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Immunocompetence and testosterone-induced condition traits in male ruffs (*Philomachus pugnax*)

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Abstract—The immune system is costly to operate, so we expect allocation to this function to relate to other indices of overall phenotypic condition. In captive male ruffs (*Philomachus pugnax*), we examine covariation between three seasonal male characteristics induced by testosterone (neck 'ruff', facial wattles, and increases in mass/size) and cell-mediated and humoral immunity. The ruff has two genetically distinct morphs of males, 'independents' and 'satellites', which differ in mating behaviour, somewhat in body size, and possibly life history strategy. Ruff length and wattle number were significantly correlated with each other and with body size, but unrelated to mass/size. Humoral immunity was weakly correlated with size, but not with secondary male traits, or with cell-mediated immunity. We did not detect differences in humoral immunity between morphs. Cell-mediated immunity was weakly correlated with ruff length, but not with wattle number, size, or mass/size. Cell-mediated immunity decreased significantly with age. Controlling for age, independents had higher cell-mediated immunity responses than satellites. The expected relationships between two measures of immune function and other measures of condition were not always present, which suggests we need more data and/or more specific predictions about how the different aspects of immune function relate to each other and to ecological variables.

INTRODUCTION

The idea that maintenance of immune function is expensive, energetically or otherwise, is supported by recent work that demonstrates trade-offs between immune function and other fitness components. For example, in collared flycatchers (*Ficedula albicolis*), experimental increases in brood size decreased parental humoral immunity on one of the two sites examined (Nordling et al., 1998). In male golden hamsters (*Mesocricetus auratus*), repeated daily exposure to the opposite

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sex, which did not necessarily include coitus, lowered primary immune responses (Krees et al., 1989; Ostrowski et al., 1989). In pied flycatchers (*Ficedula hypoleuca*), females given an immune challenge decreased the amount of maternal care (Ilmonen et al., 2000). In house sparrows (*Passer domesticus*), an immune challenge generally led to decreased feeding rates (Bonneaud et al., 2003). Therefore, there is increasing evidence that activities such as courting, mating and offspring-rearing can have detrimental effects on immunocompetence and vice versa. Our current challenge is to understand under what circumstances responses are, or are not, detected.

One approach is to manipulate the independent variable (e.g., brood size) and then observe the effects on immune function. An unmanipulated animal is expected to allocate available resources to all required functions optimally, neither neglecting nor devoting undue resources to any one system, whereas, in the manipulated system, a negative correlation is expected: in the unmanipulated situation the same energetic trade-offs predict a positive correlation between immune function and other condition indices (Lochmiller and Deerenberg, 2000). Even in such unmanipulated situations, immunocompetence is expected to differ between classes of animals (e.g., sexes or ages) because of differences in physiology (e.g., sex hormones — Schuurs and Verheul, 1990; stress hormones — Sapolsky, 1992) or, at the ultimate level, life history (Folstad and Karter, 1992).

Noting that testosterone causes the development of secondary sexual traits in most male vertebrates, and that it also depresses immune function, Folstad and Karter (1992) proposed the ‘immunocompetence handicap hypothesis’, which argues that individual males face a testosterone-mediated trade-off between the allocation of resources to secondary sexual traits or to immune function. The hypothesis fits within the more general theory of ‘indicator traits’ or ‘honest signals’, in predicting that an individual animal’s allocation of resources to the development of secondary sexual characters should correlate positively with other aspects of condition (Zahavi, 1977; Kodric-Brown and Brown, 1984; Andersson, 1986), including immune function (Folstad and Karter, 1992; Lozano, 1994; Blount et al., 2003; Saino et al., 2003).

Several studies have addressed differences in immunocompetence between the two sexes (reviewed by Grossman, 1985; Schuurs and Verheul, 1990). In this study, we examine the humoral and cell-mediated immunity within males in the ruff (*Philomachus pugnax*), a bird in which there are two genetically, morphologically, and behaviourally different types of males, ‘satellites’ and ‘independents’, which may also differ in life history strategies (Hogan-Warburg, 1966; van Rhijn, 1991; Lank et al., 1995, 1999; Widemo, 1998).

In this study, we assessed the responses of 28 adult males to two immunological challenges in relation to their body size, age, morph, and three characters directly induced by testosterone levels at the onset of the breeding season (Lank et al., 1999): an increase in body mass/size and the extent of development of two seasonal male secondary sex characteristics: ornamental plumage and facial wattles. We predicted that birds that had initiated development of ornamental feathers earlier would be in better condition (e.g., Piersma and Jukema, 1993). We also predicted that immunocompetence would be positively correlated with the degree of development of these testosterone-induced characters. Finally, it has been suggested that the morphs have somewhat different life histories, with independents being more successful per breeding bout, but satellites starting to breed at younger ages and/or living longer (Widemo, 1998). If satellites live longer than independents, one would expect them to invest more on self-maintenance; therefore, we also tested whether satellites had stronger immune responses than independents.

MATERIALS AND METHODS

Study animal

The ruff is a sandpiper that breeds across the lower Palearctic and winters primarily in Africa (van Rhijn, 1991). It is unique among birds in having two behaviourally and genetically distinct kinds of males, termed independents and satellites (Hogan-Warburg, 1966; van Rhijn, 1991). This dimorphism among males results from a single-locus two-allele autosomal gene (Lank et al., 1995, 1999). Independents are slightly larger and heavier than satellites, but there is extensive overlap in size (Höglund and Lundberg, 1989; Bachman and Widemo, 1999). Independents establish leks with tightly packed courts, which they defend against other independents; satellites join independents in transitory alliances that attract females to co-occupied courts (van Rhijn, 1991; Hugie and Lank, 1997; Widemo, 1998). Females visit leks, mate with one or more males (Lank et al., 2002), and nest and rear young on their own. As is typical for lekking species, there is a strong skew in reproductive success among males, but satellites and independents achieve equal mean reproductive success, as required to maintain a genetic dimorphism. An equilibrium morph ratio of ca. 84% independents and 16% satellites is maintained (van Rhijn, 1991; Hugie and Lank, 1997; Widemo, 1998; Thuman, 2003).

Prior to and during northward migration, male ruffs moult into a breeding plumage that includes two tracts of elaborate display feathers: the ‘ruff’, a semicircular tract of display feathers around the neck, and ‘head tufts’, which arise from occipital tracts. During the breeding season, male ruffs display highly polymorphic plumages, both in coloration and pattern (Cramp and Simmons, 1983), with independents more heavily pigmented than satellites (Hogan-Warburg, 1966). Towards the completion of ruff growth, males lose feathers on their faces and develop variable numbers of hard papilla-like structures, referred to as ‘wattles’, around the eyes and at the base of the bill (Cramp and Simmons, 1983). At the end of the breeding season, ruff feathers are dropped, wattles ‘wither’ and the area is covered by new feathers. The number of ruff feathers, the timing of onset of their growth, and the extent of wattle growth (number and area on the rostrum) increase with male age, and are retarded and reduced for males in poor body condition (Lank, unpubl.). Wattle development and ruff growth do not occur in castrated males (van Oordt and Junge, 1936), and both characteristics are directly stimulated by experimental increase in testosterone level in females (Lank et al., 1999) and in males (Lank, unpubl.).

Territorial independent males (i.e., 'residents') lose mass during the breeding season (Bachman and Widemo, 1999). Correcting for body size, non-territorial independents are heavier and fatter than territorial independents and satellites. These patterns suggests that stored body reserves early in the season facilitate maintaining territory ownership, which in turn is positively correlated with reproductive success among independents (Lank and Smith, 1987; Hill, 1991; Widemo, 1997). Hence, mass relative to size is important to fitness, at least for independents. Male body mass increases in response to experimental increase in testosterone level (Lank, unpubl., and see Lank et al., 1999).

General methods

Birds used in this study were part of a captive breeding flock originally derived from eggs collected near Oulu, Finland, in 1985, 1989 and 1990. The flock has been maintained outdoors in Burnaby, British Columbia, Canada, under natural photoperiod since 1994, with ad libitum food and water. For this experiment, 28 males, consisting of 19 independents and nine satellites, were housed together as a flock, which allowed for extensive male-male social interactions. None were inbred. The males were aged 3-9 years; in the wild, few males return to the same area as breeders for more than 3-5 years (van Rhijn, 1991), and mean life span for ruffs past the chick stage has been estimated at 4.4 years (Scheufler and Stiefel, 1985).

All morphological measurements were taken the same day, 13 May, hence capturing the considerable variation that exists in the developmental timing of ruff feathers and wattles early in the breeding season. Measures of body size were mass, tarsus, and culmen, which were also combined into a single measure (PC1) using a principal components analysis. To index relative development of secondary sexual traits, we measured ruff length and counted facial wattles. Ruff length was measured with a semicircle of circular graph paper mounted on cardboard with the central 2.54 cm half circle cut out. The male's neck was held firmly in the cut-out by placing a finger across the opening, ruff feathers were flattened against the graph paper, and the longest feather was measured to the nearest 1.27 cm (half inch). The number of facial wattles on the right side of the head, which ranged from 0 to 80, was estimated to the nearest five.

Measures of immune condition

In vertebrates, immune function is divided into natural (innate, or non-adaptive) and acquired (or adaptive) immunity; the latter is subdivided into cell-mediated and antibody mediated (humoral) immunity (Roitt et al., 1996). Here responses to two immunological challenges were assessed concurrently: a sheep red blood cell (SRBC) hemagglutination test (Wegmann and Smithies, 1966; Aitken and

Parry, 1974; Klasing, 1988; Deerenberg et al., 1997) and a subcutaneous delayed hypersensitivity response (Glick, 1986; Corner and DeLoach, 1990; Roitt et al., 1996) to assess humoral and cell-mediated acquired immunity, respectively. The SRBC hemagglutination assay entails exposing the animal to SRBC and quantifying the resulting production of SRBC-antibodies. The delayed hypersensitivity test consists of an injection of a mitogen that causes T-lymphocytes to mobilise to and proliferate at the area of injection.

Immunological challenges started on 13 May, after measuring morphological traits. Each bird was injected intraperitoneally with SRBC (Sigma Product No. R 3378) in Phosphate Buffered Saline (PBS, $\mathrm{pH} = 7.2$, approximately 5×10^8 cells/ml, hematocrit $\sim 2\%$), using a dosage of 0.1 ml/100 g of body mass. One week later, a 0.5-1.0 ml blood sample was taken from each bird. Samples were centrifuged to separate the plasma, which was stored at under $-20°\mathrm{C}$ until analysis (Wegmann and Smithies, 1966; Aitken and Parry, 1974).

Following the SRBC injection, each bird was given a sub-dermal injection of 0.05 ml Phytohemagglutinin (PHA-P Sigma Product No. L8754) solution (2 mg PHA/ml PBS) in the patagium of one wing, and as a control, the other wing was injected with 0.05 ml of PBS. PHA is positively chemotactic and mitogenic with respect to T-lymphocytes, and causes a small swelling at the site of injection. The thickness of the PHA-injected and control patagia were measured 24 h post-injection with a pressure-sensitive micrometer. Three measurements were taken from each patagium and averaged to obtain a single measure per patagium per bird (repeatability > 0.9, Zuk and Johnsen, 1998; Smits et al., 1999; Lozano and Lank, 2003). The difference in thickness of the PHA-injected versus the PBS-injected side indicates the strength of the response (Glick, 1986; Corner and DeLoach, 1990; Roitt et al., 1996).

Hemagglutination assay

Twenty-five μl of plasma from each sample was serially diluted with 25 μl of PBS, using a V-shaped 96-well microtiter plate, resulting in a two-fold dilution series (i.e., $2^{-1}, 2^{-2}, 2^{-3}, 2^{-4}, \ldots$). Twenty-five μl of a suspension of 2% SRBC in PBS was then added to each well. The plate was incubated at 41°C for 1 h and examined visually to determine the highest dilution (= lowest concentration) of plasma that led to an agglutination of SRBC. Results are expressed as the negative of the base-2 dilution factor (Wegmann and Smithies, 1966; Aitken and Parry, 1974).

Statistical analyses

Pearson's correlations were used to test associations between continuous variables, and t-tests and ANCOVAs were used to examine differences between the two morphs. One-tailed tests were used where indicated when examining directional predictions involving expected differences between independent and satellite males, based on previous knowledge and theory, as outlined above, and to test the positive relationships expected among measures of conditions in this observational study. All analyses followed the standard tests for homoscadasticity and homogeneity of variance when appropriate. Statistical significance was accepted at $P < 0.05$.

RESULTS

Size and condition

Tarsus length did not differ significantly between the two morphs, but independents had longer culmens (35.9 vs. 35.1 mm, *t*-test: $t_{26} = 1.94$, one-tailed $P = 0.03$) and were more massive (182.7 vs. 168.3 g, $t_{26} = 2.29$, one-tailed $P = 0.015$) than satellites.

Independents were more massive than satellites even when accounting for body size, either by means of an ANCOVA with tarsus as the covariate (Morph $F_{[1,25]} = 4.92$, one-tailed $P = 0.018$, Tarsus $F_{[1,25]} = 0.42$, one-tailed $P = 0.74$, interaction NS; fig. 1), or simply comparing mass/tarsus ($t_{26} = 2.193$, one-tailed $P = 0.019$, fig. 2).

The two morphs did not differ in the developmental stage of seasonal secondary sexual characters. Ruff lengths ranged from 1.0 cm to 8.0 cm, but all but one fell between 5.5 cm and 8.0 cm. Excluding this outlier, the two morphs did not differ significantly ($t_{25} = 0.03$, $P = 0.98$). Wattle number varied from 0 to 80 and, again, the two morphs did not differ significantly ($t_{26} = 0.59$, $P = 0.56$). Neither

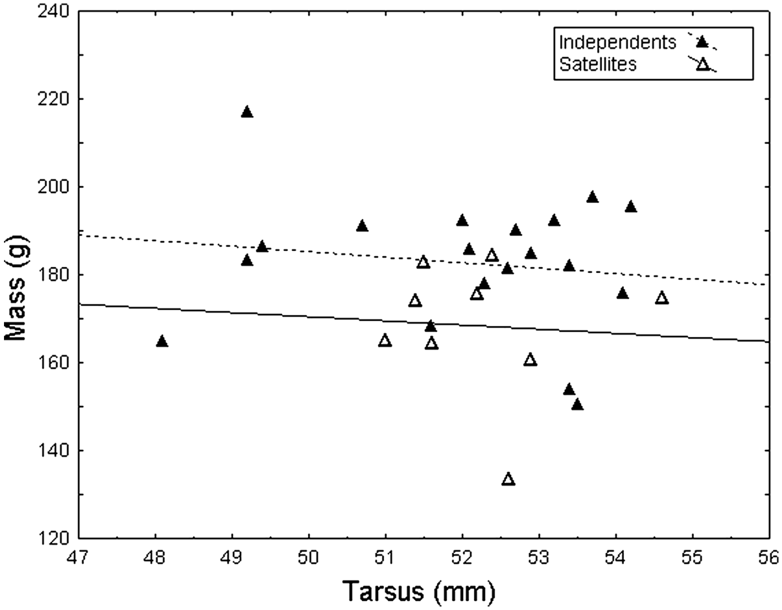

Figure 1. Mass vs. tarsus length of independent and satellite male ruffs. We used the ratio of these measurements as one potential indicator of general condition. Solid line: independents, dashed line: satellites.

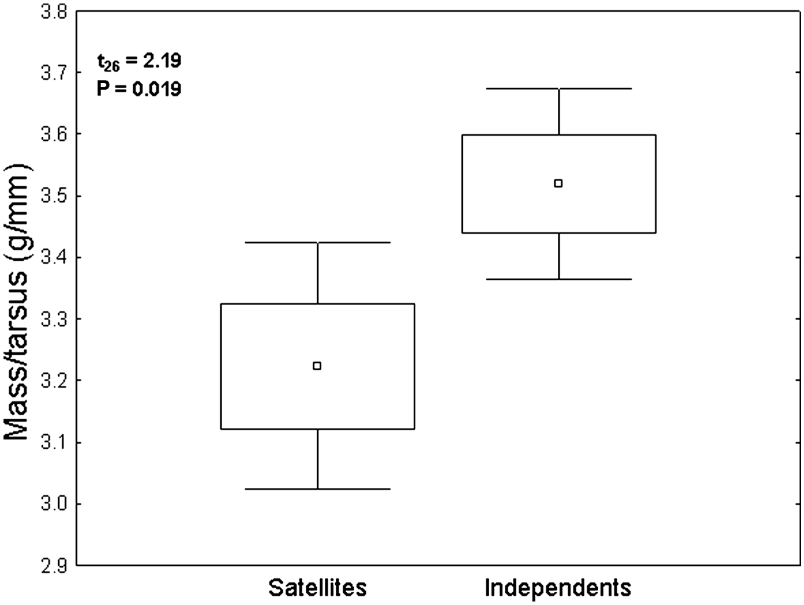

Figure 2. Differences in condition (mass/size) between independents and satellites. Box plots indicate the mean, s.e., and 1.96*s.e.

Table 1.
Correlations of size (PC1), mass/tarsus, ruff length, wattle number, and two measures of immunocompetence in male ruffs (n = 28). CMI = cell-mediated immunity. Each cell includes the respective Pearson correlation coefficient and associated P-value, two-tailed for correlations involving size, one-tailed for all others (see text). Asterisks indicate statistical significance. Parentheses indicate significance using sequential Bonferoni.

	Mass/tarsus	Ruff[a]	Wattles	SRBC	CMI
Size (PC1)	−0.300	0.429	0.549	0.346	0.028
	0.121	0.025*	(0.002)*	0.057	0.886
Mass/tarsus		0.029	−0.036	0.1264	0.055
		0.442	0.573	0.261	0.39
Ruff[a]			0.568	0.049	0.346
			(0.001)*	0.405	0.038*
Wattles				0.107	0.122
				0.294	0.268
SRBC					−0.160
					0.209

[a] n = 27, one outlier excluded.

wattle number nor ruff length was significantly correlated with mass/tarsus, but they covaried positively with each other and were also positively correlated with body size (table 1).

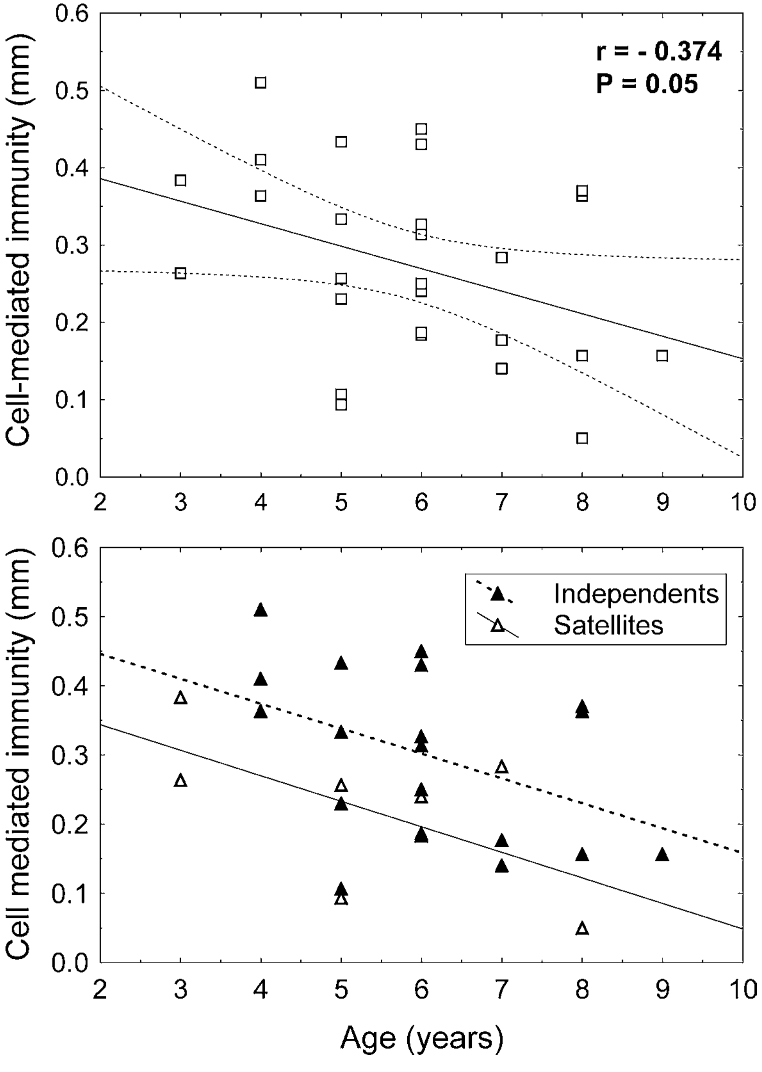

Figure 3. Decrease in cell-mediated immunity, measured in mm of response to PHA injection (see text), with age in male ruffs. Upper: all birds included, n = 28, linear regression and 95% C.I. Lower: Separately for resident and satellite morphs (ANCOVA: morph $F_{[1,25]} = 6.03$, $P = 0.021$; age $F_{[1.25]} = 7.5$, $P = 0.011$; morph-age interaction NS).

Cell-mediated immunity

Cell-mediated immunity was not significantly correlated with size, mass/tarsus, or wattle number, but was weakly related to ruff length (table 1). There was a decrease in cell-mediated immunity with increasing age (fig. 3; $r = -0.37$, n = 28, $P = 0.05$), which was still present, but not quite significant, when morphs were analysed separately (independents: $r = -0.43$, n = 19, $P = 0.06$; satellites: $r = -0.61$, n = 9, $P = 0.081$). With age as a covariate, satellites did not have

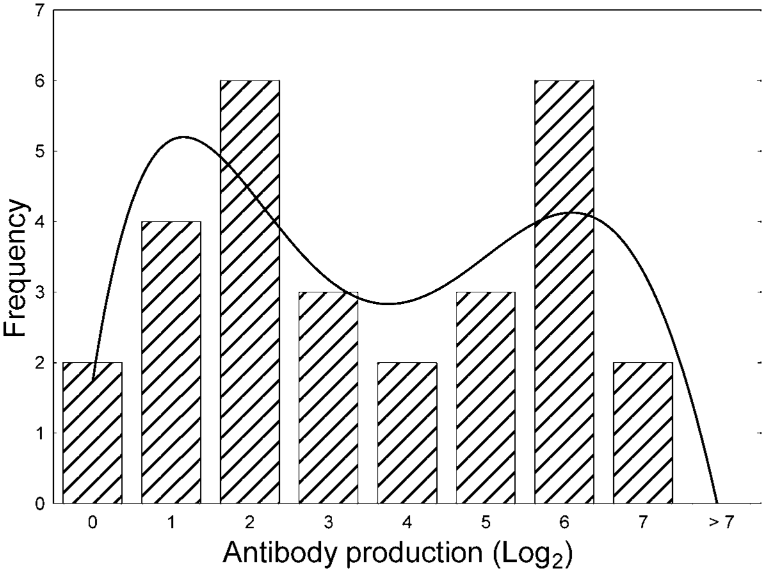

Figure 4. Frequency distribution of humoral immunity measured with an index of antibody responses (see text) in male ruffs, and the 2nd degree polynomial fit.

stronger cell-mediated immunity responses than independents, as we had predicted based on life-history theory; in fact, an opposite relationship was found (ANCOVA Morph $F_{[1,25]} = 6.03$, opposite tail, $P = 0.02$, fig. 3).

Humoral immunity

SRBC values ranged from 0 to 7 (fig. 4) in a seemingly bimodal fashion. This variation was not significantly correlated with morph, age, mass/tarsus or secondary sexual traits, but may have weakly increased with body size (table 1). Humoral immunity was not significantly correlated with cell-mediated immunity, and there were no differences in humoral immunity between the two morphs. There was a suggestion of a decrease with age among independents only ($r = -0.40$, n = 18, $P = 0.09$).

DISCUSSION

Ruff length and wattle number were positively correlated with each other, as expected if these traits signal general condition (Kodric-Brown and Brown, 1984; Dale, 2001; Dale et al., 2001), and testosterone stimulates the seasonal development of both traits (Lank et al., 1999). However, neither of these traits was significantly correlated with measures of mass relative to body size. Despite sufficient variability, the two immunocompetence responses were not significantly correlated with each other (table 1), results that are both contrary (e.g., Velando et al., 2001) and in agreement (Ewenson et al., 2001) with previous work. Cell-mediated immunity increased with ruff length, and the relationship between humoral immunity and body size was suggestive, but not significant (table 1). Taken together, these results indicate that at least early in the breeding season, different parts of the immune system may vary independently from each other and that they may not be necessarily strongly correlated with other potential measures or signals of body condition.

The lack of significant correlations between immunological responses and secondary sex traits may have occurred for at least three reasons of increasing interest. First, we may have simply lacked sufficient statistical power. However, our sample size was 40% larger than that of recent studies addressing similar questions and using the same techniques (e.g., McGraw and Ardia, 2003; Blount et al., 2003). Given our data's variance, even tripling of the sample size would not have produced any additional significant correlations, other than for humoral immunity and body size, one relationship that already approached significance (table 1). Second, different secondary sex characteristics may in fact signal different aspects of condition ('multiple messages', Møller and Pomiankowski, 1993; Johnstone, 1994), and we might have, by chance, measured unrelated characteristics. However, our two principal male characteristics were positively correlated (table 1), and are indeed 'condition' dependent, since they are: (1) expressed more extensively by older birds; (2) the timing and extent of their development can be delayed by limiting a male's food intake (Lank, unpubl.); and (3) the onset of their development is induced by a rise in testosterone levels in spring (Lank et al., 1999). The lack of a relationship with mass/tarsus could reflect our static assessment of this character. Whereas ruff length and wattle number were monotonically increasing when assessed, any correlated increase in mass/tarsus with an earlier pulse of testosterone may not have been reflected in the masses occurring when we assessed the birds. Finally, despite their condition dependence, ruff length and wattle number may nonetheless function primarily as Fisherian traits, which would weaken their usefulness as indicators of general condition (Iwasa and Pomiankowski, 1994; Kokko, 2001), immunological or otherwise.

The two immunological measures were not significantly related, which is puzzling, but not atypical of the current literature. Despite appeals to the contrary (e.g., Zuk and Johnsen, 1998), most immunoecological studies still rely on single measures of immune function. Studies that have used multiple indices (reviewed by Lochmiller and Deerenberg, 2000; Norris and Evans, 2000) typically resulted in different indices giving somewhat different results (e.g., Zuk and Johnsen, 1998; Ewenson et al., 2001; Møller and Petrie, 2002). The fact is that all measures of immune condition are not equally sensitive (Vos, 1980; Luster et al., 1988); different components of the immune system have different roles, and are not necessarily equally responsive or expected to change similarly. In this study we did not have any a priori reasons to expect any one aspect of immunity to be more responsive or more strongly correlated with other condition-dependent traits. However, our results highlight the need to measure several parts of the immune system and perhaps have several indices for each.

The concept of trade-offs, central to life-history theory, is based on the assumption of energetic or resource constraints. Ad libitum food probably dampens the magnitude and variability of these trade-offs relative to what might occur in the wild (Hoi-Leitner et al., 2001; Alonso-Alvarez and Tella, 2001; Ewenson et al., 2001). Hence, whereas positive correlations between the extent of plumage development and similar measures of body condition were documented in a related species in the wild (Piersma and Jukema, 1993), ad libitum food might explain why these correlations were not significant in our study. Despite captive conditions, however, males maintained substantial variability in the timing and extent of development of plumage and wattle traits, and mass, including a difference in 'condition' between morphs parallel to that seen in the wild (Bachman and Widemo, 1999). While it could be argued that trade-offs should not exist when food is not limited, studies continue to demonstrate otherwise (e.g., Bilbo et al., 2002; Casto et al., 2002; Cuthill et al., 1997; Prendergast and Nelson, 2001). Trade-offs come in many forms: metabolic, physiological, social, genetic, phylogenetic, and whereas ad libitum food might understandably shift the equilibrium, it does not remove all costs. In our case, males were housed communally, engaged in extensive social interactions, and began to develop their seasonal sexual traits at different times and to different maximum extents. The concept of trade-offs, central to life-history theory, is actually based on the assumption of **evolutionary adaptations** to energetic or resource constraints; adaptations that do not necessarily cease to exist once the constraints are removed.

The stable genetic polymorphism of ruffs requires mean per capita lifetime reproductive success to be equal in the long run. Widemo (1998) observed that independents obtained a higher per capita and per annum proportion of matings at leks, and suggested, as one possibility, that the morphs have different life-history strategies. Satellites, which do not defend territories, may adopt a lower cost, lower annual benefit strategy involving breeding at a younger age and/or living longer than independents. Positive correlations between different condition measures would still be expected within each morph, but because of differences in life history, satellites would be expected to allocate more to self-maintenance than independents, and hence be in better immune condition. Humoral immunity responses were bimodal, and both cell-mediated and humoral immune responses were highly variable. Although our power was limited by sample size, we have no indication at all that this variation was related to the bird's morph.

Contrary to the prediction based on life history differences, when corrected for age, cell-mediated immunity was higher for independents than for satellites (fig. 3). It has been suggested that cell mediated immunity is particularly important in wound healing (Zuk and Johnsen, 1998). If this is indeed so, the fact that during the breeding season independents are more pugnacious and are more likely to sustain external injuries than satellites might predict a higher cell-mediated immunity by independents, but further work is needed. In any case, these results do not support the idea that satellites have a more conservative life-history strategy than independents, at least as evidenced by their relative investment on immune function. Accordingly, a recent study of the relative annual reproductive success of residents and satellites, a study based on paternity analysis rather than copulation rates observed at leks, concluded that annual per capita success of the two morphs were indeed similar (Thuman, 2003).

The age-related decrease in immune condition was not specifically anticipated in this study. Using all birds, which come from the middle to upper end of the age distribution compared to wild birds, the effect was significant for cell-mediated immunity, similar for both morphs, and it paralleled differences in condition. An age-related decrease in cell-mediated immunity also occurs during the non-breeding season, for both male and female ruffs (Lozano and Lank, 2003). The fact that the immune system generally deteriorates with age is well documented in humans and in laboratory and domestic animals, so it is not necessarily surprising to find the same pattern in our study. Senescence of immune function would have important consequences to life history and population demographics, but we have only begun to document it the wild (Cichón et al., 2003; Saino et al., 2003). Captives usually live much longer than their free-living counterparts, and hence may provide a useful system with which to examine potential effects of immunosenescence in wild animals.

In summary, we found that neither cell-mediated nor humoral immunity was related to other condition traits. However, cell-mediated immunity was higher in independents than in satellites, and it decreased significantly with age in both morphs. These results indicate that we need more specific predictions about how different aspects of the immune system relate with each other, particularly when they interact with other ecological variables, such as food availability, age, and reproductive condition.

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