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Seasonal trade-offs in cell-mediated immunosenescence in ruffs (*Philomachus pugnax*)

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The immune system is an energetically expensive self-maintenance complex that, given the risks of parasitism, cannot be carelessly compromised. Life-history theory posits that trade-offs between fitness components, such as self-maintenance and reproduction, vary between genders and age classes depending on their expected residual lifetime reproductive success, and seasonally as energetic requirements change. Using ruff (*Philomachus pugnax*), a bird with two genetically distinct male morphs, we demonstrate here a decrease in male immunocompetence during the breeding season, greater variance in immune response among males than females, immunosenescence in both sexes and male morphs, and a seasonal shift in the age range required to detect senescence. Using a phytohaemagglutinin delayed hypersensitivity assay, we assessed cell-mediated immunity (CMI) of males of typical breeding age during the breeding and non-breeding seasons, and of a larger sample that included females and birds of a greater age range during the non-breeding period. CMI was higher for breeding-aged males in May than in November, but the increase was not related to age or male morph. In November, mean CMI did not differ between the sexes, but the variance was higher for males than for females, and there were no differences in mean or variance between the two male morphs. For both sexes and male morphs, CMI was lower for young birds than for birds of typical breeding ages, and it declined again for older birds. In males, senescence was detected in the non-breeding season only when very old birds were included. These results, generally consistent with expectations from life-history theory, indicate that the immune system can be involved in multifarious trade-offs within a yearly cycle and along an individual's lifetime, and that specific predictions about means and variances in immune response should be considered in future immunoecological research.

Keywords: immunoecology; life history; immunosenescence; age; ruff; *Philomachus pugnax*

1. INTRODUCTION

The concept of trade-offs between fitness components is an axiom of life-history theory (Williams 1966; Stearns 1992; Roff 1992). Trade-offs have been found between viability and mating success (e.g. Da Silva & Bell 1992), offspring quality and quantity (e.g. Wheelwright *et al.* 1991), self-maintenance and parental activities (e.g. Moreno *et al.* 1999), reproduction and predator avoidance (e.g. Candolin 1998) and other such fitness component dyads. Given finite time, energy and other resources, such pairwise trade-offs are not only a theoretical expectation but also a physical necessity. Therefore, an underlying and perhaps implicit goal of these studies is not merely to determine whether these pairwise trade-offs exist, but to identify different currencies with which to gauge such trade-offs. During the past decade the immune system has begun to be considered as a potentially useful currency with which to examine these trade-offs.

The ubiquity of parasites and the negative consequences that they can have on host fitness ensure that the integrity of the immune system cannot be carelessly compromised. Nevertheless, immune function may decrease under several situations. Among birds—for example, female collared flycatchers (*Ficedula albicolis*)—raising experimentally enlarged broods produced fewer antibodies than control birds following a challenge with a novel antigen (Cichón *et al.* 2001). Similarly, young serins (*Serinus serinus*) raised under food-limited conditions had lower immunocompetence than control birds (Hoi-Leitner *et al.* 2001). An increase of *ca.* 10% basal metabolic rate occurred following an immune challenge in great tits (*Parus major*) (Ots *et al.* 2001). Cold-stressed blue tits (*Parus caeruleus*) decreased their humoral immune response, which also had a similar energetic cost (Svensson *et al.* 1998). Hence, energetic constraints can lead to temporary and potentially dangerous reductions in immune function (Godfrey-Faussett *et al.* 1993).

Energetic requirements (e.g. cold stress, reproduction, migration) and exposure to parasites often vary predictably during a year, leading to seasonal changes in immune function (Nelson & Demas 1996). Ironically, exposure to conspecifics, and therefore parasites, is often greatest during the breeding season, a time during which energetic demand can be at its highest (Drent & Daan 1980). If the immune system is considered as essentially a homeostatic system that generally enhances survival, one would also expect immunocompetence to be positively associated with residual lifetime reproductive success (Williams 1966; Pianka & Parker 1975; Roff 1992), leading to immunosenescence and to differences among individuals or groups with different life-history strategies. Species with sexually dimorphic life histories should have sex-specific differences in immunocompetence, and its variance should mirror variance in lifetime fitness.

Several recent studies on immunoecology have considered immunity at a single point in time, but few have

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obtained age-specific profiles or repeated measures of the same individuals at different stages in their yearly cycles. In vertebrates, immune fuction is generally divided into innate and adaptive immunity; adaptive immunity is further divided into humoral and cell-mediated immunity (CMI) (Roitt *et al.* 1996). Here, we examine within-individual changes in CMI between breeding and non-breeding seasons, and age-related patterns outside the breeding season in ruff (*Philomachus pugnax*), a species with two types of male that may differ in their life-history strategies.

The ruff has females and two genetically distinct types of males: 'independents' and 'satellites' (Hogan-Warburg 1966; Van Rhijn 1991; Lank *et al.* 1995, 1999). Independents, which constitute *ca.* 84% of males, establish leks with tightly packed courts, which they defend against other independents; satellites do not set up mating courts and are free to move among and within leks, and join independents in transitory alliances that appear to attract females to co-occupied courts (Hugie & 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 typical for lekking species, there is a strong skew in reproductive success among males, and as is required to maintain a genetic dimorphism, both morphs achieve equal mean reproductive success (Van Rhijn 1991; Hugie & Lank 1997; Widemo 1998). However, the two male morphs may differ in life-history strategy, with independents being more successful per annual breeding season, and satellites perhaps starting to breed earlier and living longer (Widemo 1998). Independent males become competitive breeders at 3 years of age (Van Rhijn 1991) and can remain in their prime for 3–4 years (D. B. Lank and C. M. Smith, personal observation).

Lozano & Lank (2003) found age-related decreases in CMI in both male morphs during the breeding season. Here, we use a larger sample that includes both genders and covers a greater age range specifically to address the following questions. (i) Does CMI differ between the breeding and non-breeding seasons? (ii) Is there further evidence of immunosenescence and/or early development of immune function? (iii) Are patterns between the sexes and morphs as expected assuming different life histories? We demonstrate a decrease in immunocompetence in males during the breeding season, greater variance in immune response among males than females, and immunosenescence in both sexes and male morphs.

2. METHODS

(a) *General methods*

The experimental birds were part of a captive breeding flock derived from eggs collected near Oulu, Finland, in 1985, 1989 and 1990. The flock has been maintained in a communal outdoor pen in Burnaby, British Columbia, Canada, under natural photoperiod since 1994, with water and food *ad libitum* (Trout Chow 'Aqua-Max Grower' Purina Nutrition International—41% crude protein).

During mid-May, at the onset of the breeding season we assessed the CMI of 28 males: 18 territorial and 10 satellites, aged 3–9 years old. The following autumn, in early November, after the post-breeding moult, the CMI of all available birds ($n = 105$) was tested again. This group comprised 54 females and 51 males, aged five months to 13 years, including 26 males previously tested.

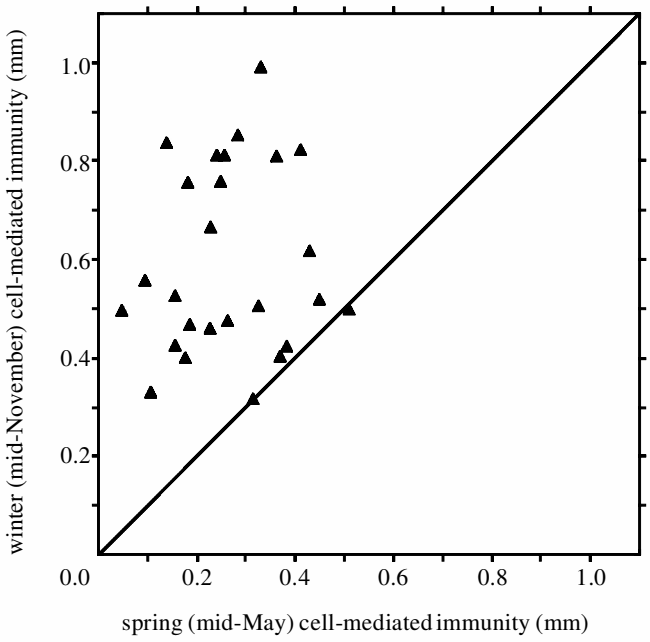

Figure 1. Within-individual seasonal increase in CMI in male ruffs. Each point represents an individual. The diagonal is the one-to-one line, around which all points would fall if there were no seasonal effect. Paired *t*-test: $t_{25} = 8.04$, $p < 0.0001$.

(b) *Cell-mediated immunity*

We assessed CMI by using a subcutaneous delayed hypersensitivity response (Corner & DeLoach 1990; Roitt *et al.* 1996). Each bird was given a sub-dermal injection of 0.05 ml phytohaemagglutinin (PHA-P) (Sigma product no. L8754) solution (2 mg PHA ml^{-1} phosphate-buffered saline (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. Twenty-four hours post-injection the thickness of the PHA-injected and control patagia were measured with a pressure-sensitive micrometer. A single value per patagium per bird was obtained by taking the mean of three measurements. The repeatability of our measurements (Lessells & Boag 1987) was similar to or higher than that reported in other studies (93%; cf. 0.94 (Zuk & Johnsen 1998); 0.99 (Smits *et al.* 1999)). The thickness of the PHA-injected patagium minus the PBS-injected side was used to indicate the strength of the response (Corner & DeLoach 1990; Roitt *et al.* 1996).

3. RESULTS

We first examine within-individual seasonal differences using 26 males that were sampled in both May and November, and then test for the expected age-, sex- and morph-related patterns in November, after the breeding season ($n = 105$).

(a) *Within-individual seasonal differences*

CMI responses were stronger in November than they had been in May (mean increase of 0.335 mm, s.e. = 0.0416, paired *t*-test: $t_{25} = 8.04$, $p < 0.0001$; figure 1). The

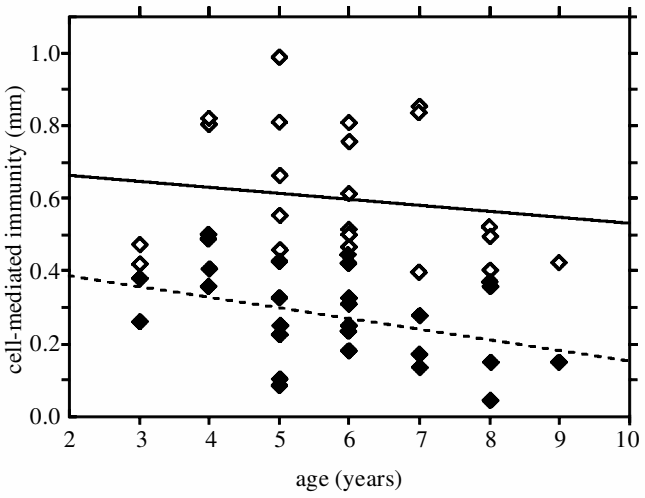

Figure 2. CMI versus age in male ruffs during the breeding and non-breeding seasons. Open diamonds, November; closed diamonds, May.

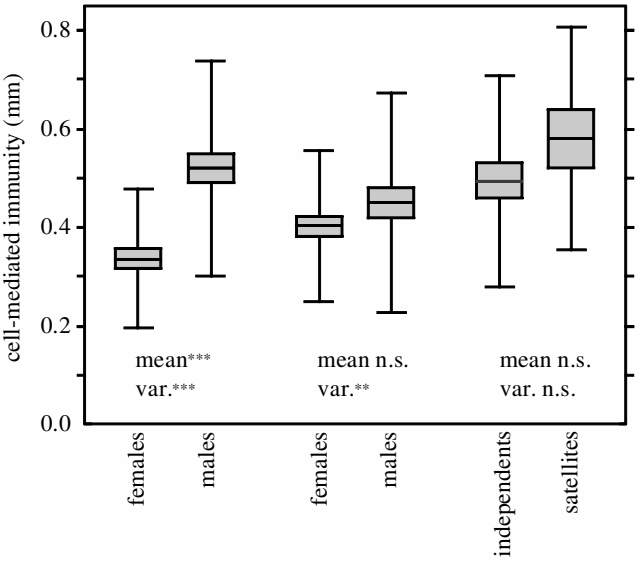

Figure 3. Sex- and morph-dependent differences in CMI in ruffs during the non-breeding season. Comparisons between the two male morphs are on the right. To account for dose effects, the two sexes were compared using raw values (left) and mass$^{1/3}$-adjusted values (middle). Plots specify the mean, s.e. and s.d. Also indicated are the p-values (* < 0.05, ** < 0.01, *** < 0.001) resulting from ANOVAs (means) and Levene's tests (variances).

increase was not related to male age or male morph (ANOVA: $F_{1,24} = 0.36$, $p = 0.55$ and $F_{1,24} = 1.27$, $p = 0.27$, respectively), and values for each male were uncorrelated between seasons ($r = 0.11$, $p = 0.58$).

In May there was an age-related decrease in CMI ('immunosenescence' (Lozano & Lank 2003) regression: $F_{1,26} = 4.24$, $p = 0.05$; figure 2). This relationship was not present when these same birds were resampled in November (regression: $F_{1,24} = 0.42$, $p = 0.52$). However, the two regression lines were not significantly different from each other ($F_{1,51} = 0.34$, $p = 0.564$).

(b) *Effects of age, sex and morph on immunity*

The seasonal increase in CMI mentioned above was not simply due to repeated exposure to PHA. Among males in the same age range as that of those tested in May, there was no difference between birds that had been previously exposed to PHA and those that were being tested in autumn for the first time (t-test: $t_{38} = 1.45$, $p = 0.16$). Therefore, birds tested in May were not excluded from the sample.

The two male morphs did not differ in their mean CMI (ANOVA: $F_{1,49} = 1.60$, $p = 0.21$; figure 3) or their variance (Levene's test for homogeneity of variance: $F_{1,49} = 0.04$, $p = 0.85$), but both mean and variance were significantly greater for males than for females (ANOVA: $F_{1,103} = 26.37$, $p < 0.0001$; Levene's test: $F_{1,103} = 11.72$, $p = 0.0009$; figure 3).

These sex-related differences could be the result of different relative dosages, given that all birds were given the same dosage but males are bigger than females. Therefore, data were reanalysed by regressing the raw CMI values, a one-dimensional response variable, against mass$^{1/3}$. Using these residuals the difference in mean CMI between the sexes vanished ($F_{1,103} = 1.64$, $p = 0.204$), but the difference in variances was still significant (Levene's test: $F_{1,103} = 8.89$, $p = 0.0036$).

Age-related differences in means and variances in CMI were tested by dividing the sample into three groups, with the middle category including birds over 3 years old but under 10 years old, as of November. This age range fortuitously encompassed most breeding ages of males in the wild, *ca*. 3-9 years old. To account for sex-differences in mean CMI, we used sex-adjusted residuals in all subsequent comparisons including both sexes. In the pooled sample, breeding-aged birds had the highest mean CMI (ANOVA: $F_{2,102} = 9.32$, $p = 0.0002$; figure 4*a*), but the variances did not differ significantly among age classes (Levene's test: $F_{2,102} = 2.38$, $p = 0.098$). Excluding 'young' birds, whose immune system may still be developing, there was a significant decrease in CMI with respect to age (ANCOVA age effect: $F_{1,83} = 4.85$, $p = 0.031$), which did not differ between the sexes (ANCOVA age × sex interaction: $F_{1,83} = 0.65$, $p = 0.42$).

Among males, the mean CMI response differed among age classes ($F_{2,48} = 4.43$, $p = 0.017$), again with birds in the middle group being significantly higher (figure 4*b*), but the variances did not differ among groups (Levene's test: $F_{2,48} = 1.84$, $p = 0.169$). Among independents, the mean CMI response also differed among age classes ($F_{2,33} = 2.68$, $p = 0.083$), but the only significant difference was between 'old' and 'breeding-aged' birds (independent contrasts: middle versus young, n.s.; middle versus old, $p = 0.048$; old versus young, n.s.), and the variances did not differ among classes (Levene's test: $F_{2,33} = 1.69$, $p = 0.20$). The equivalent analysis could not be conducted for satellites because there was only one 'young' satellite.

Consistent with the results for males, among females the mean CMI response differered significantly among age classes ($F_{2,51} = 5.70$, $p = 0.006$); in this case, the difference lay only between 'young' and 'breeding-aged' birds (figure 4*c*). Variances in CMI response did not differ significantly among age classes (Levene's test: $F_{2,51} = 1.27$, $p = 0.29$).

4. DISCUSSION

CMI was higher in breeding-aged male ruffs during the winter than in the breeding season. The CMI test that we

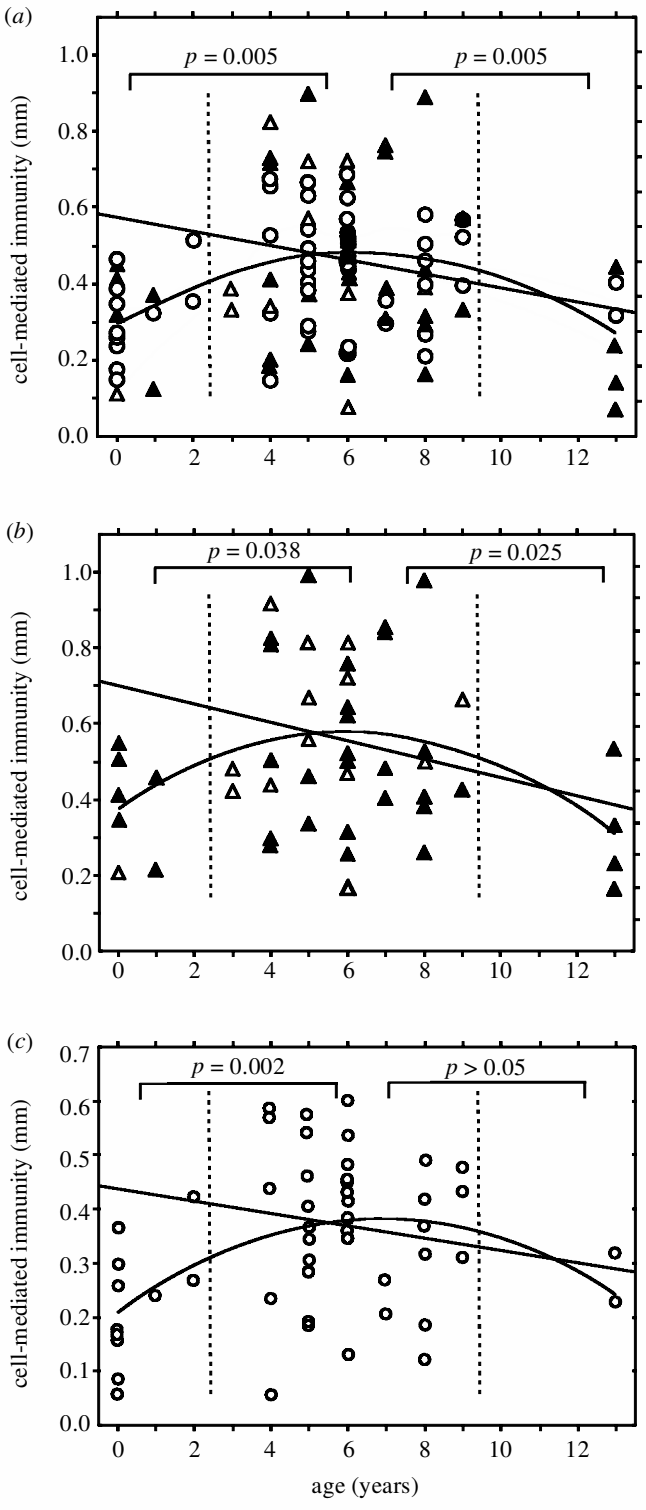

Figure 4. CMI of ruffs versus age. Vertical dashed lines separate the three age groups. Horizontal lines indicate independent contrasts between group means and the associated *p*-values. Variances did not differ significantly. Curved lines represent second-degree polynomial fits. Straight solid lines are linear regressions, which exclude birds younger than 3 years old, and are not significantly different between the sexes ($F_{1,83} = 0.65$, $p = 0.42$). (*a*) All birds, using sex-adjusted residuals (closed triangles, independents; open triangles, satellites; open circles, females), (*b*) males (closed triangles, independents; open triangles, satellites), and (*c*) females.

used does not rely on immunological memory (Corner & DeLoach 1990; Roitt *et al.* 1996) and furthermore, the November response did not differ between birds that were tested for the first time and those that had already been tested in May; therefore, the increase was not simply due to prior exposure. A depression in immunocompetence during the breeding season is congruent with results from other studies on birds and mammals (John 1994; Nelson & Demas 1996; Prendergast & Nelson 2001).

During the breeding season, animals are usually in closer and more intimate contact with conspecifics (e.g. Sheldon 1993), hence exposure to parasites or injuries is probably higher than in the winter. Therefore, if viewed in isolation, a decrease in immunocompetence during the breeding season cannot be considered adaptive; the most likely explanation is that maintaining the immune system is indeed costly (Lochmiller & Deerenberg 2000), and that during the breeding season animals allocate fewer resources to immune function (e.g. Deerenberg *et al.* 1997; Hoi-Leitner *et al.* 2001).

This reallocation is the result of resources generally being limited, and hence it is more likely to be detected under such conditions. For example, an immune challenge decreased survival of starved bumble-bees (*Bombus terrestis*), but had no effect on bees with food *ad libitum* (Moret & Schmid-Hempel 2000). Similarly, yellow-legged gulls (*Larus cachinnans*) in a food restriction treatment had lower CMI responses, and the decrease in CMI from the start to the end of food restriction period correlated positively with mass loss (Alonso-Alvarez & Tella 2001). In our study, we obtained a lower response in May than in November, even though all birds had *ad libitum* access to a high-protein food. Although such access probably dampens the magnitude of the difference in responses relative to what might be expected in the wild, the fact that it was still detected suggests that a robust seasonal allocation programme persists even for birds in good condition.

A second seasonal difference is that independents had higher age-specific levels of CMI than satellites during the breeding season (Lozano & Lank 2003), but despite a larger sample size, there were no differences during the non-breeding season (figure 4). CMI may be important in the healing of external injuries (Zuk & Johnsen 1998). If so, this seasonal difference in CMI responses between male morphs could occur because independents have a higher risk of injury than satellites, but only during the breeding season, due to the fighting associated with their territorial behaviour.

At the proximate level, these immunological trade-offs are probably mediated by testosterone (Folstad & Karter 1992), which in male ruffs also induces morphological and behavioural changes during the breeding season (Lank *et al.* 1999), but at the ultimate level they are an adaptive solution to the problem of resource allocation under energetic constraints and lifetime fitness expectations, a problem faced by both sexes. In contrast to what would be expected if male morphs differed substantially in life history, CMI means and variances did not differ between morphs, given the power available from our sample. Neither did we find the expected differences between the sexes in mean response, which may be because our study was conducted during the non-breeding season, when energetic requirements and hence short-term resource

allocation solutions may not differ between the sexes. However, long-term considerations, namely variance in expected lifetime reproductive success, still differ between the sexes in non-breeding birds, particularly in lekking species. Despite similar mean CMI responses, variance was significantly higher in males than in females. Studies on sex-related differences in mean immunocompetence are now customary (reviewed by Schuurs & Verheul 1990; Klein 2000), but to the best of our knowledge, this is the first study that examines differences in the variance in immune response.

Life history also predicts that older individuals with a lower expected lifespan should invest relatively less on self-maintenance. Accordingly, CMI was generally higher in breeding-aged birds than in old or young birds. The initial age-related increase was consistent in both sexes and in the pooled dataset, but could not be found for each morph separately, probably due to low statistical power. Lower immunocompetence in young and immature birds is common among birds, at least given the limited number of species that have been studied (Apanius 1998), and it is the result of at least two factors: developmental constraints and trade-offs in energy allocation.

An essential property of the immune system is its ability to discriminate self from non-self. Perhaps surprisingly, in vertebrates this ability is not genetically controlled, but it is learned during an organism's embryonic and neonate stages. Tolerance, a state of unresponsiveness for a particular antigen, is induced by prior exposure to the same antigen, which prevents immune responses against self-antigens or common non-pathogenic antigens (Kuby 1992; Roitt *et al.* 1996). In fact, humans raised in less sterile environments during childhood—including larger families, pets and rural settings—have a reduced incidence of allergic reactions (Svanes *et al.* 1999; Kilpelainen *et al.* 2000; Nafstad *et al.* 2001). The benefit of this mechanism is that it allows for the potential recognition of a virtually infinite number of proteins, most of which are never encountered, while preventing immune reactions to innocuous or self antigens. The drawback is that the immune system cannot be fully functional at the time of hatching, which is not strictly a developmental constraint, but rather a result of adaptive design. This drawback can be partially compensated by the transfer of acquired immunity from mothers to offspring (Patterson *et al.* 1962; Williams 1962; Carroll & Stollar 1983), something that is only beginning to be examined from an ecological perspective (Heeb *et al.* 1998; Lozano & Ydenberg 2002).

Even if developmental or design constraints did not exist and the potential ability to repeal pathogens did not differ between young and adults, immature animals should still invest relatively less on immune function than non-breeding adults. Immature animals also have other considerations, such as predator avoidance and growth, that are not as pressing for adults; consequently, immatures may be willing to compromise immune condition in favour of growth, just as adults in the breeding season balance immunity against reproduction. Therefore, the lower immunocompetence of neonates and young may result from adaptive design constraints and/or optimal allocation of resources, weighed by the prospects of continued survival.

Consistent with the idea that investment in self-maintenance should decrease with residual reproductive success, we documented immunosenescence in both sexes and morphs during the non-breeding season. Among breeding-aged males, the age-related decrease in CMI present during the breeding season was no longer apparent in these same birds in the winter, but it was again detected when older birds were included. Therefore, immunosenescence was more evident during the more energetically demanding time of the yearly cycle. Although senescence is seldom visually apparent in free-living birds, age-dependent decreases in reproductive output have been documented in many species (e.g. Collias *et al.* 1986; Dhont 1989; Lozano & Lemon 1999; reviewed by Sæther 1990; Forslund & Pärt 1995). However, the physiological basis for this reproductive senescence has been difficult to ascertain (Galbraith *et al.* 1999). Our study is unique in its scope in that it addresses senescence of immune condition in a wild bird, and it suggests that for birds in the prime of their lives, physiological indicators of senescence may be only evident during certain parts of the breeding cycle.

In summary, we demonstrate that in ruffs, male immunocompetence decreases during the breeding season, immunocompetence is more variable in males than it is in females, immunosenescence occurs on both morphs and sexes, and the age range required to detect immunosenescence is smaller during the breeding season. This work provides further evidence that immunocompetence can be investigated from a life-history context, and indicates that trade-offs occurring within a yearly cycle or along an individual's lifetime can have predictable and different effects on the mean immune response and its variance, something that will have to be addressed in future research on immunoecology.

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