

Do social mating systems limit maternal immune investment in shorebirds?

D.B. Edwards, M. Haring, H.G. Gilchrist, and A.I. Schulte-Hostedde

Abstract: Across mating systems, females differ in the amount of resources they invest in offspring. For example, polyandrous females invest in acquiring multiple matings rather than providing parental care. We examined how the amount of maternal immune investment, measured as immunoglobulin Y and lysozyme activity in eggs, was influenced by female role across three social mating systems (polyandry, polygyny, and monogamy) in shorebirds. We predicted that polyandry should impose the greatest costs on the ability to provision eggs and monogamy, where females receive benefits from biparentality, the least. Contrary to our predictions, levels of maternally derived egg immune constituents were consistently high across measures in the polyandrous species and low in the monogamous species. Our results may support a link with pace-of-life where developmental costs are greater than the energetic costs of provisioning eggs, and (or) a role for sexual selection acting on maternal immune investment.

Key words: life history trade-offs, immunoglobulins, lysozyme, pace-of-life, polyandry, polygyny, monogamy, Charadriiformes, Scolopacidae.

Résumé : La quantité de ressources investie par les femelles dans leur progéniture varie selon le système d'accouplement. Par exemple, les femelles polyandres investissent des ressources dans l'obtention d'accouplements multiples plutôt que dans les soins à leur progéniture. Nous avons examiné, chez des oiseaux de rivage, l'influence du rôle de la femelle dans trois systèmes d'accouplement (polyandrie, polygynie et monogamie) sur l'ampleur de l'investissement immunitaire maternelle, établie en mesurant la concentration d'immunoglobuline Y et l'activité du lysozyme dans les œufs. Nous avons prédit que la polyandrie imposerait le coût le plus élevé sur la capacité d'approvisionner les œufs et que ce coût serait le plus faible pour la monogamie, dans laquelle les femelles tirent des avantages de la biparentalité. Contrairement à ces prédictions, les niveaux de constituants immunitaires provenant de la mère dans les œufs étaient uniformément élevés chez les espèces polyandres et faibles chez les espèces monogames. Nos résultats pourraient appuyer l'existence d'un lien avec le rythme de vie selon lequel les coûts du développement seraient plus grands que les coûts énergétiques associés à l'approvisionnement des œufs, ou encore un rôle de la sélection sexuelle dans l'investissement immunitaire maternel. [Traduit par la Rédaction]

Mots-clés : compromis associés au cycle biologique, immunoglobulines, lysozyme, rythme de vie, polyandrie, polygynie, monogamie, charadriiformes, scolopacidae.

Introduction

Mothers confer direct genetic benefits to offspring, but females also influence offspring quality via indirect avenues that are likely to have important consequences for the offspring's phenotype. In birds, eggs are provisioned with all the components required by the offspring until the time of hatching, including hormones (Gil 2008), antioxidants (Williamson et al. 2006), and immune factors (Boulinier and Staszewski 2008), and the extent of this initial maternal investment is expected to affect offspring quality (Mousseau and Fox 1998).

The avian immune system is not fully developed at the time of hatching and, as a result, maternally derived immune constituents provide an important level of defence for the offspring at hatch (Grindstaff et al. 2003). Immune factors can influence offspring quality in several ways: by directly preventing infections that occur in the egg, by enhancing immune ability after hatching, and by promoting growth and development by delaying the need for early maturation of the offspring's immune system

(Grindstaff et al. 2003). In a variety of species, maternal immune investment influences offspring immunocompetence, growth, and survival (Rollier et al. 2000; Saino et al. 2002; Hasselquist and Nilsson 2009; Abad-Gómez et al. 2012).

Reproduction is costly for females, both in terms of egg production and incubation (Partridge and Harvey 1985; Piersma et al. 2003; Williams 2005). Energy constraints prior to egg laying result in lower immunoglobulin levels in hatchlings (Pihlaja et al. 2006; Kilpimaa et al. 2007; Karell et al. 2008; Moreno et al. 2008), because females are unable to adequately provision eggs when resources are limited. Therefore, we expect there to be marked differences in the degree of maternal immune investment across species where female reproductive roles differ. Such would be the case across mating systems, where females face different energetic demands during pre- and post-laying that could influence their ability to provision offspring, specifically through investment in eggs.

For example, the production of multiple clutches in polyandrous shorebirds is a considerable expense not faced by polygynous or

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monogamous species (Ross 1979). Similarly, females of polyandrous species arrive early to the breeding grounds when conditions are more harsh (typically the opposite for other mating systems) and initiate courtship displays and chase flights, which are energetic expenses unique to polyandrous females. As a consequence of polyandry, prelaying female Red Phalaropes (*Phalaropus fulicarius* (L., 1758)) are immunosuppressed relative to males (D.B. Edwards, G. Burness, A.I. Schulte-Hostedde, and H.G. Gilchrist, unpublished data)—another pattern opposite to most species. In particular, female Red Phalaropes have lower levels of plasma immunoglobulin Y, which is an important immune component transferred to the egg by females (Grindstaff et al. 2003). Because plasma immunoglobulin levels correlate with levels allocated to eggs (Hasselquist and Nilsson 2009), reduced levels in female Red Phalaropes likely have consequences for offspring. As a consequence of these unique factors, we predict females of polyandrous species to have low levels of maternal immune investment. In contrast, we expect biparental (monogamous) species to have a greater capacity to buffer initial maternal investment because males share in reproductive duties enabling females to more easily meet energetic demands (Reid et al. 2002). As such, we predict monogamous species will have high maternal immune investment.

Shorebirds are income breeders that rely on energy stores acquired while on the breeding grounds (Klaassen et al. 2001). Uniparental shorebirds, where only one sex incubates, must supplement energy stores with feeding shifts during incubation and in doing so suffer costs associated with reheating eggs (Monaghan and Nager 1997). Additionally, more frequent or longer feeding breaks in incubating females can slow development and ultimately affect quality of offspring (Martin and Schwabl 2008). In Blue Tits (*Cyanistes caeruleus* (L., 1758)), females mated to polygynous males laid eggs with fewer antimicrobial proteins (D'Alba et al. 2010), possibly as a cost saving measure to balance reduced paternal care. Therefore, we predict females of polygynous species to have intermediate levels of maternal immune investment because they provide sole parental care but lack the costs to polyandrous females.

We tested for differences in egg immune constituents in three species of shorebirds (Charadriiformes: Scolopacidae) representing monogamous, polygynous, and polyandrous social mating systems. This is an appropriate group to study for several reasons. First, the group has large variation in mating strategies within a narrow phylogenetic group; all three species are contained within a subgroup of Scolopacidae (Thomas et al. 2004). Second, precocial shorebirds produce large and well-provisioned eggs and the costs for doing so are considerably higher than for altricial species (King 1973; Ricklefs 1974). Third, clutch size in shorebirds is relatively invariable (Colwell 2010), and as a consequence, maternal provisioning of individual eggs may be an important avenue to improve reproductive success.

We measured yolk immunoglobulins (IgY) and albumen antimicrobial activity as measures of maternal immunological investment in eggs. IgY is allocated to the yolk as a means of bolstering the naïve immune system of offspring when endogenous production of antibodies is minimal (Grindstaff et al. 2003). Moreover, antibodies passed to the offspring reflect previous disease exposure of the mother (Gasparini et al. 2002; Baintner 2007), so that females can convey specific immunity to the local environment that persists beyond hatching. Females also confer antimicrobial peptides, chief among them being lysozyme. Lysozyme is a key component of the albumen with important antibacterial properties, particularly towards gram-positive bacteria (Wellman-Labadie et al. 2008), the effects of which can also persist beyond hatching (Saino et al. 2002).

Materials and methods

Study site and species

Our samples were collected at the East Bay Migratory Bird Sanctuary (63°59'13.5"N, 81°41'48.3"W) on Southampton Island, Nunavut, Canada, in June–July 2011. We collected eggs from three species that represented the three major social mating systems: Ruddy Turnstones (*Arenaria interpres* (L., 1758)) are monogamous and both sexes share in incubation–chick-rearing and territorial duties; White-rumped Sandpipers (*Calidris fuscicollis* (Vieillot, 1819)) are polygynous, meaning that males provide no parental support to incubating females; Red Phalaropes are polyandrous, and as a consequence, females lay the eggs then depart to find new mates, providing no parental support to the incubating male.

These species typically lay four eggs per clutch and so investment at the level of clutch size does not differ between species (Colwell 2010). At our site, the microhabitat used by Red Phalaropes and White-rumped Sandpipers for nesting completely overlaps and they are both generally found throughout our study site. Ruddy Turnstones tend to nest nearer the coast on rockier and more sparsely vegetated substrate; however, White-rumped Sandpipers and Red Phalaropes use this habitat as well for nesting. All three species migrate to overwintering areas, but White-rumped Sandpipers (Parmelee 1992) tend to migrate farther, and unlike Ruddy Turnstones (Nettleship 2000) and Red Phalaropes (Tracy et al. 2002), they commonly take terrestrial routes. Ruddy Turnstones and White-rumped Sandpipers overwinter in coastal habitat, whereas Red Phalaropes occupy near- and off-shore areas. Whereas life-history differences could contribute to differences in parasite risk, we have failed to detect any blood parasites in these species at our field site in the past ($n > 50$ for all species; D.B. Edwards, A.I. Schulte-Hostedde, and H.G. Gilchrist, unpublished data). Additional information on the species in this study, including incubation periods and adult body mass, is included in the supplementary Table S1.¹

Sample collection and field preparation

We commenced nest searching when we began to observe mating behaviour (i.e., copulations and territorial defence) in our focal species. We surveyed our study site (twelve 1 km² plots) over the course of the breeding season and located nests either by flushing the bird off their nest or watching a bird returning to their nest to incubate. At this time, we collected one egg from the nest. Collecting in this manner, we could not control for laying order, but we collected a total of 10 eggs per species to reduce the likelihood that species differences were biased as a result of laying order. For White-rumped Sandpipers and Ruddy Turnstones, the eggs were collected from independent females.

Among the three species in this study, Red Phalaropes are the most likely to lay multiple clutches within a season because they are polyandrous. With this said, actual rates of polyandry, which depends upon the availability of males, can be relatively low in Red Phalaropes (typically <50% of females; Tracy et al. 2002). To increase the probability of including eggs laid only to first clutches, we collected eggs laid early in the season starting from the first Red Phalarope nest that was discovered (25 June). Backdating nests we collected from confirmed that we were consistently collecting from the earlier nests laid this season, and also from a relatively narrow window of time, making it unlikely that we collected from the same female more than once. For White-rumped Sandpipers and Ruddy Turnstones, we could confirm that we collected from independent females because nests were temporally overlapped.

Our goal was to collect eggs as early in development as possible. In chickens, the concentration of serum IgY increases due to changes in overall water content, but remains relatively stable for

¹Supplementary Table S1 is available with the article through the journal Web site at <http://nrcresearchpress.com/doi/suppl/10.1139/cjz-2013-0122>.

the first half of the developmental period (Kowalczyk et al. 1985). Because of this, we limited our collections to eggs in the first half of their developmental period. Age was known for eggs collected from nests found with incomplete clutches. We waited until the clutch was completed to prevent abandonment and so the eggs were collected within the first 1 or 2 days of incubation ($n = 8$). For the remaining eggs, age was estimated by measuring the angle that they floated at in water following Liebezeit et al. (2007).

Eggs were measured and weighed whole. We used length and breadth measures to calculate egg volume (Narushin 2005). Subsequently, we separated yolk from albumen using a commercial egg-yolk separator and weighed each to the nearest 0.001 g. Samples were frozen at -20°C for about a month until they were processed in the laboratory.

IgY extraction and analysis

Our protocol for extracting yolk IgY closely follows that used by Addison et al. (2009). Yolk samples were thawed overnight at 4°C . We vortexed 0.1 g of yolk sample with a solution of 3.5% PEG 6000 in 0.9% NaCl to remove lipid from the sample. This was incubated overnight at 4°C then centrifuged for 30 min at 8000g. The supernatant was run for 90 min at 90 V on an 8% SDS-PAGE gel. To visualize IgY concentration, gels were stained using gelcode blue staining reagent (Pierce Biotechnology) for 1 h, then left overnight in distilled water. We scanned gels using a flatbed scanner and determined band densities using the gel tool in Image J (National Institutes of Health, Bethesda, Maryland, USA; available from <http://imagej.nih.gov/ij>). We converted sample values returned by Image J to concentrations (mg/mL) using a standard curve generated for each run using known concentrations of chicken IgY (purified polyclonal; Sigma Aldrich) as controls. Species were randomly interspersed among gels.

Antimicrobial activity

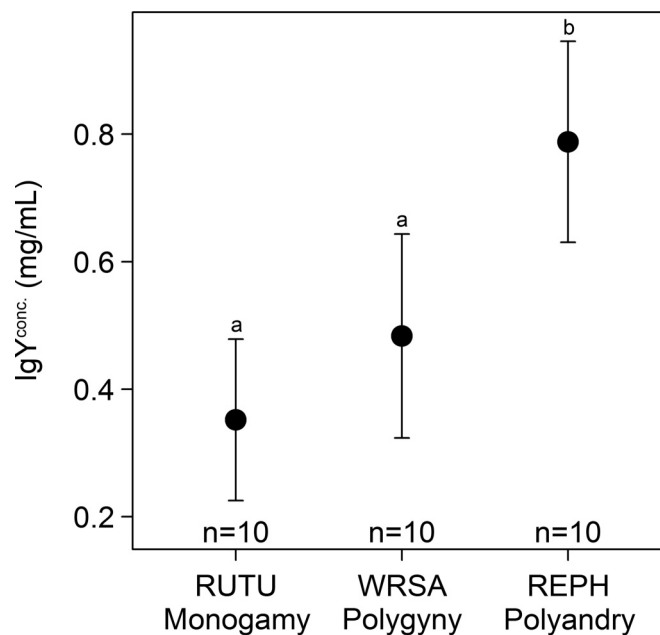
We assessed egg white antimicrobial activity in triplicate in the presence of *Micrococcus lysodeikticus* (ATCC 4689). We added 25 μL of sample to 175 μL of 0.075% solution of bacterial solution in a 0.1 mol/L phosphate citrate buffer at pH 5.8. Samples were read immediately (T_0) at 450 nm in a Molecular Devices microplate reader (Molecular Devices, Sunnyvale, California, USA), then mixed frequently for 5 min and read a second time (T_5). Activity is expressed as the change in absorbance over time ($T_0 - T_5$). As more bacterial cells are lysed, the solution becomes less murky and so large values represent high antimicrobial activity. Antimicrobial activity of albumen results from several compounds, chief among them is lysozyme (Baron and Réhault 2007). Because of this, we use the term “lysozyme” throughout the study when referring to this assay, but we acknowledge that other compounds with antimicrobial activity may contribute to species differences in antimicrobial activity observed in this study.

Statistical analysis

We identified two ways in which costs of maternal deposition of immune components could be influenced by mating system: (1) benefits to the offspring and (2) costs to the mother. For IgY, we measured costs to the offspring by representing IgY in terms of concentration in the yolk (mg/mL), assuming that a higher concentration would result in a greater immunoenhancing effect. Alternatively, we expressed the total IgY as mass-specific IgY ($\text{mg}^{\text{IgY}}/\text{g}^{\text{adult body mass}}$), assuming in this case that larger female body size relative to yolk volume should reduce the costs of high levels of IgY for mothers. We hereafter referred to these measures as $\text{IgY}^{\text{conc.}}$ and IgY^{mass} , respectively.

To determine IgY^{mass} , we calculated the amount of IgY in the yolk based on the concentration and the volume of yolk. We obtained body mass for only a very small number ($n = 4$) of individuals whose eggs we collected for this study. To correct for body mass, we used female mass measured from breeding individuals

at our site from 2008 to 2010 (see supplementary Table S1).¹ Within species, we randomly ascribed adult body mass for all individuals to 2011 samples in a bootstrap procedure (1000 replications). Species values for $\text{IgY}^{\text{conc.}}$, IgY^{mass} , and lysozyme activity were compared using ANOVA and Tukey's post hoc tests. We included the percentage of incubation complete (incubation) as a cofactor for all analyses. All statistical analyses were performed in R (R Core Team 2012).



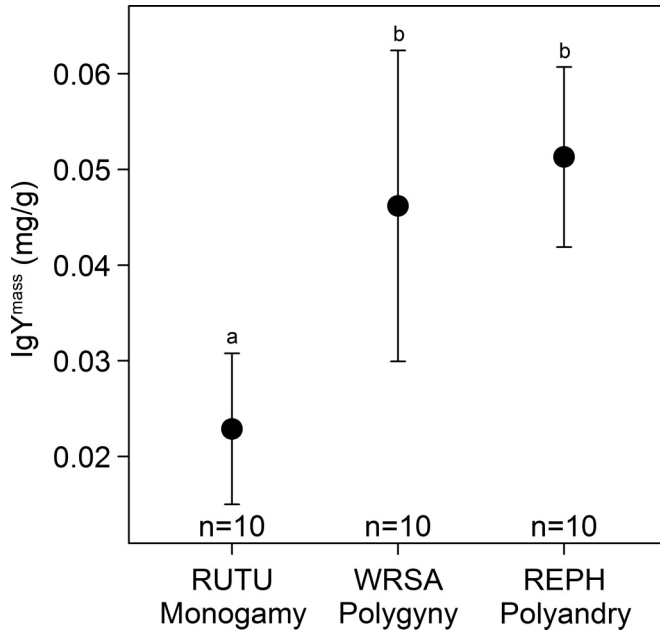
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Results

Our three measures of maternal immune investment produced similar results. Differences in yolk $\text{IgY}^{\text{conc.}}$ (species: $F_{[2,26]} = 11.56$, $P < 0.001$; incubation: $F_{[1,26]} = 1.01$, $P = 0.32$; Fig. 1) did not conform to our predictions based on mating systems. Red Phalaropes (polyandrous) had higher yolk IgY concentration than Ruddy Turnstones (monogamous) or White-rumped Sandpipers (polygynous). Although not statistically distinguishable from White-rumped Sandpipers, Ruddy Turnstones had the lowest values.

IgY^{mass} , yolk IgY expressed as milligrams per gram of adult body mass, was lowest in Ruddy Turnstones and highest in White-rumped Sandpipers and Red Phalaropes (species: $F_{[2,27]} = 8.49$, $P = 0.002$; incubation: $F_{[1,27]} = 1.22$, $P = 0.29$; Fig. 2). Both measures of IgY show marked differences between Ruddy Turnstones and Red Phalaropes, with IgY^{mass} being high in Red Phalaropes despite laying small eggs for their body size. On the other hand, in White-rumped Sandpipers, our conclusion about the level of provisioning was dependent upon the way we measured IgY. White-rumped Sandpipers had low levels of yolk IgY ($\text{IgY}^{\text{conc.}}$), but a high rate of provisioning after correcting for yolk volume and body size (IgY^{mass}). Lysozyme activity was greatest in Red Phalaropes and indistinguishable between Ruddy Turnstones and White-rumped Sandpipers (species: $F_{[2,24]} = 8.62$, $P = 0.002$; incubation: $F_{[1,24]} = 0.14$, $P = 0.71$; Fig. 3). The three species varied a great deal in the amount of variation in lysozyme activity, and the extent of variation appeared to be related to the amount of activity. Red Phalaropes had the highest levels of lysozyme activity and values were

Fig. 2. Mass-specific yolk immunoglobulin (IgY^{mass}) was lowest in the monogamous Ruddy Turnstone (*Arenaria interpres*; RUTU). Using a correction for mass and egg size, IgY deposition was not different for Red Phalaropes (*Phalaropus fulicarius*; REPH) and White-rumped Sandpipers (*Calidris fuscicollis*; WRSA). Plot was created using mean species values, although statistical tests were done using a bootstrap procedure of all breeding females caught at our site in previous years. Bars represent 95% confidence limits and lowercase letters denote statistical significance following post hoc tests performed on mean species mass.



quite variable, with several individuals having very high rates of clearance. White-rumped Sandpipers, on the other hand, had very low rates of clearance and little variation in lysozyme activity.

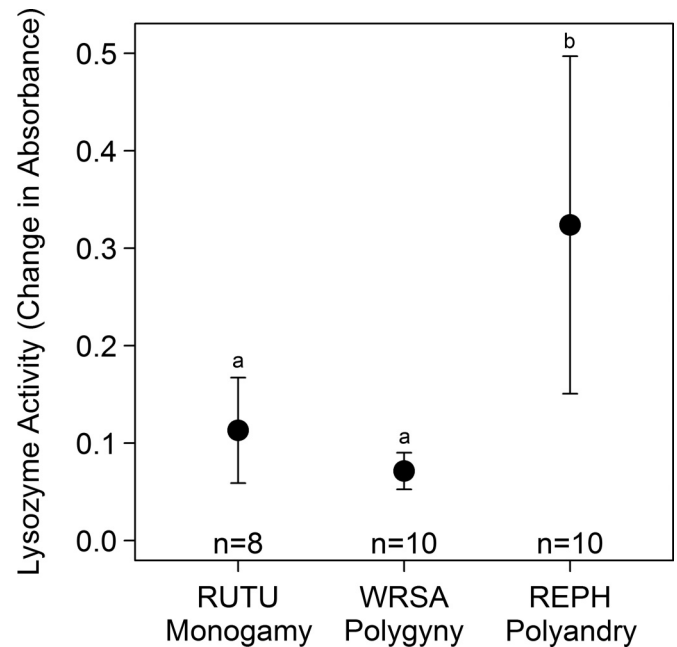
Discussion

We predicted that maternal investment, as measured through egg immune constituents, should vary across species in relation to mating system. Our results were in fact opposite to our predictions, with polyandrous Red Phalaropes having the highest levels of immune investment in eggs for all measures and Ruddy Turnstones (monogamous) generally having the lowest levels. In White-rumped Sandpipers (polygynous), the concentration of IgY in the yolk (IgY^{conc}) and the activity of lysozyme was low and indistinguishable from Ruddy Turnstones. However, the ability to provision the eggs with IgY , which we measured as the total amount of IgY in the egg correcting for body size (IgY^{mass}), was high and indistinguishable from Red Phalaropes.

Our prediction for Red Phalaropes was the clearest to formulate. Females possess several traits or behaviours that are likely to influence trade-offs with maternal provisioning. Consequently, we expected maternal investment in Red Phalaropes to reflect a quantity versus quality trade-off typically applied to males (Bateman 1948; Einum and Fleming 2000). However, for all measures, Red Phalarope eggs had high levels of immune traits. Our argument relies upon the notion that maternally derived immune constituents are costly to provision. Although there may be high energy requirements to synthesize immune components (Klasing 1998), to date there is mixed evidence that immune function, such as antibody responses, is universally costly (e.g., Eraud et al. 2005).

In terms of IgY , as follicles mature there is a short-term increase in IgY production to meet the demands required for deposition in the eggs, suggesting that the addition of IgY is not a passive pro-

Fig. 3. Red Phalarope (*Phalaropus fulicarius*; REPH) albumen had higher lysozyme activity than Ruddy Turnstones (*Arenaria interpres*; RUTU) and White-rumped Sandpipers (*Calidris fuscicollis*; WRSA). Bars represent 95% confidence limits and lowercase letters denote statistical significance following post hoc tests.



cess, but rather demands energy and resources over a short period of time (Grindstaff et al. 2003). In support of the cost of antibody production during reproduction, chicken lines that produce high levels of antibodies have lower egg production (Siegel et al. 1982; Martin et al. 1990). Although these two studies do not explicitly demonstrate a link between egg production and provisioning eggs with antibodies, they nevertheless show that high antibody production and high egg production are not compatible, and why we expected low levels of IgY in Red Phalaropes.

Our results are not compatible with the view that the costs of uniparental incubation and polyandry result in reduced maternal immune investment, but rather could be explained by differences in pace-of-life (POL); species with a fast POL have shorter developmental periods, high reproductive potential and, reduced lifespan (Promislow and Harvey 1990). Adult immune investment across species is frequently related to POL (Tieleman et al. 2005; Palacios and Martin 2006; Lee et al. 2008; Edwards 2012), although the nature of the relationships can vary, making predictions difficult. Addison et al. (2009) found that faster paced species have higher rates of IgY deposition in eggs, which is consistent with the differences found between Ruddy Turnstones (slow) and Red Phalaropes (fast; White-rumped Sandpipers are closer to Red Phalaropes in terms of POL) in the present study (see supplementary Table S1).¹ Because polyandry constrains egg size (Liker et al. 2001)—at our field site, Red Phalarope eggs are 15% lighter than those of White-rumped Sandpipers, despite adult female Red Phalaropes being 28% heavier—high rates of provisioning could be a relatively inexpensive way to offset the costs of uniparental incubation or, in particular, small egg size in Red Phalaropes. Addison et al. (2009) outline an argument for developmental costs of maternal antibody deposition based on the idea that high maternal antibody deposition can impede the development of offspring immunity by inhibiting B-cell development (Carlier and Truysens 1995; Siegrist 2007). Along the POL continuum, it is argued that for faster paced species that have a shorter lifespan, initial growth rate is more important than adult immune function (Lee 2006). Because Red Phalaropes lay small eggs for their body size, the advantages to them of high

maternal immune investment could be higher than for other species if this investment facilitates faster growth. Although studies have demonstrated that the benefits of maternal immune investment wane prior to fledging (e.g., Karell et al. 2008), studies that demonstrate long-term influences generally support a positive effect (Gasparini et al. 2006; Reid et al. 2006). As a result, more study is needed to explain the negative relationship that has been described between maternal immune investment and POL.

POL studies also emphasize different predictions for innate and adaptive immune functions (Lee 2006): species with faster paced lifestyles should exhibit greater investment in innate immunity relative to antibody-mediated functions. In the present study, innate (lysozyme) activity and IgY levels in the egg were both high in Red Phalaropes and conversely both low in Ruddy Turnstones, perhaps demonstrating that across the three species no such trade-off was occurring.

An alternative, but not mutually exclusive, explanation for high levels of maternal immune investment in Red Phalaropes is that the rate of provisioning by females is an avenue for sexual selection. Females often choose to mate with more immunocompetent males (e.g., Ekblom et al. 2005) and so sexual selection can promote higher immune investment across species (Edwards 2012). However, in species where females provide no parental care, such as in polyandrous Red Phalaropes, male mate choice may be acting upon the level of initial maternal investment in a similar way, particularly if the level of maternal investment is related to plumage coloration of individual females. A benefit to mating with a bright female could be immunologically well-provisioned eggs, and selection in this manner could result in polyandrous Red Phalaropes having high levels of maternal immune investment.

Finally, Addison et al. (2009) found that larger bodied species allocate more IgY to eggs than smaller species based on yolk IgY concentration, possibly due to reduced marginal costs resulting from larger body size. In the present study, we found the opposite pattern suggesting that our results are unlikely to be due to a body size effect. Furthermore, when we used IgY corrected for body mass and egg size (IgY^{mass}) as a way to adjust for the simple mechanics of a relatively large-bodied bird provisioning a relatively small yolk, we found similar results to using yolk IgY concentration and lysozyme activity.

In summary, we did not find evidence for energetic costs of mating systems relating to maternal immune investment. Rather, our results may support maternal immune investment as a function of developmental costs associated with POL trade-offs in which faster paced species invest in higher levels of maternal immune investment, and (or) as a sexually selected trait. Whereas we might predict that slow-paced species with high adult immune investment also deposit higher levels in eggs than fast-paced species, we are the second study to report the opposite pattern. This pattern may simply reflect the tendency of fast-paced species to invest more in current reproduction, in which high rates of maternal immune investment is one way to do so. Because provisioning eggs with immune components is an active process, this pattern suggests that fast-paced species achieve this by more substantial production and deposition of immune components than do slow-paced species. Thus, to clarify this relationship, it will be fruitful to determine to what degree rates of maternal immune investment are influenced by costs to adults or by costs to offspring. In future studies, we seek to expand to a larger comparative framework while also including similar immune measures in adults.

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