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Immune and oxidative stress trade-offs in four classes of Ruffs (*Philomachus pugnax*) with different reproductive strategies

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Abstract: Immunity and resistance to oxidative stress are two mechanistically related aspects of self-maintenance that are usually not studied together in connection to ecological or evolutionary relevant variables. Whereas many studies compare two sexes, here we use Ruffs (*Philomachus pugnax* (L., 1758)), a species in which males have three alternative reproductive morphs: independents, satellites, and faeders. Previous work suggested that immune function in Ruffs depends on energetic constraints or potential of injuries. Based on their behaviour and life history, the three male morphs and females can be placed on an ordinal scale with independents at one end and females at the other, and these two explanations predict opposite patterns along this continuum. Innate and cell-mediated immunity decreased along this axis from independents to females, supporting a risk-of-injury explanation over the energetic constrains hypothesis. No such pattern was evident for oxidative stress or resistance, and no relationship was detected between immunity and oxidative resistance or stress. Hence, during the breeding season immunity reflected the risk of injury, with faeders located in the immunological continuum between females and other male morphs. Species with alternative reproductive strategies provide particularly useful systems in which to address the evolution and ecology behind physiological mechanisms.

Key words: Philomachus pugnax, Ruff, alternative mating strategy, immunoecology.

Résumé : L'immunité et la résistance au stress oxydatif sont deux aspects de l'entretien reliés sur le plan mécaniste qui, en ce qui concerne leurs liens avec des variables écologiques ou évolutionnaires pertinentes, ne sont normalement pas étudiés ensemble. Alors que de nombreuses études comparent deux sexes, nous nous penchons sur les combattants variés (*Philomachus pugnax* (L., 1758)). une espèce dont les mâles se présentent sous trois formes reproductrices distinctes, à savoir, les indépendants, les satellites et les « faeders ». Des travaux antérieurs ont indiqué que la fonction immunitaire chez les combattants variés pourrait dépendre de contraintes énergétiques ou du potentiel de blessure. À la lumière de leur comportement et de leur cycle de vie, les trois formes de mâles et les femelles peuvent être disposées sur une échelle ordinale sur laquelle les indépendants occupent une extrémité et les femelles, l'autre. Or, les deux explications susmentionnées prédisent des distributions contraires le long de ce continuum. L'immunité innée et à médiation cellulaire diminue, le long de cet axe, des mâles indépendants vers les femelles, appuyant l'explication reliée au risque de blessure plutôt que l'hypothèse des contraintes énergétiques. Une telle tendance n'est pas observée en ce qui concerne le stress oxydatif ou la résistance à l'oxydation, et aucun lien n'a été détecté entre l'immunité et la résistance à l'oxydation ou le stress oxydatif. Ainsi, durant la période de nidification, l'immunité reflète le risque de blessure, les faeders se situant, dans le continuum immunologique, entre les femelles et les autres formes de mâles. Les espèces présentant des stratégies de reproduction alternatives constituent des systèmes particulièrement utiles pour étudier l'évolution et l'écologie des mécanismes physiologiques. [Traduit par la Rédaction]

Mots-clés : Philomachus pugnax, combattant varié, stratégie d'accouplement alternative, immunoécologie.

Introduction

Life-history theory is based on the fact that all fitness components cannot be simultaneously maximized, so organisms necessarily face a myriad of trade-offs at several levels of complexity and temporal and spatial scales (Williams 1966; Pianka 1976; Stearns 1992; Roff 2002). In general, resources, time and energy, can be allocated towards growth, reproduction, and self-maintenance. Investment in immune function is essentially a choice of self-maintenance over growth and reproduction, and as such, it ought to vary predictably with the organism's ecology and life history (Lochmiller and Deerenberg 2000; McDade 2003; Lee 2006; Sadd and Schmid-Hempel 2009).

Interest in immunoecology over the 20 years arose with a seductively simple proposed mechanism linking testosterone, immunity, and sexual signals: the immunocompetence handicap hypothesis (Folstad and Karter 1992). Although the hypothesis has generally not been supported (reviewed by Roberts et al. 2004; Muehlenbein and Bribiescas 2005), it brought forth a new appreciation for the immune system in evolutionary ecology, and interest soon extended into many other areas, such as the relationship between immune function and sexual attractiveness (Clotfelter et al. 2007), diet (Grether et al. 2004), age (Cichoń et al. 2003), pollution (Smits and Williams 1999), stress (Martin 2009), maternal effects (Lozano and Ydenberg 2002), and reproductive effort (Deerenberg et al. 1997).

Another aspect of self-maintenance, physiologically linked to immunity, is an organism's ability to mitigate damage from oxidative stress (Surh and Packer 2005). Oxidative stress is defined as an imbalance between the production of prooxidants and the organism's ability to mitigate their negative effects with antioxi-

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dants (Surh and Packer 2005). Oxidative damage is caused by free radicals, which are produced by normal metabolic processes, and also specifically as a defence against microbes, and are harmful to DNA, proteins, and cell membranes (Surh and Packer 2005; Halliwell and Gutteridge 2007; Matata and Elahi 2007). Free-radical oxidation is one of the main mechanistic hypotheses of aging (Harman 1956; Beckman and Ames 1998), and it is involved in most diseases, either causally, or, more often, as an associated consequence (Surh and Packer 2005; Halliwell and Gutteridge 2007; Matata and Elahi 2007). To counter the negative effects of oxidative damage, animals rely on various endogenous and exogenous antioxidants (Krinsky et al. 2005; Halliwell and Gutteridge 2007).

In ecology, particular attention over the past 15 years has been paid to exogenous antioxidants because they are often used in sexual signals, which leads to several multifaceted trade-offs involving foraging, immune function, parasites, health, and mate attraction (Lozano 1994; Svensson and Wong 2011). Not surprisingly, interest expanded from antioxidants to oxidative stress, and mirroring the immunoecological work, recent studies (reviewed by Costantini 2008) have examined the links between oxidative stress and sexual attractiveness (Torres and Velando 2007), diet (Pike et al. 2007), age (Torres and Velando 2007), pollution (Pérez et al. 2010), stress (Costantini 2008), maternal effects (Wang et al. 2001), and reproductive effort (Ots and Hõrak 1996).

Nevertheless, two aspects have not been well addressed. First, immune function and oxidative resistance are linked. Although the exact mechanisms linking them remain intriguing physiological questions, from an evolutionary perspective, the conceptual relationships are not clear. Other than the links between immunity, oxidative stress, and carotenoids, which some workers have addressed (Isaksson and Andersson 2008; Pérez-Rodríguez et al. 2010; Simons et al. 2012), the rationales of immunoecological and oxidative ecology studies are surprisingly similar. Hence, it might be useful to measure both immune function and resistance to oxidative stress simultaneously, not to merely test whether they are related to each other as they are known to be (Surh and Packer 2005; Costantini and Møller 2009), but rather to examine their relationship with other ecological or evolutionary relevant variables. Second, it has long been recognized that two sexes have different life histories (Bateman 1948), and whereas most studies are limited to comparisons between the two sexes, species with alternative reproductive morphs provide additional within-species comparison groups that can be used to separate otherwise intercorrelated effects (Miles et al. 2007). Here, we address these two issues and examine immune function and resistance to oxidative stress of breeding Ruffs (*Philomachus pugnax* (L., 1758)), a species in which males have three alternative reproductive strategies.

Three male morphs exist in Ruffs. Two of these morphs, independents and satellites, have elaborate courtship behaviour and plumage (Hogan-Warburg 1966; van Rhijn 1991). The third morph, first reported in 2006, is a female mimic called a "faeder" (Jukema and Piersma 2006). Hereinafter, we shall refer to two sexes, three male morphs, and four genders. During the breeding season independents establish lek mating courts and fiercely defend them against other independents. Satellites do not fight but are pecked and chased by independents if they fail to behave submissively (Höglund and Lundberg 1989; Hill 1991; van Rhijn 1991). At the other extreme, females do not incur the costs or benefit from the consequences of displaying and fighting in reproductive contexts (Widemo 1998), nor do faeders. Hence, both the energetic demands and potential for injuries owing to territory establishment and defence during the breeding seasons are highest for independents and lowest for females.

Immunoecological work conducted before the discovery of the faeders showed that in male Ruff's cell-mediated immunity (CMI) is higher during the nonbreeding season than during the breeding season (Lozano and Lank 2003), and during the breeding season independents have stronger CMI than satellites (Lozano and Lank 2004). The seasonal differences support the idea that immune function decreases in the breeding season due to energetic constraints, whereby investment in immune function is traded off against reproduction. In contrast, differences during the breeding season support the idea that investment in immune defence depends on the actual or potential exposure to injuries incurred while defending territories. These two alternative explanations yield contrasting predictions when all four genders are compared.

In this study, conducted during the breeding season, we measured all three arms of the immune system, antioxidant capacity, and oxidative stress. We expected the immune and oxidative stress profiles of faeders to be similar to those of females and different from those of independents and satellites. Specifically, we tested the following:

(1) The energetic constraints hypothesis predicts that mean immune responses and oxidative resistance should be lowest for independents, followed by satellites, faeders, and females, in that order.
(2) In contrast, the risk-of-injury hypothesis predicts the exact opposite, that mean immune responses and oxidative resistance should be lowest for females, followed by faeders, satellites, and independents, in that order.
(3) In Ruffs, variance in immune response was higher for males than for females (Lozano and Lank 2003), reflecting the typical sex-specific variance in reproductive effort and reproductive success, so here we also examine the differences in variability among the genders.
(4) Innate immune defences partially rely on inflammatory responses that produce free radicals (Roitt et al. 1996; Clark 2008; Martin 2009), so here we test whether there is a positive relationship between the strength of innate immunity responses and the antioxidant capacity, and a negative one between innate immunity and oxidative stress.

Materials and methods

Study organism

Independents establish lek mating courts, defend them from other independents, and try to attract into their courts not only females but also satellites (Höglund and Lundberg 1989; Hill 1991; van Rhijn 1991). Independents benefit from having satellites in their territories because females find co-occupied courts more attractive, and satellites benefit because they obtain copulations from visiting females without having to establish and defend courts (van Rhijn 1991; Hugie and Lank 1997; Widemo 1998). Faeders look like females, and during the breeding season behave like females, spending time among females and visiting leks unchallenged. Nevertheless, faeders readily copulate when females solicit copulations from independents, satellites, or perhaps even faeders. It is unknown whether females or other males recognize faeders as males, or if they do, whether they do so all the time.

The dimorphism between independents and satellites results from a single-locus two-allele autosomal gene (Lank et al. 1995). Independents and satellites are maintained at an equilibrium ratio of about 84% independents and 16% satellites (van Rhijn 1991; Hugie and Lank 1997). In the wild, faeders make up about 1% of males (Jukema and Piersma 2006; Jaatinen et al. 2010), but they are more common in our captive population because of selective breeding. The genetics behind the faeder morph are only now beginning to be worked out.

General procedures

The birds were part of a captive flock in Burnaby, British Columbia, Canada, maintained outdoors since 1994. The flock originated from eggs collected near Oulu, Finland (65.0°N, 25.3°E),

in 1985, 1989, and 1990, and supplemented by the addition of two adult faeders in 2006, soon after the faeder morph was first reported (Jukema and Piersma 2006). Before 2006, the population may have included some faeders, or faeder alleles. Birds were maintained with ad libitum water and food (Trout Chow "Aqua-Max Grower" Purina Nutrition International with 41% crude protein, plus Purina Turkey Starter feed with 26% protein). The study was conducted from 22 May to 14 June, during the peak and second half of the breeding season. Birds younger than 3 years of age and older than 9 years of age have lower immune responses (Lozano and Lank 2003), so only birds in their prime were used.

Birds were placed in groups of 16, consisting of 5 independents, 5 females, 3 faeders, and 3 satellites. These groups were separated from the rest of the flock, in pens measuring 17.5–18.5 m^2. The aim was to balance the numbers of each comparison group given the numbers of birds available, and to allow the birds to interact with conspecifics as they normally would, which they did. Four such groups were set up, staggered by a few days to spread the workload. Hereinafter these groups shall be referred as "leks" 1 through 4, and shall be used as statistical blocks when appropriate. On the third day after the leks were formed, baseline blood samples (approximately 1 mL) were taken, and the birds were weighed and injected with a liposaccharide (LPS) antigen. On the 4th day, the delayed hypersensitivity test was started, and it was concluded on the 5th day. On the 7th day, the birds rested. A suitable humoral response occurs after 7 days (Lozano and Lank 2004), hence on the 10th day, 7 days after the LPS injection, the birds were weighed again, a second 1 mL blood sample was taken, and the lek was disbanded. These blood volumes are within the recommended maxima for birds of this size (Campbell and Dein 1984; Stangel 1986). Serum was separated and stored at under –70 °C until analysis.

A broad picture of both immune function and oxidative stress was obtained. Innate or "nonadaptive" immunity was assayed using the haemolysis–haemagglutination assay on the initial, baseline blood samples. Furthermore, both aspects of adaptive immune system were assessed; CMI was quantified using a subcutaneous delayed hypersensitivity response and the haemolysis–haemagglutination assay on the after-exposure blood samples. Humoral immunity was assessed by qualifying antibody response following an immune challenge. Plasma antioxidant capacity was measured using a total antioxidant capacity (TAC) assay and total oxidant status (TOS) of plasma was measured using a colorimetric assay.

Innate immunity (haemolysis–haemagglutination assay of before-injection samples)

Innate, non-induced (constitutive) immunity was assayed using the haemolysis–haemagglutination assay (Matson et al. 2005). Briefly, 20 μL samples were serially diluted with PBS across a 96-well, round-bottomed tissue culture plate and incubated for 90 min at 37 °C with an equal volume (20 μL) of 1% rabbit red blood cell (RBC) solution. The plate was tilted for 20 min at room temperature to ease scoring. Titre scores were assigned as the $-\log_2$ dilution of the last well exhibiting agglutination or lysis. The degree of agglutination is a measure of antibodies, mostly IgM, interacting with RBC antigens. The degree of lysis is a measure of antibodies and complement working in conjunction.

Cell-mediated immunity (haemolysis–agglutination and delayed hypersensitivity)

The aforementioned haemolysis–haemagglutination assay on the after-injection blood samples was used to estimate B-cell proliferation resulting from exposure to LPS. A delayed hypersensitivity test was also used as a second measure of CMI. It consists of an injection of a mitogen (phytohemagglutinin) derived from the common bean (*Phaesolus vulgaris* L.) that causes T lymphocytes to mobilize to and proliferate at the area of injection (Breuer et al. 1976; Corrier and DeLoach 1990). Each bird was injected in the patagium of one randomly chosen wing with 0.05 mL of phytohemagglutinin (PHA-P; Sigma-Aldrich product No. L8754) dissolved in phosphate-buffered saline (PBS; Sigma-Aldrich product No. P3813) with a concentration of 2 mg PHA/mL PBS. As a control, the other wing was injected with 0.05 mL of PBS. The thickness of the PHA-injected and control patagia were measured 24 h after injection with a pressure-sensitive micrometer (Dyer model No. 304-196). The difference in thickness of the PHA-injected side minus the PBS-injected side indicates the strength of the response. This technique has been used by other researchers and by ourselves in previous work with Ruffs.

Antibody-mediated immunity (anti-PHA and anti-LPS IgY)

Several antigens could be used to elicit and measure antibody production. To minimize the effects of prior exposure, it is possible to use an antigen that none of the birds has been exposed to or, at the other extreme, an antigen to which it can be reasonably assumed all birds have been exposed (Salvante 2006; Boughton et al. 2011). We chose the latter: LPS from the cell wall of the gram-negative bacterium *Escherichia coli* (serotype O55:B5; Sigma product No. L5418). This method has been used successfully in Ruffs (Nebel et al. 2013). On the third day, a 1 mL blood sample was first taken, to be used as a baseline level and each bird was injected with 0.1 mL of a solution of LPS on PBS (concentration 0.1 mg/mL). Seven days later, a second blood sample was taken. Previous work indicated that 7 days is adequate to elicit a humoral response (Lozano and Lank 2004). Furthermore, taking advantage of the CMI test, we also quantified anti-PHA immunoglobulin, as well as the amount of after-LPS injection anti-PHA and anti-LPS plasma IgY, relative to the before-injection pooled plasma standard.

A sandwich ELISA protocol was used. Nunc maxisorp 96-well plates were coated overnight with 100 μL of 2 μg/mL PHA or LPS in carbonate–bicarbonate buffer (Sigma), washed 4× in PBS-Tween, and then blocked for 2 h at room temperature with 1% bovine serum albumin (BSA) in PBS, and again washed. Then 100 μL of sample plasma diluted 1:99 in PBS were incubated 1 h at room temperature. IgY was detected using goat anti-bird IgG with horseradish peroxidase conjugate (Bethyl Labs product No. A140-110P) in 1% BSA blocking buffer and developed with TMB peroxidase substrate (Sigma-Aldrich product No. T3405), stopped with 50 μL of 2 mol/L sulphuric acid. Plates were read on a PowerWave 340-plate reader at room temperature. All samples were run in duplicate; coefficients of variation over 0.1 were rerun and outliers discarded from concentration calculations. Each sample concentration is expressed as a value proportional to a standard pool made up of 5–10 μL of plasma from each sample (Addison et al. 2010).

Total antioxidant capacity (TAC) and total oxidative stress (TOS)

The initial sample provides a baseline measure, the second sample indicates the condition after an immune challenge, and the difference between before and after vaccination (i.e., $TAC_{before} - TAC_{after}$ and $TOS_{before} - TOS_{after}$) measures the oxidative stress caused by the treatment (ΔTOS) and the individuals' ability to quench that stressor (ΔTAC).

Plasma antioxidant capacity was measured using a total (trolox-equivalent) antioxidant capacity (TAC) assay, modified from Erel (2004). This assay uses a colorimetric reaction where the green ATBS cation is reduced to its colourless uncharged form, and antioxidants in the plasma sample accelerate this reaction at a rate proportional to their concentration. A standard curve is generated using a trolox standard and sample values are expressed as mmol trolox equivalent/L. The assay was modified to use 220 μL of reagent mixture and 10 μL of plasma sample. The reaction was read on a Powerwave 340-plate reader at 660 nm. In

disagreement with Erel (2004), an anonymous reviewer suggested that this test only measures the "nonenzymatic" antioxidant capacity.

Total oxidant status (TOS) of plasma was measured using a colorimetric assay, modified from Erel (2005). The assay measures the oxidation of (Fe^{2+}) ferrous ion-o-dianisidine complex by plasma oxidants to ferric ion (Fe^{3+}) that reacts with xylenol orange, changing colour from yellow to red. Hydrogen peroxide (H_2O_2) was used as a standard, and plasma values are expressed as μmol H_2O_2 equivalent/L. The assay was modified by using 65 μL of reagent mixture and 5 μL of plasma sample. The reaction was read on a plate reader at 560 nm.

Samples were run in lines with all wells in a line completed under 5 min., and the reaction read on the plate reader 5 min after finishing each line. Baseline readings were done immediately before completing each line. All samples for both assays were run in duplicate and duplicates with large coefficients of variation (greater than 0.15) were rerun.

Statistics

Parametric statistics were used when possible. In all parametric tests, "orthogonal" or type III sum of squares were used. The four leks were used as a blocking, random factor and were removed from the models when not significant. Similarly, interactions were examined and removed if not significant. The predictions called for not only differences between groups, but ordered differences, so two approaches were used: (1) ANOVAs in which the four genders were used as groups and (2) regressions in which the four genders were coded along an ordinal scale, with independents at one end, followed by satellites, faeders, and finally females (Robertson et al. 1988; Hirschenhauser et al. 2003). Both analyses have drawbacks that must be kept in mind during the interpretation of the results: the ANOVA ignores order and treats the independent variables as equal categories, while the regression assumes the fout genders equidistant along the ordinal scale, so the direction of the slope is relevant, but its value is immaterial.

Results

Innate immunity (haemolysis–haemagglutination assay)

Neither agglutination nor lysis differed among the four genders (ANOVA—lysis: $F_{[3,60]} = 2.51$, $P = 0.07$; agglutination: $F_{[3,60]} = 2.24$, $P = 0.09$). In a multivariate comparison, again, the four genders did not differ significantly ($F_{[3,59]} = 0.75$, $P = 0.53$), agglutination titres were significantly higher than lysis titres ($F_{[1,59]} = 80.4$, $P < 0.001$), and the interaction was not significant ($F_{[3,59]} = 2.57$, $P > 0.06$). Variances did not differ between the two sexes or among the four genders (Levene's test: $P > 0.05$ for all tests; Fig. 1). Regressions with independents, satellites, faeders and females, in that order, showed that haemolysis titres decreased significantly from independents to females ($b_{[1]} = -0.307$, $r^2 = 0.094$; $t_{[61]} = 2.52$, $P = 0.01$), whereas agglutination titres did not ($P > 0.05$; Fig. 1).

Cell-mediated immunity (haemolysis–haemagglutination and delayed hypersensitivity)

CMI was estimated with two assays. The agglutination titres from the haemolysis–haemagglutination assay on the before-vaccination blood samples were used to estimate B-cell proliferation resulting from exposure to LPS. In a multivariate analysis, there is still no differences among the four genders ($F_{[3,58]} = 0.9$, $P = 0.44$); as expected, the agglutination titres were significantly higher than the lysis titres ($F_{[1,58]} = 266$, $P < 0.001$), and the change between agglutination and lysis titres differed significantly among the four groups (interaction: $F_{[3,58]} = 7.75$, $P < 0.0002$; Fig. 2). Variances did not differ between the two sexes or among the four genders (Levene's test: $P > 0.05$ for all tests). Regressions showed that haemolysis titres decreased significantly from independents to females (haemolysis titres decreased from independents to females ($b_{[1]} = -0.402$, $r^2 = 0.161$, $t_{[60]} = 3.39$, $P = 0.001$), whereas agglutination titres did not ($P > 0.05$; Fig. 2).

Fig. 1. Ruff (*Philomachus pugnax*) baseline hemolysis and hemagglutination titres. Values are least-square means with 95% confidence intervals (CIs). Haemolysis titres ($b_{[1]} = -0.307$, $r^2 = 0.094$, $t_{[61]} = 2.52$, $P = 0.01$). Agglutination titres ($P > 0.05$). ANOVAs ($P > 0.05$).

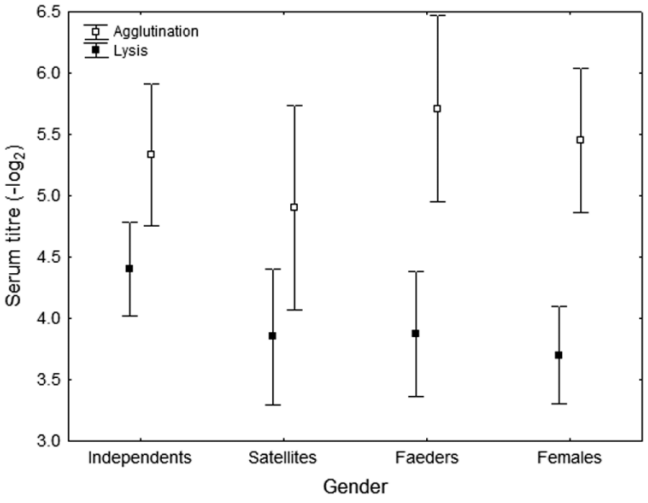
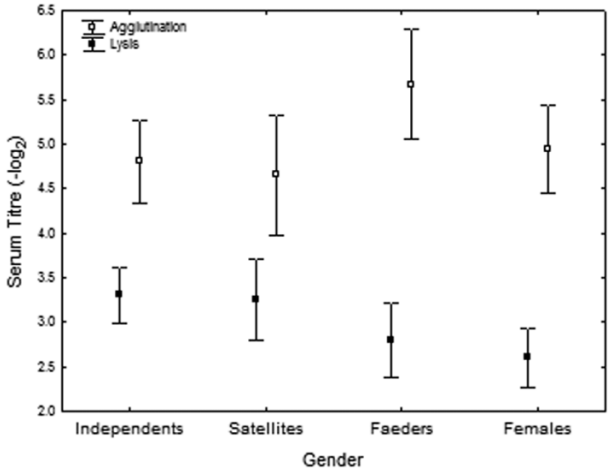


Fig. 2. Ruff (*Philomachus pugnax*) hemolysis and hemagglutination titres 7 days after an LPS injection. Values are least-square means with 95% confidence intervals (CIs). Haemolysis titres ($b_{[1]} = -0.402$, $r^2 = 0.161$, $t_{[60]} = 3.39$, $P = 0.001$). Agglutination titres ($P > 0.05$). ANOVAs ($P > 0.05$).



In the delayed hypersensitivity test, the mean time between injection and measurement was 23 h and 42 min (SD = 45 min). Responses to PHA were significantly different among the 4four genders (two-way ANOVA—gender: $F_{[3,55]} = 6.09$, $P = 0.001$; lek: $F_{[3,55]} = 4.37$, $P = 0.007$). Mean response by females was significantly lower than that of independents (Fisher LSD; df = 55, $P = 0.007$) and satellites (Fisher LSD: df = 55, $P = 0.004$). Regressions showed that responses decreased significantly from independents to females ($b_{[1]} = -0.340$, $r^2 = 0.115$; $t_{[60]} = 2.79$, $P = 0.007$; Fig. 3). Variances did not differ between the two sexes or among the four genders (Levene's test: $P > 0.05$ for all tests).

Antibody-mediated immunity (anti-PHA and anti-LPS IgY)

Anti-PHA and anti-LPS IgY were low, leading to a positive skew (anti-PHA: skewness = 2.36, SE = 0.308; snti-LPS: skewness = 1.154, SE = 0.311) and hence non-normally distributed (Lilliefor's test:

Fig. 3. Ruff (*Philomachus pugnax*) delayed hypersensitivity responses by gender. Values are least-square means with 95% confidence intervals (CIs) (two- way ANOVA—gender: $F_{[3,55]} = 6.09$, $P = 0.001$; lek: $F_{[3,55]} = 4.37$, $P = 0.007$). Females < independents (Fisher LSD: df = 55, $P = 0.007$) and females < satellites (Fisher LSD: df = 55, $P = 0.004$). Regression: $b_{[1]} = -0.340$, $r^2 = 0.115$, $t_{[60]} = 2.79$, $P = 0.007$.

$P < 0.01$ for both). Transformations (square root, ln, or $\log_{10}$) were not sufficient to attain normality, so nonparametric tests were used.

Anti-PHA and anti-LPS IgY were significantly correlated (Spearman's correlation: $r = 0.535$, $P < 0.05$). Neither anti-PHA (Kruskal–Wallis ANOVA by ranks: $H_{[3,60]} = 2.57$, $P = 0.463$) nor anti-LPS (Kruskal–Wallis ANOVA by ranks; $H_{[3,59]} = 4.12$, = 0.249) differed among the four genders. Regressions were not significant ($P > 0.05$). Variances did not differ between the two sexes or among the four genders (Levene's test, $P > 0.05$ for all tests).

Total antioxidant capacity and total oxidative stress

Response variables were tested for normality using Lilliefors test and morphs were compared using ANOVAs or Kruskal–Wallis tests, as appropriate. In all cases, before-, after-, and Δ- TAC and TOS did not differ significantly among the four genders (least-squares means—ΔTAC in mmol trolox equivalent/L: independents = 0.22, satellites = 0.27, faeders = −0.38, females = −0.12; ΔTOS in μmol H_2O_2 equivalent/L: independents = 0.024, satellites = 0.010, faeders = −0.020, females = −0.014). Also, there were no significant patterns with the genders on an ordinal scale (independent, satellite, faeder, female), as analysed with regression.

Innate immunity versus oxidative capacity (TAC) and oxidative stress (TOS)

Baseline TAC was not correlated with baseline agglutination titre, a measure of antibodies, but it was negatively correlated with baseline lysis titre ($r = -0.44$, $n = 24$, $P = 0.03$), which is a measure of antibodies and complement. However, the relationship between TAC and lysis titre neither differed among the four genders (ANOVA for homogeneity of slopes interaction effect: $F_{[3,17]} = 0.75$, $P = 0.5$) nor between the two sexes (ANCOVA main effects: $F_{[3,20]} = 1.23$, $P = 0.33$). No significant relationships occurred between oxidative stress and immunity, before or after the LPS injection.

Discussion

The salient results of this study were (*i*) innate immunity and two measures of CMI decreased along the axis from independents to females, providing support to the risk-of-injury explanation for the allocation of resources towards immune function; (*ii*) no such pattern was evident for oxidative stress or resistance; and (*iii*) no relationships were detected between immune function and oxidative resistance or stress.

Whereas most studies are confined to comparisons between two sexes, we compared four genders with different life histories, behaviour, and ecology. Variances and mean responses were examined. Previously, we showed immune responses during the nonbreeding season are more variable among males than females (Lozano and Lank 2003), so here we tested for but failed to find differences in variance in either immune responses, antioxidant capacity, or oxidative stress. These results were not as expected, but because the current study was conducted during the breeding season, the results do not necessarily disagree with our previous work. Nevertheless, we cannot think of any reason why differences in the variance of immune responses should be more pronounced during the nonbreeding period. In fact, if anything, it ought to be the other way around; differences between the genders should be more prominent during the breeding period. Seasonal differences in mean immune responses are not uncommon (reviewed by Nelson and Demas 1996), but here we reiterate that based on life history, we can and should also make predictions about variances, not only means.

Two hypotheses provided contrasting predictions about mean responses. If immune responses depend on energetic constraints, independents should be less able to invest in immune function, followed by satellites, faeders, and finally females. On the other hand, the risk-of-injury argument predicts the exact opposite pattern, with independents spending the most on immune defence and females the least. For wing-web thickness, the baseline and day 7 haemolysis titres were highest in independents and lowest in females, both at the start of the study and 7 days after injection with LPS. We did not measure energy expenditure, so the "energetic constraints" hypothesis cannot be completely dismissed, but these results nonetheless support the risk-of-injury alternative. This is consistent with our previous work showing that in the breeding season, independents had stronger immune responses than satellites (Lozano and Lank 2004), and extends the generality of this result to females and faeders.

Innate immune defences partially rely on inflammatory responses, which produce free radicals (Roitt et al. 1996; Surh and Packer 2005; Clark 2008), but we did not find the expected relationships between innate immunity and either antioxidant capacity or oxidative stress. In fact, the one significant correlation indicated that for blood samples taken at the start of the study, lysis titre (one of the measures of innate immunity) was negatively correlated with antioxidant capacity. This might be expected for individuals with a recent history of infection, whereby lysis of invading cells leave free radicals that must be neutralized by antioxidants, temporarily yielding high lysis titres and low antioxidant capacity. However, this correlation occurred in the before-challenge samples, which might reflect the unknown infection history in our flock, or that we simply do not know enough about the effects of seasonal infection cycles on antioxidant dynamics in this species. This is the first oxidative ecological study on a species with alternative reproductive strategies; so much remains to be learned.

Both the risk-of-injury and the energetic constraints hypotheses have been supported in other immunoecological studies on species with alternative reproductive strategies. For instance, in the Azorean rock pool blenny (*Parablennius parvicornis* (Valenciennes, 1836)), males start adulthood as satellites or sneakers (M–) and in their second or third year switch to either floaters or territory owners (M+) (Santos and Almada 1988). M– males have more injuries and stronger adaptive immunity than M+ males (Ros et al. 2006). In contrast, in Side-blotched Lizards (*Uta stansburiana* Baird and Girard, 1852), there are two female morphs: orange females that produce large clutches of small eggs and yellow females that produce small clutches of large eggs. Crowding weakens immune responses in both morphs but occurs more sharply in orange

females. Furthermore, the strength of the immune response is negatively related to subsequent survival in orange females, whereas it is positively related in yellow females (Svensson et al. 2001). Hence, the morph adapted to fast reproduction, i.e., the orange morph, has a more susceptible immune system and is less capable of activating it without suffering negative consequences. Similarly, in the peacock blenny (*Salaria pavo* (Risso, 1810)), older territory owners and floaters have lower immunity than younger female-mimicking sneakers (Ros and Oliveira 2009). Our previous and current work with Ruffs paint a more complex picture: energetic constraints are a better explanation for seasonal differences, but during the breeding season, investment in immune function is dependent on the risk of injury.

Immunoecology continues to be an exciting area of research in evolutionary ecology (reviews by Nelson and Demas 1996; Lochmiller and Deerenberg 2000; McDade 2003; Sadd and Schmid-Hempel 2009). Evolutionary ecologists are often seduced by the technical complexity of the reductionist approach, but integration is a two-way street that will not truly occur until immunologists also start applying evolutionary concepts. The most palatable outcomes and exciting prospects will occur as we accept that the two approaches are immiscible to some degree, and require conscientious and repeated mixing to remain suitably emulsified.

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