small tree finch, <i>Camarhynchus parvulus</i>
YW	yellow warbler, <i>Setophaga petechial</i>

(2008) also measured natural antibody and complement activity in one species of shorebird], so it remains unknown whether and how other measures of innate immune function are impacted by handling stress in wild birds.

A second issue in interpreting field data on immune function is that it remains unknown whether immune function in wild animals varies over the course of the day. Several studies have shown that glucocorticoid levels vary with time of day in a variety of species (Breuner et al., 1999; Dhabhar and McEwen, 1997; Dickstein et al., 1991; Janssens et al., 1995; Rich and Romero, 2001). Given the ample literature linking glucocorticoid levels to immune function (reviewed in Martin, 2009), it is possible that immune function could also vary with time of day. Proper interpretation of immune data acquired under field conditions requires that we better understand the impact of acute handling stress and the time of sampling on immune function in wild animals.

Here, I used observational field data to test the hypotheses (1) that innate immune function varies with handling stress and (2) that innate immune function changes over the course of the day. I tested these hypotheses in five species of passerine, Galápagos fly catchers [*Myiarchus magnirostris* (GFC), Gould], medium ground finches [*Geospiza fortis* (MGF), Gould], small ground finches [*Geospiza fuliginosa* (SGF), Gould], small tree finches [*Camarhynchus parvulus* (STF), Gould] and yellow warblers [*Setophaga petechial* (YW), (Linnaeus)]. In these species, I focus on four measures commonly used in ecological immunology studies to evaluate the strength of individual immune defenses (Boughton et al., 2011): natural antibodies, complement, haptoglobin-like PIT54 acute phase protein and lysozyme acute phase protein.

**RESULTS**

I sampled 100 GFC, 132 MGF, 240 SGF, 92 STF and 81 YW. Because of the small volume of the blood samples obtained, I was not able to run each immune test on every individual so sample sizes vary by immune metric (see Table 1).

Several immune measures varied significantly with handling time (Table 1); this variation was often substantial, but the period during which these measurements reached their peak varied by immune parameter and by species (Figs 1 and 2). Natural antibody and complement activity varied significantly with handling time in SGF. There was no statistically discernible difference in natural antibody activity in SGF in the first 15 min after capture, nor was there a discernible difference in complement activity in the first 25 min after capture; natural antibody and complement activity increased 15 and 25 min post-capture, respectively (Fig. 2). In each case the change was substantial, with levels of natural antibodies nearly half as high again and levels of complement nearly doubling when measured at later time points. Natural antibody and complement activity followed a similar, though not statistically significant, pattern (increasing ~15–20 min post-capture) in several other species (MGF, STF, YW in the case of natural antibodies, and STF, YW, GFC in the case of complement) (Table 1, Figs 1 and 2).

Levels of the PIT54 acute phase protein varied significantly with handling time in GFC, but not in the other study species. In GFC,

there was no statistically discernible variation in plasma levels of PIT54 within 35 min of capture, but individuals sampled 35:01–40 min after capture showed levels of PIT54 almost four times higher than at previous time points, and this difference was statistically significant (Table 1, Fig. 2).

Lysozyme levels showed the most consistent variation across species, showing statistically significant variation with handling time in three species: MGF, SGF and STF (Table 1, Fig. 2). MGF showed no statistically discernible variation in lysozyme levels within 20 min of capture, but showed a significant decrease in lysozyme levels 20:01–35 min post-capture, followed by a significant increase 35:01–40 min post-capture (Fig. 2). In SGF, lysozyme levels followed a qualitatively similar pattern to that in MGF, showing no statistically discernible variation within 15 min of capture, at which time lysozyme levels dropped significantly, and then later increased, though the subsequent increase was not statistically discernible in SGF (Fig. 1, Table 1). In STF, lysozyme levels dropped significantly 5 min after capture, showed no statistically discernible difference 5:01–20 min post-capture, then dropped significantly again after 20 min post-capture (Fig. 2). Lysozyme levels also showed the largest variation with handling time, with levels measuring 2.5–10 times as high in early time points compared with later time points (Table 1, Fig. 2).

Immune measures also varied significantly depending on the time of day that individuals were captured, with immune measures generally peaking in birds that were sampled in the afternoon (Fig. 3). This was statistically significant in the case of some, but not all, immune measures and the impact of time of day on a given immune measure varied by species (Table 1).

Natural antibody activity varied significantly with capture time in STF and SGF; STF showed significantly higher levels from 14:01 h to 16:00 h, while SGF exhibited significantly lower levels from 12:01 h to 14:00 h compared with other times of day (Table 1, Fig. 4). Complement activity varied significantly with time of day in SGF, with levels decreasing mid-morning then increasing again in the afternoon, whereas in GFC, complement levels were fairly constant throughout most of the day and then increased significantly in the afternoon (Table 1, Fig. 4). PIT54 acute phase protein levels did not vary with the time of day that individuals were sampled (Table 1). Lysozyme acute phase protein levels varied significantly with time of day in four out of five species, generally peaking from late morning through to the early afternoon; lysozyme levels were as much as three times higher during these times compared with other times of day (Table 1, Fig. 4). Interestingly, SGF showed variation with time of day across more measures of innate immune function than the other species.

**DISCUSSION**

In this study, I have shown that measures of innate immune function vary with handling stress over relatively short periods typical of field handling times, and that these measures also vary with the time of day that an individual is sampled. Below, I discuss these findings from an ecological perspective and end with a summary of the practical implications of these findings for field studies of ecological immunology.

**Handling time and innate immune function**

Previous studies examining the impacts of handling stress on innate immune function, specifically bacterial killing ability, have reported mixed results, with some species exhibiting a subtle difference in bactericidal ability 30 and/or 60 min post-capture as compared with 3 min post-capture and others showing no

Table 1. Models of variation in immune function with time of day and handling time

Immune parameter	Species	$R^2_{adj}$	F-ratio	N	d.f.	P-value	Overall mean	Range of means	Max. absolute difference	Max. % difference
Natural antibodies	<b>GFC</b> model	0.0	0.41	94	93	0.97				
	Effect									
	Sex		0.32			0.72				
	Time of day		0.42			0.83	8.18	7.58–8.80	1.22	16
	Handling time		0.29			0.96	8.18	7.40–8.58	1.18	16
	<b>MGF</b> model	0.02	1.18	132	131	0.30				
	Effect									
	Sex		1.28			0.28				
	Time of day		1.84			0.11	5.55	4.43–6.38	1.95	44
	Handling time		0.94			0.48	5.55	5.09–7.20	2.11	41
	<b>SGF</b> model	0.08	2.38	232	231	<b>0.0043*</b>				
	Effect									
	Sex		0.78			0.46				
	Time of day		1.44			0.21	<b>5.45</b>	<b>3.89–6.27</b>	<b>2.38</b>	<b>61</b>
	Handling time		3.15			<b>0.0034*</b>	<b>5.45</b>	<b>4.4–6.57</b>	<b>2.17</b>	<b>49</b>
	<b>STF</b> model	0.10	1.62	89	88	<b>0.089</b>				
	Effect									
	Sex		0.41			0.75				
	Time of day		2.66			<b>0.029*</b>	<b>5.06</b>	<b>4.07–7.0</b>	<b>2.93</b>	<b>72</b>
	Handling time		1.48			0.19	5.06	3.50–6.15	2.65	76
	<b>YW</b> model	0.0	0.62	77	76	0.84				
Complement	Effect									
	Sex		0.46			0.64				
	Time of day		0.71			0.62	5.75	4.40–6.43	2.03	46
	Handling time		0.73			0.65	5.75	5.00–7.50	2.50	50
	<b>GFC</b> model	0.09	0.36	94	93	0.92				
	Effect									
	Sex		1.13			0.33				
	Time of day		3.52			<b>0.0064*</b>	<b>3.46</b>	<b>2.40–5.18</b>	<b>2.78</b>	<b>116</b>
	Handling time		0.66			0.70	3.46	3.00–4.10	1.1	37
	<b>MGF</b> model	0.0	0.81	132	131	0.65				
	Effect									
	Sex		0.18			0.84				
	Time of day		1.19			0.32	1.46	1.26–2.21	0.95	75
	Handling time		0.69			0.69	1.46	1.15–1.8	0.65	57
	<b>SGF</b> model	0.08	2.46	232	231	<b>0.0031*</b>				
	Effect									
	Sex		0.13			0.89				
	Time of day		3.36			<b>0.006*</b>	<b>1.20</b>	<b>0.64–1.62</b>	<b>0.98</b>	<b>153</b>
	Handling time		1.88			<b>0.07</b>	<b>1.20</b>	<b>0.64–1.71</b>	<b>1.07</b>	<b>167</b>
	<b>STF</b> model	0.01	1.06	89	88	0.41				
PIT54 acute phase protein	Effect									
	Sex		0.87			0.46				
	Time of day		0.92			0.47	1.10	0.71–1.30	0.59	83
	Handling time		1.53			0.17	1.10	0.63–1.58	0.95	151
	<b>YW</b> model	0.0	0.50	77	76	0.92				
	Effect									
	Sex		0.18			0.84				
	Time of day		0.73			0.60	1.38	0.60–1.64	1.04	173
	Handling time		0.48			0.85	1.38	1.0–2.25	1.25	125
	<b>GFC</b> model	0.21	2.69	90	89	<b>0.0030*</b>				
	Effect									
	Sex		0.36			0.70				
	Time of day		1.30			0.28	0.68	0.39–1.22	0.83	193
	Handling time		4.15			<b>0.0007*</b>	<b>0.68</b>	<b>0.47–2.26</b>	<b>1.79</b>	<b>380</b>
	<b>MGF</b> model	0.0	0.83	77	76	0.64				
	Effect									
	Sex		0.65			0.53				
	Time of day		1.19			0.33	0.80	0.63–1.12	0.49	78
	Handling time		0.68			0.69	0.80	0.56–0.93	0.37	66
	<b>SGF</b> model	0.04	1.49	166	165	0.12				
	Effect									
	Sex		2.0			0.14				
	Time of day		0.77			0.57	0.81	0.64–0.95	0.31	48
	Handling time		1.7			0.11	0.81	0.40–0.95	0.55	222

Continued

Table 1. Continued

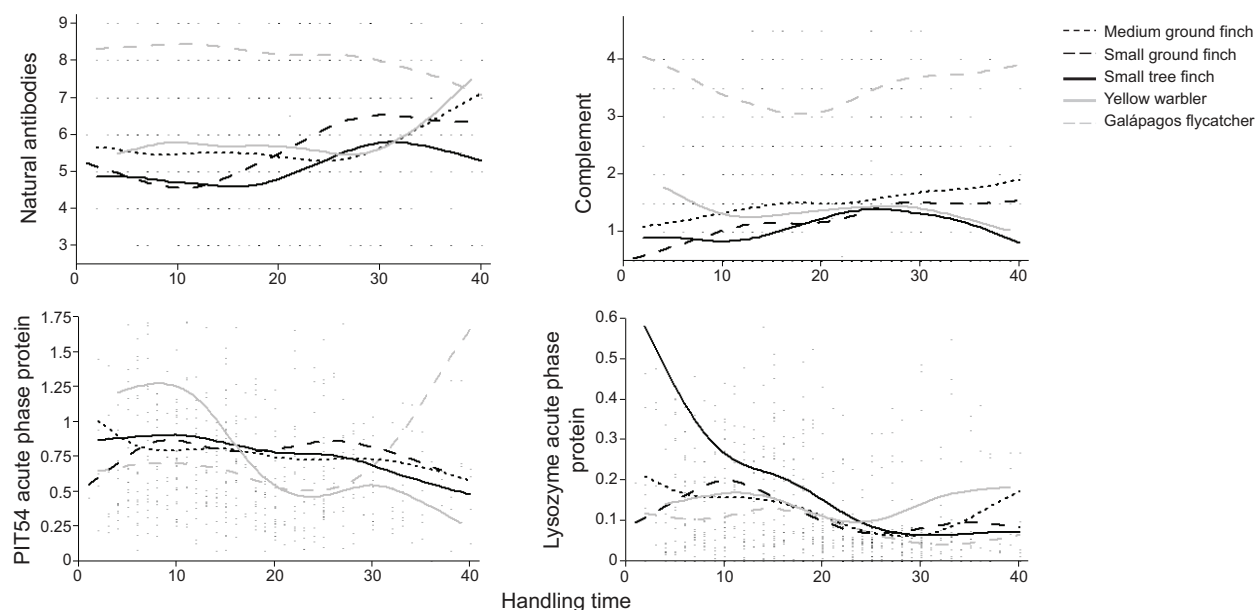
Immune parameter	Species	$R^2_{adj}$	F-ratio	N	d.f.	P-value	Overall mean	Range of means	Max. absolute difference	Max. % difference
Lysozyme acute phase protein	<b>STF model</b>	0.0	0.89	91	90	0.57				
	Effect									
	Sex		0.34			0.71				
	Time of day		1.00			0.42	0.78	0.61–0.92	0.31	51
	Handling time		1.04			0.41	0.78	0.43–0.96	0.53	123
	<b>YW model</b>	0.05	1.31	81	80	0.23				
	Effect									
	Sex		1.39			0.26				
	Time of day		0.85			0.52	0.73	0.25–1.28	1.03	412
	Handling time		1.43			0.21	0.73	0.35–1.46	1.11	317
	<b>GFC model</b>	0.03	1.22	100	99	0.27				
	Effect									
	Sex		1.10			0.34				
	Time of day		1.01			0.42	0.092	0.060–0.21	0.15	250
	Handling time		0.79			0.60	0.092	0.027–0.18	0.15	57
	<b>MGF model</b>	0.24	3.86	128	127	<b>&lt;0.0001*</b>				
	Effect									
	Sex		0.61			0.55				
	Time of day		5.23			<b>0.0002*</b>	<b>0.13</b>	<b>0.057–0.30</b>	<b>0.24</b>	<b>421</b>
	Handling time		2.90			<b>0.008*</b>	<b>0.13</b>	<b>0.021–0.23</b>	<b>0.21</b>	<b>995</b>
	<b>SGF model</b>	0.13	3.59	240	239	<b>&lt;0.0001*</b>				
	Effect									
	Sex		0.39			0.68				
	Time of day		4.45			<b>0.0007*</b>	<b>0.13</b>	<b>0.080–0.29</b>	<b>0.21</b>	<b>263</b>
	Handling time		3.18			<b>0.0031*</b>	<b>0.13</b>	<b>0.059–0.21</b>	<b>0.15</b>	<b>256</b>
	<b>STF model</b>	0.54	8.17	92	91	<b>&lt;0.0001*</b>				
	Effect									
	Sex		0.46			0.71				
	Time of day		6.08			<b>&lt;0.0001*</b>	<b>0.18</b>	<b>0.089–0.29</b>	<b>0.20</b>	<b>226</b>
	Handling time		12.09			<b>&lt;0.0001*</b>	<b>0.18</b>	<b>0.062–0.26</b>	<b>0.20</b>	<b>319</b>
	<b>YW model</b>	0.19	2.26	76	75	<b>0.015*</b>				
	Effect									
	Sex		0.24			0.79				
	Time of day		4.46			<b>0.0016*</b>	<b>0.14</b>	<b>0.088–0.35</b>	<b>0.26</b>	<b>298</b>
	Handling time		1.34			0.25	0.14	0.066–0.19	0.12	19

For each model, the table gives overall  $R^2_{adj}$ , F-ratio, sample size (N), degrees of freedom (d.f.) and P-value. In addition, for each model effect, the table provides the F-ratio and P-value. Bold font indicates values significant at the  $\alpha=0.1$  level, asterisks indicate values significant at the  $\alpha=0.05$  level. Max. % difference is calculated as the (maximum absolute difference/minimum mean)×100.

difference in bactericidal activity with handling time (Buehler et al., 2008; Matson et al., 2006; Millet et al., 2007). In contrast, I found strong and substantial differences (as much as 4- to 10-fold changes; Table 1) in multiple measures of innate immune function with handling time (although the impact of handling time on changes in innate immune function varied both with immune measure and by species). The difference between my findings and those of previous studies on different measures of immune function could suggest that the measures I used are more sensitive to handling stress. However, it could be a result of the different time frames over which this and other studies were conducted. Out of the six measures of innate immune function that varied significantly with handling time in my study, three of them no longer showed a statistically discernible difference 35:01–40 min post-capture compared with 0–5 min post-capture (this is true of natural antibodies in SGF, and lysozyme in MGF and SGF); if the observed differences reflect changes in immune function over time with handling stress, then it appears that innate immune function may change quickly in response to handling stress and then revert to normal levels in a relatively short period of time. However, I did not collect samples more than 40 min post-capture; therefore, more data are needed to ascertain whether these data do indeed capture a reversion to baseline immune function levels or whether they represent a small window on to an ongoing change in

immune function over time in response to capture stress. In addition, the amount of time elapsed between capture and blood collection in this study was largely determined by field conditions; therefore, it is possible that the variation in immune function with handling time reflects influences other than handling stress. For example, average time until blood sample collection increased with the number of birds captured at a given time. Therefore, a relationship between gregariousness and immune function could help explain the observed variation in immune function with handling time. An experimental study utilizing repeated measures of the same individuals across multiple time points from 0 to 40 min post-capture would help to elucidate this issue.

Interestingly, out of the four immune parameters that showed statistically discernible changes over time, three (PIT54 acute phase protein, natural antibody and complement activity) increased with handling time. These findings are in contrast to the common assumption in the ecological literature that stress is necessarily immunosuppressive, but are in keeping with many studies of the impacts of stressors on domestic animals (Martin, 2009). This observed increase in immune parameters in response to a stressor is in keeping with the idea that the ‘stress response’ is best viewed as a mechanism by which individuals can maintain homeostasis in the wake of unpredictable events (Wingfield et al., 1998). Some of the most important stressors that a wild animal will

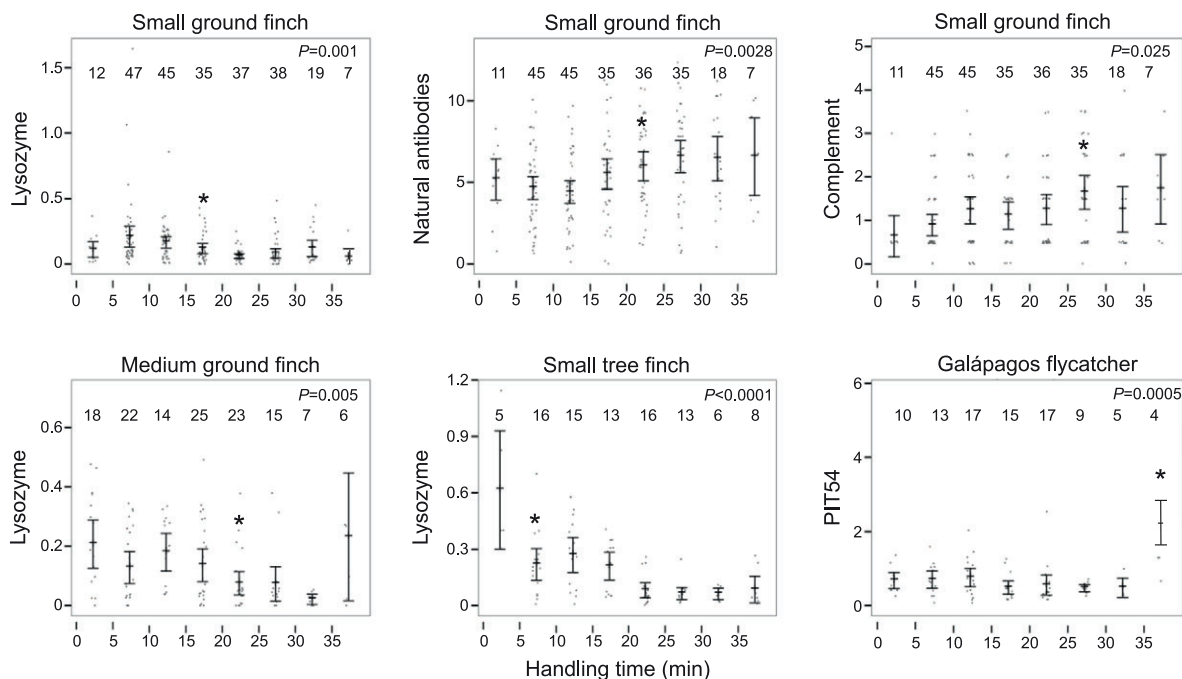


**Fig. 1. Change in innate immune function metrics with handling time.** Splines show the change in immune parameters over time by species ( $\lambda=1000$ ). Note: in all cases, the x-axis has been adjusted so that splines are discernible; therefore, not all data points contributing to the splines are visible.

encounter include failed predation attempts, conflicts over territories or access to mates that escalate to physical aggression, wounds and infections (Martin, 2009). In this context, it would be counter-productive, and potentially even fatal, to suppress the very system needed to heal wounds or avoid colonization by pathogens (Dhabhar and McEwen, 1997). Indeed, in support of the idea that immune function changes adaptively in response to immediate increases in infection risk, stressors lasting just minutes or days tend to elevate immune function, while only long-term stressors

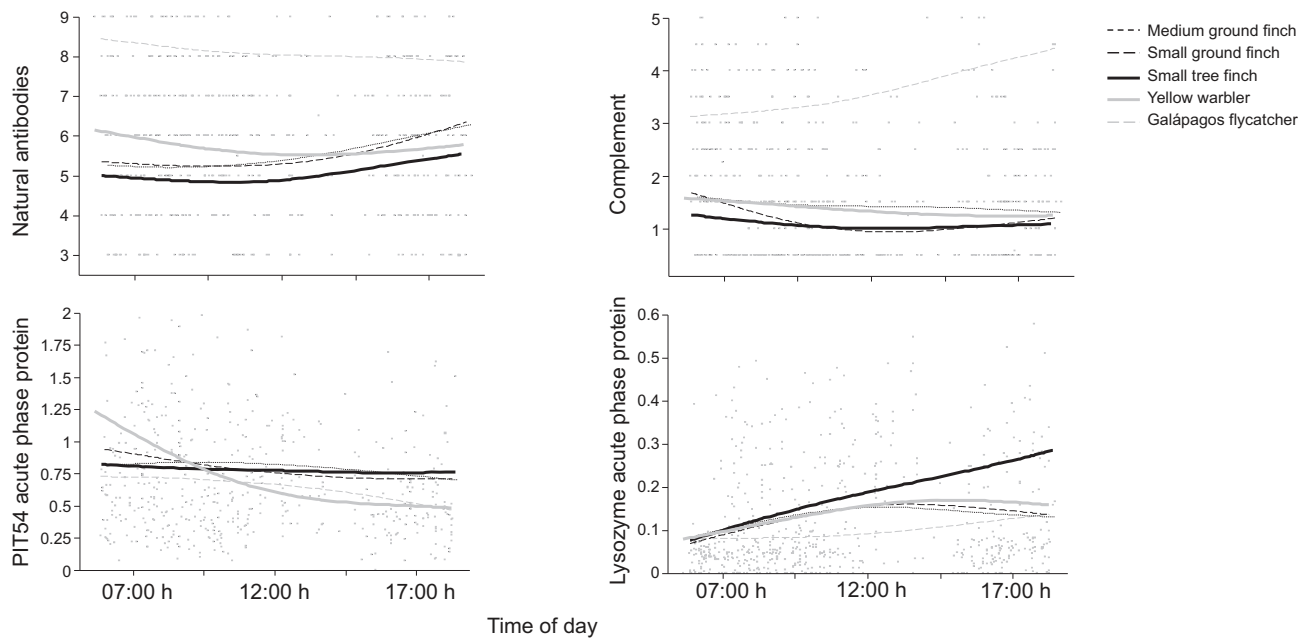
suppress immune function. Furthermore, it is innate immune responses that tend to be elevated most quickly following exposure to an acute stressor (Martin, 2009), preparing an individual to fight off nascent and novel infections.

Lysozyme is the one measure of innate immunity that decreased with handling time and it did so in three species (MGF, SGF and STF). However, in both SGF and MGF, this decrease only lasted 10–15 min, after which time lysozyme levels had returned to baseline levels in MGF, and had begun to increase in SGF. Only



**Fig. 2. Variation in immune function with handling time.** The figure includes only those parameters that showed statistically discernible differences between groups (Table 1); an asterisk indicates the first time period that is statistically different from a preceding time period at the  $\alpha=0.05$  level after Holm–Bonferroni correction for multiple tests. Sample size ( $N$ ) is indicated at the top of each graph, along with the ANOVA  $P$ -value testing for differences between groups. Group mean is indicated by a horizontal black line and bars around this line show the 95% confidence interval.





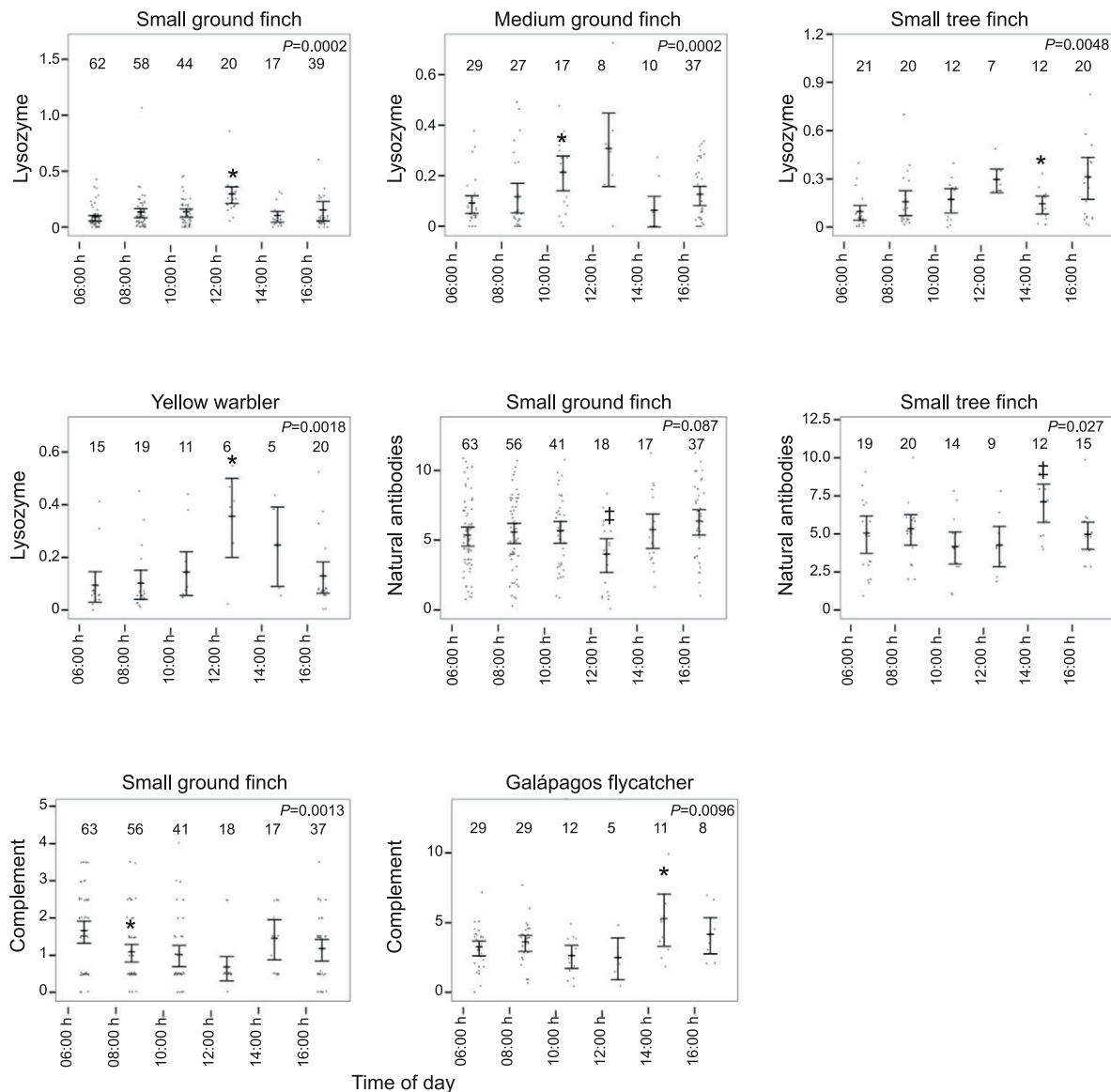
**Fig. 3. Change in innate immune function metrics with time of day.** Splines show the change in immune parameters over the day by species ( $\lambda=0.1$ ). Note: in all cases, the x-axis has been adjusted so that splines are discernible; therefore, not all data points contributing to the splines are visible.

STF showed a long-lasting decrease in lysozyme, which remained in effect until the final time point, 35:01–40 min after capture. It has been suggested that decreases in specific immune parameters as a result of acute stress are not representative of overall decreases in immune function, but rather represent a redistribution of resources in order to maximize the efficacy of an immune response (Braude et al., 1999). This may explain why we see increases in some immune parameters (natural antibodies and complement) and decreases in others (lysozyme) within a single species (SGF). Furthermore, the rapid recovery that we saw in lysozyme levels in SGF and MGF is theoretically in keeping with immune redistribution, which has the potential to change rapidly in response to the needs of the individual (Braude et al., 1999; Dhabhar and McEwen, 1997). In contrast, stress-mediated suppression of the immune system is not in keeping with the rapid rebound in lysozyme levels that I observed in MGF and SGF, though it remains a possible explanation for the decrease in lysozyme seen in STF. In the case of SGF and MGF, the acute stress that individuals were experiencing had not ended at the time that lysozyme levels rebounded (Wingfield et al., 1995) and, as a result, stress-induced increases of corticosterone would not have abated; this makes it unlikely that a stress-related suppression of the immune system would have lasted for the short period of time that lysozyme was observed to decrease in MGF and SGF.

The non-linear variation in immune function in response to a stressor documented here suggests, at a minimum, that caution must be used in choosing sampling windows when measuring innate immune function at a single time point and that sampling should be highly standardized. More importantly, the observed pattern of differences in immune parameters and individual variation in this change profile are likely to reflect biologically meaningful differences between individuals and species; this suggests that studies of innate immune function may benefit from measuring immune time series, much as 'stress series protocols' have become common in the study of glucocorticoids because of the additional information gained from examining the change in an individual's

glucocorticoid levels over time (Wingfield et al., 1995). For example, the magnitude of the change in certain immune parameters may be important to limit colonization by a pathogen, while the speed at which these parameters return to normal, or the relationship between these two measures, could be an important factor in determining the risk of self-harm due to the immune response (Boughton et al., 2011; Haine et al., 2008; Koenen et al., 2002; Korner and Schmid-Hempel, 2004).

More work is needed to examine curvilinear immune responses across species (hereafter termed the 'immune response profile'), to identify the optimal sampling points to capture these immune response profiles, and to determine the implications of individual variation in the immune response profile on the outcome of exposure events (i.e. the impact of variation in the immune response profile on an individual's ability to resist colonization or clear infection). In examining the impact of immune response profiles on the risks of self-harm stemming from activation of the immune response, it will be important to consider that self-harm can manifest in a variety of ways (Boughton et al., 2011), including but not limited to auto-reactive damage (Sadd and Siva-Jothy, 2006), auto-immunity (Day et al., 2007), increased use of important resources (i.e. energy, nutrients or antioxidants) (Klasing, 1998; Lee et al., 2006; Martin et al., 2003), opportunity costs (i.e. decreased defense, foraging, mating, investment in offspring) (Owen-Ashley and Wingfield, 2007; Zylberberg, 2014; Zylberberg et al., 2014) or increased sickness behavior (Zylberberg et al., 2013a). Furthermore, it is interesting that different species exhibit different immune response profiles. It is possible that this reflects variation in life history strategies of the different species studied here. Indeed, it has been suggested that there may be differences in immune responses to handling stress between tropical and temperate birds (Buehler et al., 2008). However, it will require comparative data from a wide variety of species before we can begin to understand the relationship between different life history strategies and immune response profiles. This is likely to be a rich topic for investigation; future



**Fig. 4. Variation in immune function with time of day.** The figure includes only those parameters that showed statistically discernible differences between groups (Table 1); an asterisk indicates the first time period that is statistically different from a preceding time period at the  $\alpha=0.05$  level after Holm–Bonferroni correction for multiple tests, while a double dagger indicates significance at the  $\alpha=0.1$  level after Holm–Bonferroni correction. Sample size (N) of groups is indicated at the top of each graph, along with the ANOVA P-value testing for differences between groups. Group mean is indicated by a horizontal black line and bars around this line show the 95% confidence interval.

studies should examine immune response profiles in a variety of species, including temperate and migratory species and species that live in different environments, exhibit different life histories, as well as in non-passerine and non-avian species to ascertain whether the patterns described here hold true across species.

#### Time of day and innate immune function

I found significant and substantial variation in several aspects of innate immune function as a result of the time of day that individuals were sampled, though this varied by species (natural antibodies in SGF and STF, complement in SGF and GFC, and lysozyme in MGF, SGF, STF, YW all varied significantly with the time of day that individuals were sampled). I know of no other study that has examined the effects of time of day on immune function in wild animals. However, these results are in keeping with studies that have shown that glucocorticoids vary with time of day in a variety of species, including passerine birds (Breuner et al., 1999; Dhabhar and

McEwen, 1997; Dickstein et al., 1991; Janssens et al., 1995; Rich and Romero, 2001). In the case of diel rhythms in glucocorticoids, it has been suggested that fluctuations in hormone levels may be beneficial for individuals in the wild by adjusting their responses to stressors in ways that are most appropriate at different times during the day (Breuner et al., 1999), possibly reflecting differences in activity level or metabolic demands. Given that fluctuations in glucocorticoid levels can alter immune function (as discussed above), and that changes in glucocorticoids can have particularly rapid impacts on innate immune function (reviewed by Martin, 2009), it is perhaps not surprising that immune function varies with time of day.

This raises an intriguing question: could diel changes in immune function be adaptive? It has been shown that variation in affiliative, aggressive and exploratory behaviors and social competition correlate with variation in innate immune function at the individual level (Hawley et al., 2006; Zylberberg, 2014; Zylberberg et al., 2013a, 2014); in this context, it has been suggested that individuals may

**Table 2. Sample collection recommendations**

Immune parameter	Maximum handling time (min)	When/how to sample to minimize variation associated with time of day
Natural antibodies	15	Morning
Complement	15	Limit sampling to a 2–4 h window
PIT54 acute phase protein	30	Any time
Lysozyme acute phase protein	5–15, depending on species	Sample before 10:00 h and/or after 14:00 h

balance investment in behavioral and immunological pathogen defenses (Zylberberg et al., 2013a). Thus, as individuals engage in different activities over the course of the day (i.e. resting versus foraging versus aggressive interactions with conspecifics), their immediate risk of encountering pathogens or being wounded changes, and allocation of immune resources may change accordingly. Interestingly, changes in temperature, which are often associated with behavioral changes in wild animals, have also been shown to impact immune function (Hangalapura et al., 2003). If changes in physiology and behavior over the course of the day alter an individual's likelihood of encountering certain stressors, which in turn alters an individual's need for and investment in certain aspects of immune function, then variation in physiology, behavior and ecology between species may explain why I see different patterns of change in innate immune function metrics in different species. Comparative data from a range of species will help to elucidate the links between species ecology, behavior, life history strategy and diel patterns in immune function. Because I did not measure specific individuals multiple times, there are alternative explanations that I cannot rule out; for example, if variation in behavioral type is correlated with the time of day that an individual is captured, this could help to explain the observed correlation between the time of day and immune function. Therefore, while my data are intriguing, further investigation is needed to ascertain whether individual immune function does indeed vary over the course of the day and, if so, how this relates to other aspects of individual biology, including hormone levels and behavior.

### Practical implications

Several measures of innate immune function vary significantly and substantially over relatively short handling periods as well as over the course of the day. Therefore, in order to make reliable comparisons, either within or between species, and to examine the relationship between immune parameters and factors of biological interest, sampling protocols must be highly standardized and must include relatively narrow sampling windows. Below, I outline specific recommendations for obtaining reliable measurements of natural antibodies, complement, PIT54 and lysozyme acute phase proteins (Table 2). However, it is important to note that these recommendations are based on just five species of passerine from an island system. It will be important to conduct similar studies in a range of species from a variety of habitats, as well as examining how factors such as life history, breeding status, age or time of year may alter the variation in immune function documented here. In addition to the recommendations outlined below, researchers should keep in mind that measures of immune function have been shown to vary between plasma and serum samples, and that periods of greater than 12 h between blood collection and plasma or serum collection may also impact immune measurements (Hoye, 2012).

Natural antibodies and complement are both easily measured with a single assay (Matson et al., 2006). Therefore, it is advisable when using these metrics to obtain blood samples within the more conservative 15 min window required for confidence in natural antibody measurements. Furthermore, the variation and lack of consistency in measurements over the course of the day suggests that sampling should be restricted to a narrow window of time to avoid introducing measurement error associated with time of day. Alternatively, if this is not possible or desirable, then care should be taken to ascertain the study species' pattern of variation over the course of the day and this should be taken into account when interpreting the resulting data.

In contrast, PIT54 appears to be a relatively robust measurement of individual innate immune function, and one that can be obtained regardless of time of day, as long as blood samples are taken within 30 min of capture.

Lysozyme showed the most consistent variation across species, changing with handling time in three species and showing similar, though not statistically discernible, patterns in the other two species studied. The consistent and substantial variation (2.5- to 10-fold changes) in lysozyme levels suggests that this measure of innate immune function should be interpreted with caution. It should only be measured in samples collected within short handling times and narrow time windows during the day. Furthermore, given the rapid drop in one species (STF), it is highly advisable to empirically determine the maximum handling time that yields a reliable measurement for individual study species.

### Conclusions

I have shown that innate immune function varies with handling stress over relatively short periods typical of field handling times, and that measures of innate immune function also vary with the time of day that an individual is sampled. Rather than being a mere artifact of measurement, I suggest that this variation is likely to have important adaptive functions. The non-linear variation in immune function in response to a stressor may represent the real and important need for individuals to limit colonization by a pathogen while at the same time limiting the risk of self-harm due to the immune response. Similarly, diel patterns in immune parameters may represent the organism's optimization of immune investment in relation to changing risks of pathogen exposure or wounding throughout the day, just as diel changes in glucocorticoid physiology may allow an individual to optimize their response to stressors depending on changing circumstances throughout the day. Much more work is needed to understand patterns of immune change over time and to elucidate the underlying reasons for individual and species-level variation in immune response profiles and diel immune variation. In conducting this and other research focusing on ecological immunology, protocols for collection of immune samples must be highly standardized and must include relatively narrow sampling windows (Table 2) in order to yield reliable comparisons, allowing us to continue to elucidate the complex and remarkable relationships between immune function, ecology and evolution.

### MATERIALS AND METHODS

I conducted this study on Santa Cruz Island, Galápagos, Ecuador (00°38'S, 90°22'W) during the breeding season (January–February 2008 and 2009). Birds were captured using passive mist netting and mist nets were actively watched. Birds were removed from the mist net and processed as quickly as possible; in the event that multiple birds entered the mist net, they were removed and kept in cloth bags until they could be banded and a blood sample collected. I banded each bird and collected blood samples using



heparinized microcapillary tubes within 40 min of capture. The time between capture and collection of a blood sample was measured as the time elapsed between when the bird was observed to enter the mist net and the time that collection of the blood sample was completed. Each sample was kept on ice until it was centrifuged, and the plasma was collected and frozen; for each sample, plasma was collected within 10 h of blood sample collection in order to minimize introduction of measurement artifacts as a result of sample handling (Hoye, 2012). I used handling time (time elapsed between capture and obtaining a blood sample) as a proxy for handling stress. Birds were processed with the approval of University of California Davis IACUC protocol 13171.

### Immune function

I measured four aspects of innate immune function in my study species: natural antibodies, complement, PIT54 acute phase protein and lysozyme acute phase protein. I chose these four aspects of the innate immune system because each plays an integral and complementary role in the first line of defense against novel pathogens (as previously discussed in Zylberberg et al., 2013b). In short, the complement system of proteins activates the lysis of foreign cells, enhances antibody activity and directly destroys viruses (Hirsch, 1982; Janeway et al., 2001). Natural antibodies bind novel pathogens, facilitate phagocytosis and promote cell lysis (Caroll and Prodeus, 1998; Casali and Schettino, 1996). The PIT54 protein minimizes self-damage during inflammation and stimulates the white blood cell response upon pathogen exposure (Quaye, 2008; Wicher and Fries, 2006). Lysozyme lyses gram-positive bacteria (Millet et al., 2007). Together, these measures afford a broad view of the innate immune system by providing information on both inducible and constitutive components of innate immunity.

While immune parameters can be expected to change with infection status, when recorded in healthy individuals these measures provide information on investment in both innate and adaptive immunity. Therefore, to simplify interpretation of the results, I limited my analysis of immune parameters to individuals showing no signs of avian pox, a common viral infection in the Galápagos avifauna (Zylberberg et al., 2012, 2013b), and no clinical signs of infection with other pathogens or other illness; for example, I excluded individuals with abnormal crusts, growths or excessive lethargy. However, it is possible that some individuals that showed no clinical signs of infection and were, therefore, included in this study were infected with parasites or pathogens. Because of the small sample sizes, I was not able to run each immune test on every individual; in cases where there was not enough plasma to run all immune assays, I randomly selected which of the immune assays to run (natural antibodies and complement, PIT54 and/or lysozyme).

I used a hemolysis–hemagglutination assay to measure levels of natural antibody and complement activity (Matson et al., 2005). I used a commercial kit (Tri-delta Diagnostics Inc., Morris Plains, NJ, USA) to determine the plasma concentration of PIT54 acute phase protein following Millet et al. (2007). I used the lyso-plate assay described in Millet et al. (2007) to measure levels of lysozyme in plasma samples. Each of these assays was carried out with previously described modifications for small sample sizes (Zylberberg et al., 2014).

### Analysis

I used a separate linear regression model for each species to determine whether immune function varied with the time of day that an individual was captured and handling time; these models had time of capture, handling time and sex as the explanatory variables. To determine whether measured immune function varied with time of day, individuals were assigned to one of five groups based on time of capture (06:00 h–08:00 h, 08:01 h–10:00 h, 10:01 h–12:00 h, 12:01 h–14:00 h, 14:01 h–16:00 h and 16:01 h–18:00 h); I used ANOVA to test for variation in immune function between these groups. Similarly, to determine whether immune function varied with handling time, individuals were assigned to one of eight groups based on time elapsed (0–5 min, 5:01–10 min, 10:01–15 min, 15:01–20 min, 20:01–25 min, 25:01–30 min, 30:01–35 min and 35:01–40 min); I used ANOVA to test for variation in immune function between these

groups. Statistical analyses were conducted using JMP 7.0.1 by SAS; Figs 2 and 4 were created in R version 3.1.1 using ggplot2 (R Core Team, 2014; Wickham, 2009).

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### Competing interests

The author declares no competing or financial interests.

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