MATE CHOICE AND IMMUNOCOMPETENCE IN OSTRICHES (*STRUTHIO CAMELUS*)

by

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Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Females of many bird species prefer to mate with males exhibiting elaborate ornamentation, which serves as an indicator of male quality. Such ornaments, called secondary sexual traits, could act as signals to females that males could confer direct and/or indirect genetic benefits (when offspring inherit superior genes), on offspring. In particular, it has been suggested that these signals relate to male ability to resist infections, as only high quality individuals are able to invest both in high immune defence and elaborate ornament expression.

The ostrich (*Struthio camelus*) is the largest living bird and is a member of the family of flightless birds, the ratites. They are sexually dimorphic, males displaying black plumage, and a pink-coloured neck and bill; whereas females display dull-brown plumage (both sexes have white feathers). Little is known about the mating system of ostriches: they are promiscuous and in the wild, males and females have multiple partners. The communal nesting system of ostriches is unique in that only the major female and major male provide parental care, in the form of incubation and guarding the offspring until independence. Furthermore, a remarkable feature of cohorts is that offspring may differ greatly in size, and these size differences are likely to have a genetic basis arising from differing parental genotypic differences.

As a trade-off between immune response and life-history traits has been documented in various bird species, I examined the relationships between male secondary sexual traits (and specifically colouration) and maternal investment; levels of immunocompetence in both parents and chicks; and chick growth. This study showed that females invest more at the egg stage in response to traits involved in the male courtship display: the colour of the neck, white and black body feathers, and the brightness of black feathers. As these traits, which are exposed during the courtship display as well as during male-male interactions, were related to male immune responses, I suggest that only high quality males will be able

to display their condition optimally. Chicks with higher growth rates were found to have intermediate responses to stimulation of their humoral immune system with diphtheria and tetanus vaccines, suggesting that not only fitness benefits, but also costs are associated with mounting an immune response; and that variation in humoral responses and growth rates relates to how individuals trade off these costs and benefits. In addition, chick humoral responses were found to be related to the humoral response of both parents, but through different antibody responses (maternal responses to tetanus and paternal responses to diphtheria), suggesting that this component of the immune system is heritable. As the colouration of white feathers predicted chick growth rates, as well as a male's ability to raise an antibody response, I suggest that this visual cue could serve as a signal to females of male humoral immunocompetence, therefore forming the basis of mate choice whereby females could increase the fitness of their offspring through higher growth rates.

OPSOMMING

Wyfies van verskeie voël spesies verkies om met mannetjies te paar wat oordadige ornamentasie vertoon en ’n aanduiding van manlike kwaliteit is. Ornamentasie kan as tekens dien vir wyfies dat mannetjies direkte en/of indirekte genetiese voordele kan bydra (as die nageslag superieure gene oorerf) tot haar nageslag. In particular, it has been suggested that these signals relate to male ability to resist infections, as only high quality individuals are able to invest both in high immune defence and elaborate ornament expression. Daar word beweer dat hierdie tekens die vermoë van mannetjies om infeksies weer te staan weerspieël, want net individuë van hoë gehalte in stand is om in beide hoë immuunbevoegdheid asook uitvoerige ornamentasie te belê.

Die volstruis (*Struthio camelus*) is die grootste lewende voël spesie en is lid van die nie-vlieënde voël familie ratites. Volstuise is geslagtelik dimorf. Die mannetjies het ’n swart en wit veredos met ’n pienk kleurige nek en snawel terwyl die wyfies ’n dowwe bruin veredos het. Min is bekend oor die parings sisteem van volstuise: hulle is promisku en in hul natuurlike omgewing het beide mannetjies en wyfies meer as een paringsmaat. Die gemeenskaplike nesmaak sisteem van volstuise is uniek in die sin dat slegs die hoof wyfie en hoof mannetjie ouersorg voorsien in die vorm van inkubasie en beskeming van die kuikens tot onafhanklikheid. Daarmee saam is die variasie in die groote van kuikens afkomstig van dieselfde nes ’n merkwaardige verkynsel wat heel moonlik ’n genetiese basis het wat spruit uit die verkille in ouer genotipes.

Aangesien ’n kompromie tussen immuunreaksies en lewensgeskiedenis eienskappe vir verskeie voël spesies aangeteken is, het ek die verwantskap tussen manlike sekondêre geslagskenmerke (meer spesifiek kleur) en materne investering, vlakke van immuunbevoegdheid in beide ouers en kuikens, en kuiken groei ondersoek.

Hierdie studie het getoon dat wyfies meer investeer tydens die eierstaduim in reaksie tot eienskappe betrokke by manlike hofmakingsvertoon: kleur van die nek, wit en swart verebedekking, en die helderheid van swart vere. Daarmee saam stel ek voor dat slegs mannetjies van hoë kwalitiet dit regkry om hul kondisie optimaal te vertoon aangesien daar bevind is dat die eienskappe vertoon tydens die hofmakings proses tesame met die gepaardgaande mannetjie tot mannetjie interaksie verband hou met manlike immuunreaksies. Verder is gevind dat kuikens met vinniger groeitempos intermediêre reaksies toon tot humorale immuunsisteemstimulasie met diphtheria en tetanus in-entings wat daarop dui dat daar nie net fiksheids voordele verbonde is aan die loods van immuunresponse is nie maar dat daar ook kostes is. Die varieërende humoral reaksies en groeitempos dui aan hoe individue die voordele en kostes teen mekaar opweeg. Daar is ook bevind dat kuikens se humorale reaksie verbind kan word tot die humorale reaksie van die ouers maar dat dit deur verkillende teenliggaam-reaksies plaasvind (materne reaksie tot tetanus en paterne reaksie tot diphtheria) en dit dui daarop dat hierdie komponent van die immuunsisteem oorerflik is. Aangesien die kleur van wit vere kuikens se groeitempo voorspel het, asook mannetjies se vermoë om teenliggame te produseer stel ek voor dat hierdie visuele aanduiding vir wyfies as ’n teken van ’n mannetjie se immuniteitsvermoëns dien en die basis vorm waarvolgens wyfies maatkeuse uitoefen om sodoende die fiksheid van hul nageslag te verhoog deur middel van ’n verhoogde groeitempo.

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CHAPTER ONE INTRODUCTION

1. The diversity of avian mating systems

Birds show considerable variation in mating systems, which relate to the number of social and sexual partners. A social partner cooperates in providing parental care and/or in defending the territory, whereas a sexual partner provides only gametes (Bennett & Owens 2002). In some species, individuals form social pairs and cooperate in raising offspring, while in others, individuals of one sex desert their offspring and social partner to seek for extra mating elsewhere. The majority of birds are socially monogamous (92% of all birds), with the remainder displaying social polygyny (2%), social polyandry (1%) and promiscuity (6%) (Lack 1968; Møller 1986; Davies 1991; Owens et al. 1999; Owens & Bennett 1997). Recently, with the advent of molecular techniques, social associations have been found to not necessarily indicate exclusive mating relationships, highlighting the need to differentiate between social mating systems and genetic mating system (Griffith et al. 2002).

1.1. Social mating systems

The variation in social mating in birds appears to be intimately linked to variation in parental care (Lack 1968; Owens & Bennett 1997), with only few species providing no post-hatching care for eggs or offspring. Ninety two percent of bird species are socially monogamous and they all show some form of biparental care (Lack 1968; Bennett & Owens 2002), with the exception of megapodes (family Megapodidae) and obligate brood parasites such as cuckoos (family Cuculidae and Coccyzidae) or cowbirds (family Fringillidae). Therefore variation in social mating systems in birds is linked to the incidence and distribution of desertion of one sex, referred as mate desertion (Davies 1991) or more recently as offspring desertion (Székely et al. 1996), offspring being directly affected by the absence of one parent.

Owens (2002) estimated that offspring-desertion by males occurs in at least 19 avian families and usually occurs in various forms of social polygyny, where the male deserts or partially deserts his offspring and mate (Lack 1968; Davies 1991; Ligon 1999; Owens & Bennett 1997). Offspring-desertion through resource-based polygyny is the widespread form of desertion and occurs among most of the passerine families as well as in other species such as owls, hummingbirds or wrens (from the family Tytonidae, Trochilidae and Certhidae respectively; Bennett & Owens 2002). Some other forms of male desertion occurs in harem polygyny, where a male defends a groups of females and copulates with each of them before they leave the harem to lay eggs and raise offspring alone (e.g. a few species of pheasants, family Phasianidae, and tinamous, family Tinamidae; Bennett & Owens 2002); and territorial polygyny, in which males provide some level of parental care, e.g. the corn bunting, *Emberiza calandra* (Hartley & Shepherd 1994).

Offspring-desertion by females is less widespread among birds and is estimated to occur in less than 1% of all species (Lack 1968; Ligon 1999, Owens 2002). Such desertion is essentially associated with polyandrous mating systems and has been found in 11 different families such as in the jacanas, (family Jacanidae: Emlen et al. 1998), rheas, family Rheidae, emus, family Casuariidae, and tinamous, family Tinamidae (Bennett & Owens 2002).

Finally, promiscuous mating systems are characterized by indiscriminate sexual relationships, usually of a brief duration. The male's investment in offspring is usually limited to sperm, and the female raises the young alone. Promiscuous mating occurs in less than 6% of species (Bennett & Owens 2002) including the reed bunting, *Emberiza schoeniculus* (Dixon et al. 1994), the superb-fairy wren, *Malurus cyaneus* (Double & Cockburn 2000), and ostriches, *Struthio camelus* (Bertram 1992; Kimwele & Graves 2003).

1.2. Cooperative mating systems

Cooperative breeding occurs when more than two individuals provide care to a single brood of offspring (Brown 1987) and has been reported in about 3% of bird species (Arnold & Owens 1998). Its frequency of occurrence varies widely between families, from being entirely absent in megapodes, tinamous and hummingbirds, to being the predominant breeding system in at least 12 families, such as in fairy-wrens and ostriches (family Maluridae and Struthionidae respectively: Bennett & Owens 2002). Various forms of cooperative breeding exist: at one extreme only a single pair of individuals mate and reproduce at any one time, e.g. acorn woodpeckers, *Melanerpes formicivorus* (Koenig & Stacey 1990); at the other more than two members of a group copulate and contribute to the clutch (e.g. Smith's longspurs, *Calcarius pictus* ; Briskie 1992).

In contrast to these systems, the communal nesting system of ostriches is unique in that even though up to 18 females lay in the same nest, only the major male and major female provide parental care, including incubation (Bertram 1992; Sauer & Sauer 1966). After hatching, chicks are supervised as a group, or "crèche" and the dominant pair even occasionally competes to gather the young of others to their group (Bertram 1992). This crèche system is also observed in other bird species such as in the South American guira cuckoo (*Guira guira*), although several individuals cooperates to the supervision of the crèche (Cariello et al. 2006).

1.3. Genetic mating systems

Before the advent of molecular techniques, socially monogamous species of birds were believed to be truly monogamous, with individuals often mating for life (Bennett & Owens 2002). Once DNA fingerprinting techniques were applied to birds, most of supposed monogamous species were actually found to participate in extra-pair copulations (EPCs). For instance, over 85% of passerine bird species presumed to be monogamous

were found to be sexually polygamous (Owens & Hartley 1998) and EPCs have now been recorded in more than 150 bird species (Birkhead & Møller 1992, 1995; Westneat et al. 1990;Griffith et al. 2002). The common pattern is that both sexes solicit EPCs with neighbouring individuals (Griffith et al. 2002; Owens & Bennett 2002). One of the most famous examples of extra-pair paternity is the superb fairy-wren, a cooperatively breeding species, where 72% of offspring may be sired by males other than the social father, and with 95% of broods containing extra-pair offspring (Mulder et al. 1994; Double & Cockburn 2000). However, even though the benefits of such behaviour are apparent to males (i.e. a direct increase in fitness), the adaptive value of multiple mating in females remains unclear. Several possible benefits have been proposed, such as direct benefits (Heywood 1989; Buchanan & Catchpole 2000; Hill 1991), fertility insurance (Sheldon 1994;Westneat et al. 1990; Birkhead & Møller 1992; Edler & Friedl 2008), genetic diversity or compatibility (Westneat et al. 1990;Tregenza & Wedell 2000, 2002; Foerster et al. 2003) or the "good genes" hypothesis (Westneat et al. 1990; von Schantz et al. 1999; Birkhead & Møller 1992 ; Richardson et al. 2005). However, as only 25% of socially monogamous species studied to date are truly genetically monogamous (Griffith et al. 2002), EPCs might also have costs. For instance, males could suffer increased parasitism (Sheldon 1993), a decrease in territory defence or parental care (Westneat 1988), whereas females could possibly risk male retaliation (Weatherhead et al. 1994; Dixon et al. 1994; Møller & Cuervo 2000), or to be injured through resistance to sexual harassment (Frederick 1987).

2. Female mate choice and the evolution of male signals

2.1. The choosy sex

Mate choice can be exercised by both sexes, but it is usually the female's domain (Andersson 1994). Females must select their mates with care since they typically invest

more time and energy in each offspring than do males, which compete for mating opportunities. Trivers (1972) advocated that this higher parental investment was the primary mechanism driving strong sexual selection on male traits and for female choice. While females may invest in prolonged care in young, males are free to move on and mate with other females or compete with other males for access to females (Andersson 1994). Therefore, selection should act on females to choose high quality mates; and on males to display their status (Guilford & Dawkins 1993; Candolin 2003).

2.2. Male signals and female choice

Animal signals are typically used by senders to increase their fitness by modifying the receiver's behaviour (Endler 2000). This is specifically important in the context of mate choice as females might use different / several cues to assess the quality of a potential mate (Møller & Pomiankowski 1993; Candolin 2003). In accordance with this, several male attributes or displays have been shown to affect the probability of a male being selected as a mate. For instance, in the pheasant (*Phasianus colchicus*) females show a preference both for spur length (which reflects condition and viability: Goransson et al. 1990), and for male display activity, which is correlated with parasite load (Johnstone 1995). Birdsong has also been widely attributed to indicate quality, where individual males exhibiting extraordinary complex songs or having an extremely large repertoire have been found to be preferred as mates (e.g. Mountjoy and Lemon 1996; Buchanan & Catchpole 1997). Furthermore, Spencer et al. (2005) found that the extent of parasitism in males had a negative effect on the male's repertoire size. This suggests that the extent of a male's repertoire could be an honest signal of his quality, and potentially be used as a cue in female choice. Recent theoretical and empirical work on the evolution of extravagant secondary sexual characters has shown that males with exaggerated ornamentation accrue mating advantages arising from female choice. This thesis investigates this phenomenon in

the ostrich, with a specific emphasis on male coloration; and fitness consequences for offspring.

2.3. Bird coloration and the evolution of male ornamentation

2.3.1. An avian perception of the world

Bird colour vision differs from that of humans in several ways, which are very likely to result in differences in their colour perception. First, birds are sensitive to the ultra-violet (UV) part of the light spectrum (wavelengths between 300nm and 400nm), to which humans are blind (Bennett & Cuthill 1994). As they can also see the entire human-visible spectrum (400-700nm), they have a wider spectral range than humans. Second, they have four cone types, as opposed to the three found in humans (Bowmaker et al. 1997), implying that birds have the potential for tetrachromatic vision (Chen & Goldsmith 1986; Jane & Bowmaker 1988; Bowmaker et al. 1997). Birds have 6 cone classes: 4 single cones and 2 "double cones" which are also found in fish and turtles, but lacking in humans (Bowmaker 1980). The function of double-cone is still unknown but could be part of hue discrimination because of their broad spectral sensitivity. The avian single cones span the avian-visible spectrum fairly evenly, but there is some variation between species in the maximum sensitivity of their visual pigments, and essentially in the pigment conferring UV sensitivity. For instance, passerines have a 'true UV' visual pigment (around 335nm-370nm) whereas other species can see in the UV only through visual pigments that absorb light between 400nm-420nm (e.g. mallard, *Anas platyrhynchos*, peacock, *Pavo cristatus*, ostrich: for a review see Bennett & Owens 2002). Finally, avian cone-cells contain light-absorbing oil droplets. In diurnal birds and other groups with densely pigmented oil droplets, their function appears to be to filter the light entering the cones (Bowmaker 1980), and thus enhance discrimination of certain classes of spectra and improve colour constancy.

In summary, tetrachromacy and the possession of oil droplets both improve the discrimination of these colours as compared to the trichromatic system (Vorobyev et al. 1998), implying that humans and birds see the colours of objects differently (Håstad & Odeen 2008).

2.3.2. The complexity of bird coloration

Coloration in birds is derived from two types of pigmentation, melanins and carotenoids, as well as a range of structural mechanisms. These pigments produce coloration by absorbing particular wavelengths of light. Melanins are responsible for most black, brown, and brick-red coloration. Melanin-based coloration is thought to be cheap to produce as it can be synthesized by the organism (Maynard Smith & Harper 1988). However, Owens & Wilson (1999) pointed out the major component of melanins is the amino-acid tyrosine, which is also an important precursor in immunological processes. Furthermore, Galvan & Alonso-Alvarez (2008) have suggested that melanin-based signals could indicate individual capacity to manage oxidative stress, a major contributor to ageing and to several degenerative diseases, such as immune disorders (Vlek et al. 2007). On the other hand, carotenoids are responsible for most bright yellow, orange and red coloration. Unlike melanins, carotenoids can not be produced by the organism and are exclusively acquired through the diet (Brush 1990). They also play an important role in many immunological and metabolic pathways (Hill 1999) as their expression has been demonstrated to be affected by parasitism (Lozano 1994).

Structural colours, by contrast to pigments, are derived from diffracting and scattering light and include most white and iridescent colours, including blue, purple and green (Andersson 1999). They have received more and more attention recently as they appear to be important source of UV reflection in male birds. Furthermore, at least one species of parrot has been found to have fluorescent plumage, which may also be used as a

cue for mate choice (budgerigar *Melopsittacus undulatus*: Arnold et al. 2001; Bennett & Owens 2002). Fluorescent plumage has the characteristic of absorbing short wavelengths of light (UV included) and retransmitting them at higher wavelengths, usually in the yellow, orange and red parts of the spectrum, and could therefore potentially be used to highlight important information, such as individual's quality.

2.3.3. Sexual dimorphism and extravagant male ornamentation

Sex differences in coloration are a particularly prominent aspect of sexual dimorphism in birds, in which the extent of sexual dimorphism is very variable. For instance, in the European swift, *Apus apus* the sexes are indistinguishable by eye, whereas male and female mallard (*Anas platyrynchus*) were initially classified as separate species (Andersson 1994). However, most species fall somewhere in between these extremes, with the majority showing some differences in plumage coloration (Bennett & Owens 2002).

Darwin (1871) was the first to claim that sexual selection was likely to play a major role in the evolution of male secondary sexual characters. These characters may incur costs, such as a lower survival. In the case of male-male competition, such costs could arise from the production and maintenance of traits which indicates the male's physiological state to his rivals and thus acts as an intimidation tactic (Møller 1987, Liker & Barta 2001). Specifically, the production of androgens required to produce secondary sexual traits has been suggested to be an important factor affecting survival, as androgens act via a complex pathway which suppresses the immune system (Folstad & Karter 1992; Buchanan et al. 2003; Owen-Ashley et al. 2004).

Furthermore, numerous studies have confirmed that males with elaborate ornamentation, or possessing certain attributes, have mating advantages arising from female choice (Andersson 1994; Møller 1994; Johnstone 1995). In particular, experimental manipulations of male attractiveness (length of the tail: Evans & Hatchwell 1992; Møller

1994; UV coloration: Bennett et al. 1997) have demonstrated the influence of specific phenotypic traits on the male's probability of being chosen as a mate.

3. Female tactics within the mate choice context

3.1. Differential maternal investment

Life-history theory predicts that females should modify their investment in a particular breeding attempt according to the likelihood of its success (Williams 1966). Females may benefit from selecting an attractive mate by increasing the viability and quality of her offspring, and if this is the case, they should invest more in reproduction when mated to attractive males than when mated with less attractive males. Evidence for this differential allocation hypothesis (Sheldon 2000; Burley 1986) has been found in studies on mallards and zebra finches (*Taeniopygia guttata*), where a differential investment in offspring in the pre-laying period was observed. In both species, females mated to attractive males lay larger eggs than females mated to less attractive males (Burley 1988; Cunningham & Russell 2000; Rutstein et al. 2004).

Furthermore, females in a number of species have been found to invest more in egg resources such as yolk immunoglobulin (Saino et al. 2002) or testosterone (Gil et al. 1999). Recent studies have emphasized the fitness consequences of laying eggs of different sizes, as nutrients and energy allocated to eggs can have a profound influence on the development of embryos, as well as the growth and survival of hatchlings. For instance chicks hatched from eggs with higher amounts of testosterone beg for food more intensely and therefore grow faster than other chicks (Schwabl 1993; Lipar et al. 1999). Furthermore, the ability of mothers to transmit antibodies to their offspring has been documented in birds (for a review see Grindstaff et al. 2003). Although maternal effects generally have their greatest impact early in development and then decrease as offspring mature (Price 1998; Wolf & Brodie 1998), maternal antibodies may continue to affect

offspring phenotype by influencing growth and developmental rates, as well as the strength and diversity of the immune response (Boulinier & Staszewski 2008;Hasselquist & Nilsson in press).

3.2. Shopping for "Good genes"

Numerous explanations have been provided for how females gain by being selective in their choice of mate; and why females prefer to mate with the most elaborately ornamented males. Females can gain direct benefits, such as paternal care in the form of territorial defence and resources (Heywood 1989; Buchanan & Catchpole 2000; Hill 1991), or indirect benefits in the form of heritable traits, which enhance offspring survival and/or reproductive success (Andersson 1994; Borgia et al. 2004).

Fisher's runaway process predicts that genes for female preference become strongly associated with genes for the male trait through linkage disequilibrium, leading to a runaway process that favours even more elaborate traits despite their effects on male survival (Fisher 1930). These traits affect only mating success of offspring, but are not adaptive in terms of their survival. On the other hand, the good genes models suggest that females use traits as signals to discriminate between the health and condition of males. The degree to which these traits are developed reflect a male's underlying genetic quality, which will be inherited by his offspring and in turn enhance their survival (Zahavi 1975, 1977; Hamilton & Zuk 1982; Folstad & Karter 1992). The condition-dependence of male ornaments has been indicated by various studies showing that the expression of traits, such as tail ornamentation or colouration, correlates with condition and survival (Andersson 1994). In addition, good genes models are specifically supported by studies showing that females increase offspring fitness by mating with more ornamented males, without obtaining any direct benefits (Møller 1994; Hasselquist et al. 1996). These models are therefore of a particular interest in the context of this study.

3.2.1. The Handicap principle

In the early 1970's, Amotz Zahavi tried to understand the question that has puzzled many evolutionary biologists since Darwin: why do animals develop such costly and conspicuous ornamentation? To answer this question, he proposed that ornaments were signals used by other individuals to estimate the overall quality of the bearer's condition and/or genetic quality. The handicap principle of sexual selection (Zahavi 1975) predicts that sexual selection promotes the evolution of honest sexual signals and that these signals express condition dependency, thereby reflecting male genetic quality. Males of high genetic quality should express greater sexual ornamentation size or display, whereas males of poorer quality are unable to bear the associated costs. The peacock's tail is perhaps the best-known example of a costly signal or Zahavian "handicap" (Petrie & Halliday 1994). However, even though the development and maintenance of secondary sexual characters may be a considerable handicap (Zahavi 1977), in terms of reducing a male's survival, such sexual characters may also act as a signal of quality by advertising strong genetic resistance to parasites (Hamilton & Zuk 1982). Although Zahavi's model was initially disputed (Maynard-Smith 1976; 1978; Kirkpatrick 1986), this principle gained wider acceptance due to supporting game theory models (Andersson 1982; Grafen 1990; Maynard-Smith 1991; Pomianskowski 1987).

3.2.2. Hamilton-Zuk Parasite Hypothesis

Individuals are affected to various degrees by the negative impact of parasites, because of inter-individual variation in genetic and non-genetic factors affecting general phenotypic condition (Nordling et al. 1998; Gonzalez et al. 1999). Therefore, one of the most plausible explanations for the evolution of sexual dimorphism and extravagant ornamentation in birds is that sexual ornaments signal an individual's ability to cope with parasites (Hamilton & Zuk 1982). Hamilton & Zuk measured the degree of plumage

ornamentation (specifically colouration) and the extent of parasite load in several species of North American passerines and demonstrated that these two measures were correlated among species. They used this correlation to suggest that the variation between species in the degree of sexual ornamentation was due to the variation between species in host-parasite interactions. Intra-specifically, assuming that the expression of these traits is condition-dependent, only males in prime condition will be able to develop the most exaggerated ornamentation (Andersson 1994), and consequently, a female that chooses heavily-ornamented males should be endowed with good genes. Despite substantial interest in this hypothesis, no consensus has been reached yet on its validity (Hamilton & Poulin 1996; Møller 1990; Møller et al. 1999; Getty 2002). First, Hamilton and Zuk's interspecific test of the hypothesis relies on a comparative analysis which might have been confounded by artefacts of phylogenetic relationships and ecological variables (Andersson 1994). Second, as previously discussed, birds do not see in the same way as humans, so their description of bird coloration is probably inadequate. Furthermore, tests of this hypothesis have led to contrasting results. Intra-specific support for this parasite-mediated mechanism of sexual selection initially came from a study on swallows *Hirundo rustica* (Møller 1990). Cross-fostering experiments and manipulation of the level of parasitism in a natural population of swallows suggested that host fitness was negatively affected by parasites, as both chick growth and adult tail size were inversely related to parasite burden. A cross-fostering experiment indicated that the level of parasitism was heritable; and as the development of male tail length, a sexually selected trait, reflected parasite loads, females choosing males with longer tails should have offspring with commensurately higher resistance to parasites. However, other intra-specific studies, such as those on the sage grouse, *Centrocercus urophasianus* (Vehrencamp et al. 1989) and the red bishop, *Euplectes orix* (Edler et al. 2004), did not find any relationship between male parasite

level, display performance and mating success, suggesting that male advertising signals may be more complex.

3.2.3. Immunocompetence handicap hypothesis

The vertebrate immune system has evolved as a defence mechanism against parasites and pathogens, and hence plays a crucial role in host survival and fitness (Goldsby et al. 2000). The immunocompetence handicap hypothesis (ICHH) proposed by Folstad & Karter (1992) incorporates both the handicap principle (Zahavi 1975) and Hamilton and Zuk's (1982) model. Immunocompetence can be defined as the ability of a host to prevent or control infection by pathogens and parasites (Norris & Evans 2000). This hypothesis states that testosterone is responsible for the production of male secondary sexual traits and is also immunosuppressive (Folsdad & Karter 1992). Therefore, the cost of being able to express sexual traits is a reduction in the immune response and consequently, only high quality individuals will be able to produce extravagant secondary traits, specifically because of the trade-offs between androgens and immune capacity. Another prediction of the ICHH is that a male should have his own optimum level of testosterone, which allows maximal trait expression, while minimizing immunosuppression. Consequently, females basing their mate choice decisions on secondary sexual traits could acquire males with better resistance to parasites, resulting in either direct benefits in species with paternal care; and/or indirect genetic benefits when offspring inherit genes for superior immunocompetence (Folstad & Karter 1992; Andersson 1994; Westneat & Birkhead 1998).

However, contrasting results have been found across a wide range of experiments which have manipulated male levels of testosterone (for a review see Roberts et al 2004). For instance, whereas on one hand an increase of testosterone increased wattle size (a sexually selected trait) and increased male aggressiveness in pheasants, *Phasianus*

colchicus (Briganti et al. 1999); on the other, male house finches, *Carpodacus mexicanus*, responded by developing duller plumage (Stoehr & Hill 2001) and there was little correlation between testosterone levels and secondary sexual traits in male red-winged blackbirds, *Agelaius phoeniceus* (Weatherhead et al. 1993). Furthermore, Roberts et al. (2004) also found little support for a relationship between elevated levels of testosterone and immunosuppression; and pointed out that testosterone might only have an effect only on behaviour, and not on immunological variables.

Several alternative explanations have been proposed for the trade-off between immune function and secondary sexual traits in male vertebrates. First, the role of glucocorticoids (such as corticosterone) in mediating the ICHH has recently received much attention, as elevated levels of glucocorticoids have been found to be immunosuppressive in some species (Råberg et al. 1998, Buchanan 2000). Second, dietary quality - and specifically the levels of amino acids - might also profoundly influence the immune response (Klasing 1996), as the functioning of T-cells is dependent on the intracellular concentrations of glutathione, which in turn may be affected by sulphur amino acid shortage (Grimble & Grimble 1998) . In accordance with this, supplementary feeding of methionine, in blue tit and magpie nestlings led to an increase in cell-mediated responses (Soler et al. 2003; Brommer 2004). However, Alonso-Alvarez & Tella (2001) highlighted that the relationship between changes in dietary proteins in food intake and cell-mediated responses may not be linear. They found that captive gulls appear to reach a threshold above which the increase in food intake did not enhance the cell-mediated response. Recently, much emphasis has been placed on the role of carotenoids in mediating both the immune response and the expression of sexually selected traits (Blount et al. 2003; Faivre et al. 2003). Finally, a complex relationship between testosterone production and the Major Histocompatibility Complex (MHC) may allow some individuals to bear the costs associated with elevated levels of testosterone, such as in the white-tailed deer (Ditchkoff

et al. 2001). The extremely polymorphic genes of the MHC play an important role in triggering the vertebrate immune response (Roitt 1997) and have been linked to mate choice across in several species, including birds (Zelano & Edwards 2002). Bonneaud et al. (2005), for example, showed that a specific MHC allele in the house sparrow was associated with higher responses to two different T-cell dependent antigens.

4. The ostrich mating system

4.1. The ostrich

The ostrich is the largest living bird and is a member of a group of flightless birds, the ratites. The species name *Struthio camelus* derives from the Greek and Latin name *Struthocamelus* (Bertram 1992), and is the only living species in the family Struthionidae. There are four sub-species of ostrich in Africa (*camelus, molybdophanes, massaicu, australis*), which have all been kept in captivity to produce meat, leather and feathers (Bertram 1992). In the wild, they prefer open habitat (short-grass plains and semi-desert), although ostriches are also found in the hot desert steppes of the western Sahara and the deserts of Namibia (Deeming 1999). Ostriches are diurnal, and spend much of the day in motion, except when dust bathing, resting or nesting (Bertram 1992).

They are seasonal breeders and primarily breed in late winter, spring and summer months (Jarvis et al. 1985). Out of the breeding season, the ostrich is a gregarious species, and they tend to form groups of mixed gender and age, particularly around water holes (Deeming 1999). They are sexually dimorphic: males have black plumage and a coloured neck and bill, whereas females have a dull-brown plumage (Bertram 1992; Deeming 1999). Interestingly, the bare shins and the beak of ostrich males change in colour from light pink to crimson red during the breeding season and as males become territorial (Bertram 1992; Lambrechts 2004).

Juvenile birds resemble the females, and can only be sexed (on the basis of plumage characteristics) from the age of two years, whereas young chicks are mottled brown, yellow, orange and cream with black quills on the back (Deeming 1999). Families of chicks are combined into crèches and are overseen by a single pair of adult birds. Little is known about the behaviour of ostrich chicks in the wild, as predation on nests is relatively high; and as it is difficult to track them in grassland (Bertram 1992).

4.2. Social and courtship behaviour

Both males and females use a repertoire of visual displays in many of which the wings play a major part, in addition to their utilitarian functions of controlling the bird's temperature; protecting eggs and young; and chasing away flies (Bertram 1992). In particular, the contrast of the white feathers against the black body feathers renders wing displays particularly conspicuous in males. Wings are typically involved in aggressive encounters with predators or opponents, in which ostriches raise both wings high above the body; or flick them alternately up and down. Most importantly, wings are involved in the courtship display (or 'kantling' behaviour), whereby the male typically sits on its legs, while his wings are held forward, directly exposed to the females, and his neck swings from side to side (Bertram 1992). Bertram (1992) also observed that males sometimes make a "booming" sound (that he describes as a 'mwoo-mwoo-mwoooo') while approaching a female. Before copulation, the female flutters her wings and holds them forward, while her head is held down accompanied with a clapping of the beak (Deeming 1999). She then drops to the ground with her tail raised and neck forward. The male responds by getting to his feet, approaching the female with his wings held forward and by stamping his feet several times on the ground before mounting the female. The kantling display is also used during antagonistic interactions between males, and is usually performed by a male who is driving a competitor away. Apart from their obvious use in

locomotion, ostrich males use their legs for striking opponents and small predators, as well as for making scrapes in the ground when establishing territories (Bertram 1992).

4.3. Mating system

Although an iconic bird of open savannas, little is known about the mating system of ostriches. They are promiscuous and in the wild, males and females have multiple partners (Bertram 1992; Kimwele & Graves 2003). During the breeding season, males hold and defend territories. Females usually have a larger home range relative to males and therefore move through several male territories regularly, and mate occasionally with the territorial male. Bertram (1992) reported that mating often occurs just before or after scrape-showing, whereby a resident male might show several different scrapes to the same female within a couple of hours; and/or to a number of different females over a period of days. However, only a few scrapes were usually used as proper nests. Typically, the first female to lay in a nest is the one who will undertake guarding and incubation of the nest, and is referred as the major female (Sauer & Sauer 1966); and the territorial male as the major male.

The ostrich communal nesting system is unique in that the major female allows minor females to lay in her nest even though they provide no parental care. Up to 18 females may lay in a nest, but only the major female and major male incubate the eggs and guard the offspring until independence (Bertram 1992; Kimwele & Graves 2003). One to six minor females usually lay between 20 and 40 additional eggs, with some clutches containing up to 67 eggs (Sauer & Sauer 1966; Bertram 1992). As more eggs are laid in the nest than can be incubated (a maximum of 20 eggs), the major female usually ejects surplus eggs from the incubated central clutch. Typically, she arranges the eggs into a central, incubated clutch and a ring of peripheral, unincubated eggs that will never develop. Kimwele & Graves (2003) demonstrated that she usually contributes a disproportionate number of fertile eggs to the central incubated clutch. Furthermore, she seems to be able to recognize

her own eggs, as Bertram (1979) found that in five nests in which major females had laid, in only one had one of her own eggs been ejected from the central clutch. In addition, eggs that did not resemble hers were more likely to be in the peripheral, unincubated clutch. Furthermore, Kimwele & Graves (2003) showed that the major male usually fertilized most of the incubated eggs of his major female, while other males fertilized only a small proportion. They also found that the major male fertilized some of the eggs of the minor females incubated in his nest, and that all males also fertilized eggs of the neighbouring clutches.

There may be costs associated with incubating more eggs than a major female has laid, as predators might be attracted by her ejecting eggs into the peripheral clutch, as they are more visible than eggs covered by the incubating bird (Bertram 1992). Several hypotheses have been erected in an attempt to understand why the major female tolerates other female eggs in her nest. To date, there is still no clear explanation for this behaviour, as minor females were not found to be closely related to the major female (Kimwele & Graves 2003). However, her fitness might be enhanced by increasing the chance of eggs escaping predation, by a dilution effect. In addition, Kimwele & Graves (2003) revealed that all major females were simultaneously minor females on the territories of neighbouring males. This may be adaptive in terms of improving their reproductive success, as nest predation is relatively high among ostriches (Bertram 1992).

4.4. Ostrich farming

Ostrich farming originally developed in South Africa in response to the increasing demand for ostrich feathers by the fashion industry during the middle of the 19th century (Deeming 1999). Despite various setbacks in the market due to frequent threats of avian influenza, the ostrich has remained a valuable animal for farmers as the modern industry not only relies on feathers, but also on leather (processed from ostrich skins) and on the

increasing popularity of ostrich meat, which is relatively low in cholesterol (Deeming 1999). In 2007, the foreign income from ostrich products in South Africa was estimated at about US$140 million per annum (South African Ostrich Business Chamber 2008).

In farmed environments, most ostrich breeders use two main methods for mating adults: they are either kept in breeding groups of birds; or occasionally, single pairs of individuals are used for mating (Deeming 1999). Because of the intensification of ostrich farming and the increase in emphasis on the welfare of animals under intensive farming conditions, commercial ostrich farmers had to integrate the behavioural requirements of ostriches into their management programmes to ensure an optimal breeding environment (Stewart 1994; Mohammed et al. 2003). Behaviour exhibited in farming conditions resembles that observed in the wild, with males displaying territorial aggression towards other males and performing the kantling display towards females (Deeming 1999). Furthermore, ostrich farmers often use the change of shin colour to crimson red, as a cue of the readiness of a male to commence breeding (Lambrechts 2004). Accordingly, farmed conditions are not that different from those in the wild situation, although eggs are usually incubated artificially and chicks reared by farmers. Only occasionally are chicks reared by foster parents (Verwoerd et al. 1999).

Ostriches are fast-growing birds and a remarkable feature of cohorts of chicks is that they differ greatly in size (Deeming & Ayres 1994; Deeming et al. 1993). After hatching, chicks are usually transferred to a chick rearing facility where they are kept in groups of 100 to 110 chicks. Most growers separate cohorts at weekly intervals into size-determined groups, in order to minimize competitive interactions between individuals of differing sizes so that smaller ones do not starve. Factors influencing growth rates in ostrich chicks are believed to include the protein content in the diet (Deeming 1996); social grouping (Deeming & Ayres 1994; Mushi et al. 1998); and disease (Deeming & Ayres 1994). However, size differences within cohorts are most likely to have a genetic basis

arising from differing parental genotypic differences. Furthermore, ostrich farmers face many difficulties in raising healthy chicks, which display highly variable mortality, reaching up to 50% at three months of age (Verwoerd et al 1999). The causes of such a high mortality in ostrich chicks are still poorly understood, but could be related to current husbandry and management practices which might not fully take into consideration variation in chick (Bubier et al. 1996; Cloete et al. 2001) and/or adult (Lambrechts 2004) behaviour.

5. Aims of the study

To date, there has been no evidence to suggest that female ostriches discriminate between males as potential mates, but the degree of dimorphism in the species and the variance in success between wild males suggest that mate choice is highly likely to occur. If some males are more attractive to females than others, then we would expect a differential investment by females in the offspring of attractive males. Furthermore, there is increasing evidence from studies of other bird species, that investment in immune defence is central to many internal trades-offs with life-history traits such as growth rates (Norris & Evans 2000) or survival, which together with differential maternal investment could potentially explain observed differences in offspring quality.

In chapter two, I investigate whether females kept in breeding flocks invest differently in egg mass, according to the degree of attractiveness of their mates. I specifically focus on male colouration, using UV-visible range spectrophotometry, as birds are sensitive to the UV part of the spectrum (Bennett & Cuthill 1994) and have the potential for tetrachromatic vision (Chen & Goldsmith 1986; Bowmaker et al. 1997; Wright & Bowmaker 2001). In chapter three, I examine whether male colouration reflects a male's ability to raise an immune response, by stimulating the two main components of the immune system, the cell-mediated and humoral systems. In addition, I investigate the

use of the heterophil/lymphocyte ratio to provide an estimate of ostrich immune status. As a trade-off between immune response and life-history traits, in particular growth rate, has been documented in various bird species, chapter four focusses on the potential relationship between variation in offspring growth rates and variation in levels of immune defence. Finally, chapter five examines whether variation in levels of immunocompetence in both parents, as well as male colouration, are related to chick growth rates.

CHAPTER TWO Investment in eggs is influenced by male coloration in the ostrich, *Struthio camelus*

(M. Bonato, M.R. Evans & M.I. Cherry)

(Animal Behaviour, in press; except for Figure 1, omitted for space reason)

ABSTRACT

Life-history theory predicts that females should modify their investment in a particular breeding attempt according to the likelihood of its success, as the investment of females in reproduction is typically higher than that of males. The ostrich mating system is promiscuous, and is thus a particularly interesting one in which to investigate differential investment by the sexes. To date, there has been no evidence that female ostriches discriminate between males as potential mates, but the degree of dimorphism in this promiscuous species and the variation in chick size within clutches suggest that differential maternal investment is likely. We investigated the relationship between egg mass and coloration of the feathers, bill, neck and legs of 15 male ostriches, maintained in a breeding flock at an ostrich farm in South Africa. Paternity was determined using microsatellite markers. We found that the colour of the neck, white and black body feathers, and the brightness of black feathers predicted egg mass. These traits are exposed during the male courtship display, so we suggest that these visual cues influence the degree of maternal investment in eggs through their influence on female perception of mate quality.

1. Introduction

Females of many species prefer to mate with males showing the most elaborate ornamentation, as by selecting an attractive mate, females might increase the viability and quality of their offspring. If this is the case, females should thus invest more in reproduction when mated to an attractive male than when mated to a less attractive male. Work on mallards, *Anas platyrhynchos*, and zebra finches, *Taeniopygia guttata*, has shown that differential investment in offspring in the pre-laying period is possible. In both species, females mated to attractive males lay larger eggs than females mated to less attractive males (Burley 1988; Cunningham & Russell 2000; Rutstein et al., 2004). In zebra finches, eggs laid by females mated to attractive males contain more testosterone than eggs laid by females paired to less attractive ones (Gil et al., 1999).

Egg size is a good indicator of maternal investment, being both energetically and nutritionally costly to females (Heaney & Monaghan 1995), and is not confounded with paternal effort. Furthermore, the potential benefit of larger eggs is that they are more likely to produce larger offspring with higher chances of survival and faster growth, especially during the first few days after hatching (Reid & Boersma 1990; Bize et al., 2002; Silva et al., 2007). This is the case for ostriches (Cloete et al., 2004).

Avian colour vision differs from that of humans in several ways. First, birds are sensitive to the ultraviolet (UV) part of the spectrum (320-400 nm) to which humans are blind (Bennett & Cuthill 1994). Second, most birds have four cone types, rather than three as found in humans, implying that birds have the potential for tetrachromatic vision (Chen & Goldsmith 1986; Jane & Bowmaker 1988; Bowmaker et al., 1997). This is also the case for ostriches (Wright & Bowmaker 2001). Finally, in contrast to humans avian cone-cells contain light-absorbing oil droplets which act as cut-off filters and reduce the overlap between cone spectral-sensitivities (Bowmaker 1980). Consequently, the assumption that birds see in the same way as humans is almost certainly invalid. Recent studies have

emphasized the need to measure colour over the bird-visible spectrum as they show that a variety of species uses UV wavelengths in decision making. In particular, females frequently use sexually dimorphic characteristics to discriminate between males during mate choice (Andersson 1994). Hunt et al (1998) found that blue tits, *Cyanistes caeruleus*, which had been classified as sexually monochromatic, were dichromatic in the UV, and that females prefer males with the brightest crest.

Although an iconic bird of open savannas, little is known about the mating system of ostriches. They are the largest living birds and are members of a group of flightless birds, the ratites. Ostriches are sexually dimorphic: males have black plumage and coloured necks and legs, whereas females have a dull-brown plumage (Deeming 1999). They are promiscuous, and in the wild males and females have multiple partners (Bertram 1992; Kimwele & Graves 2003). The ostrich communal nesting system is unique in that even though up to 18 females lay in the same nest, only the major male and major female provide parental care, including incubation (Bertram 1992; Sauer & Sauer 1966). Furthermore, Kimwele & Graves (2003) discovered that major females were simultaneously minor females to neighbouring males, suggesting that this is a strategy to improve their reproductive success. Eggs are laid in the late afternoon or late morning, and the clutch build up over a period of up to 30 days (Bertram 1992). Hatching takes place over 2-3 days and families of chicks are combined into creches, overseen by the major male and female. The ostrich mating system is thus a particularly interesting one in which to investigate differential investment by the sexes. In farmed environments, most ostrich breeders use two main methods for mating adults: they are either kept in breeding groups of six females and four males; or maintained in camps containing very large groups of birds with the same sex ratio. Only occasionally are single-pairs of individuals used for mating. Therefore farmed conditions are not that different from the wild situation, although eggs are incubated artificially and chicks reared by farmers.

To date, there has been no reported evidence that female ostriches discriminate between males as potential mates, but the degree of dimorphism in this promiscuous species and the variation in chick size within clutches suggest that differential maternal investment is highly likely. Thus, our aim in this study was to investigate whether and to what extent females kept in breeding flocks invest differently in egg mass, according to the degree of attractiveness of their mates. For this purpose, using UV-visible range spectrophotometry, we measured the colour of the beak, neck, feathers and legs of 15 males maintained within the breeding flock. In particular, the neck and body feathers are exposed during the male courtship display, and we suggest that these visual cues could influence the degree of maternal investment in eggs if they are used by females to assess male quality.

2. Materials and Methods

2.1. Population

We studied South African black ostriches, *S. camelus var. domesticus*, maintained at an experimental farm in Oudtshoorn, South Africa, from August 2005 to March 2006. The breeding flock consisted of two groups in 8 ha camps containing 7 males and 12 females; and 8 males and 11 females respectively. Eggs were collected on a daily basis, identified according to their camp, weighed and stored in electronic incubators until hatching at a temperature of 36°C. We recorded only egg mass using an electronic balance (Mercer), and not egg volume, both for practical reasons and because egg mass is usually highly correlated with egg volume in birds (Christian 2002). Females laid eggs in four nests in camp 1; and three nests in camp 2. As eggs were removed daily, and parentage could not be determined for unhatched eggs, clutch size *per se* could not be recorded . Only eggs that hatched were considered for subsequent analysis (59% of eggs, resulting in a total of 398 chicks in total: 201 males and 197 females). Each chick was sexed, marked, and weighed

on hatching, and again a month later. All chicks were allowed to dry off for 8-10 h after hatching, and subsequently transferred to an extensive chick rearing facility where they were kept in groups of 100-110 chicks. Only 99 chicks (44 males and 54 females) were weighed at one month of age. As we could not assess parentage with certainty in our camp, 50ul of blood was collected from the jugular vein of both the adult birds and the day-old chicks and stored in Vacutainer tubes kept at 4°C until parentage analysis could be conducted. Ethical clearance for this work was granted by the Stellenbosch University ethics committee.

2.2. Parentage determination

DNA was extracted from blood samples using a standard protocol with overnight digestion with proteinase K and phenol/chloroform extraction. The amount of DNA was estimated and dilutions were made to approximately 1ng / µL.

Genotyping was determined for six primers selected from literature: CAU1, CAU7, CAU14, CAU17, CAU64, CAU76 (Tang et al. 2003). All polymerase chain reactions (PCR) were realized in a total reaction volume of 10 µL containing 1 µL of the DNA solution, 1µM of each primer , 0.25 units of YB-*Taq* Polymerase in the manufacturer's buffer, 0.2nm of each dNTP and 1.5-3mM $MgCl_2$ (YorBio, York, U.K.). The reaction profile was 95°C for 5 min, then 95°C for 30s, then T_a for 30s, then 72°C for 30s for 35 cycles, and finally 72°C for 10 min, where T_a is the annealing temperature, adapted to obtain optimal reactions. All PCR were performed in a Px2 Thermal Cycler (Thermo Electron Corporation, Waltham, MA, USA).The PCR products were diluted with 50µl of ddH20 and multiplexed into two sets: set 1 CAU1, CAU76 and CAU14; set 2 CAU7, CAU17 and CAU64. For each set, a mixture of 1ul of the products and 9µl of loading buffer containing Genescan 500 LIZ size standard and formamide (volume of 0.15 and 8.85 respectively) (Applied Biosystem, Foster City, CA, USA) was made and denatured

for 5min at 95°C. Plates were then run in 3130xl Genetic Analyser (Applied Biosystems). If the reaction failed, PCR were re-run with modification of $MgCl_2$ concentration or annealing temperature.

Genemapper 4.0 (Applied Biosystems) was used for allele scoring and the parentage assignment was conducted via CERVUS 3.0 (Kalinowski et al. 2007).This programme performs an allele frequency analysis by using exclusion and likelihood-based approach. Because neither parent was known, CERVUS recommends a two-step analysis with the first step running the group of parents with fewer candidates (fathers in this case), and the second step to running the analysis with the mothers using the results of the first step. Both camps were analysed separately for the parentage assignment, as some adults in camp 1 and in camp 2 were related. From the 38 adults genotyped, 7-15 alleles per locus were detected with an observed heterozygosity of 0.567-0.926 (Table 1). An exact Hardy-Weinberg equilibrium test found no significant deviation from expectations ($P > 0.05$). The total exclusion probabilities for first and second parents in camp 1 were 0.991 and 0.994 respectively; and in camp 2, 0.993 and 0.996. Paternity and maternity were both assessed at the 95% confidence interval (95 and 83 assignments respectively in camp 1; 161 and 134 assignments in camp 2) and at the 80% confidence interval (81 and 93 in camp 1; 61 and 88 in camp 2). Marshall et al (1998) suggest that any locus with a null allele frequency greater than 0.05 should be excluded from the analysis. In our study there were no null alleles with a frequency greater than this.

Table 1: Allelic variation of the 6 loci used to genotype 38 adult ostriches and 398 ostrich chicks. Data are given for camp 1/camp 2

Locus	No alleles	No Individuals typed	Observed heterozygosity
CAU1	12 / 13	195 / 236	0.774 / 0.886
CAU7	8 / 10	194 / 242	0.567 / 0.674
CAU14	9 / 8	191 / 237	0.738 / 0.730
CAU17	9 / 9	196 / 242	0.658 / 0.723
CAU64	10 / 7	196 / 241	0.837 / 0.722
CAU76	15 / 15	195 / 242	0.800 / 0.926

2.3. Colour measurements

Reflectance spectra between 300 and 700nm were recorded using an Ocean Optics USB 2000 spectrophotometer and a PX-2 xenon lamp (Ocean Optics, Dunedin, Florida, U.S.A.) on five traits (bill, neck, black feathers, white feathers and legs) on the 15 males. As each trait appeared uniform in colour, it was measured 10 times in randomly allocated places. Reflection was recorded using a probe held normal to the surface, collecting light from a spot of 6mm in diameter. A white reference (Spectralon 99% white standard) and a dark reference were taken in between measuring each trait for calibration.

2.4. Statistical analysis

A principal component analysis (PCA) was performed on the reflectance spectra for all five traits for the 15 males, and reduced a number of highly correlated variables (reflectance at 2.4nm intervals) to a small number of independent variables.

We performed three general linear mixed models (GLMM) in which egg mass; hatchling mass; and mass at 1 month old were entered as the respective response variables. Parental mass and age; as well as scores for each principal component of the father's spectrophotometric measurements were entered as explanatory variables while offspring sex, date of laying, laying order, and the number of young hatched by each female (*in lieu* of clutch size) were entered as fixed factors. To account for individual female effects on the response variables, we treated the identification of each mother, nested by camp, as a random factor. All statistics were performed using SPSS 16 (SPSS Inc., Chicago, IL, U.S.A.)

3. Results

3.1. Parentage determination

The pattern in both camps was similar, showing reproductive skew in both sexes where each male and female mated with several partners, but a few combinations of parents sired more chicks than others (goodness of fit with Poisson distribution: camp 1: females: χ^2_{11}= 25.71, p = 0.001, males: χ^2_6 = 60. 32, p = 0.001, N = 176; camp 2: females: χ^2_{10} = 98.29, p = 0.001, males: χ^2_7 = 112.29, p = 0.007, N = 222; Fig. 1).

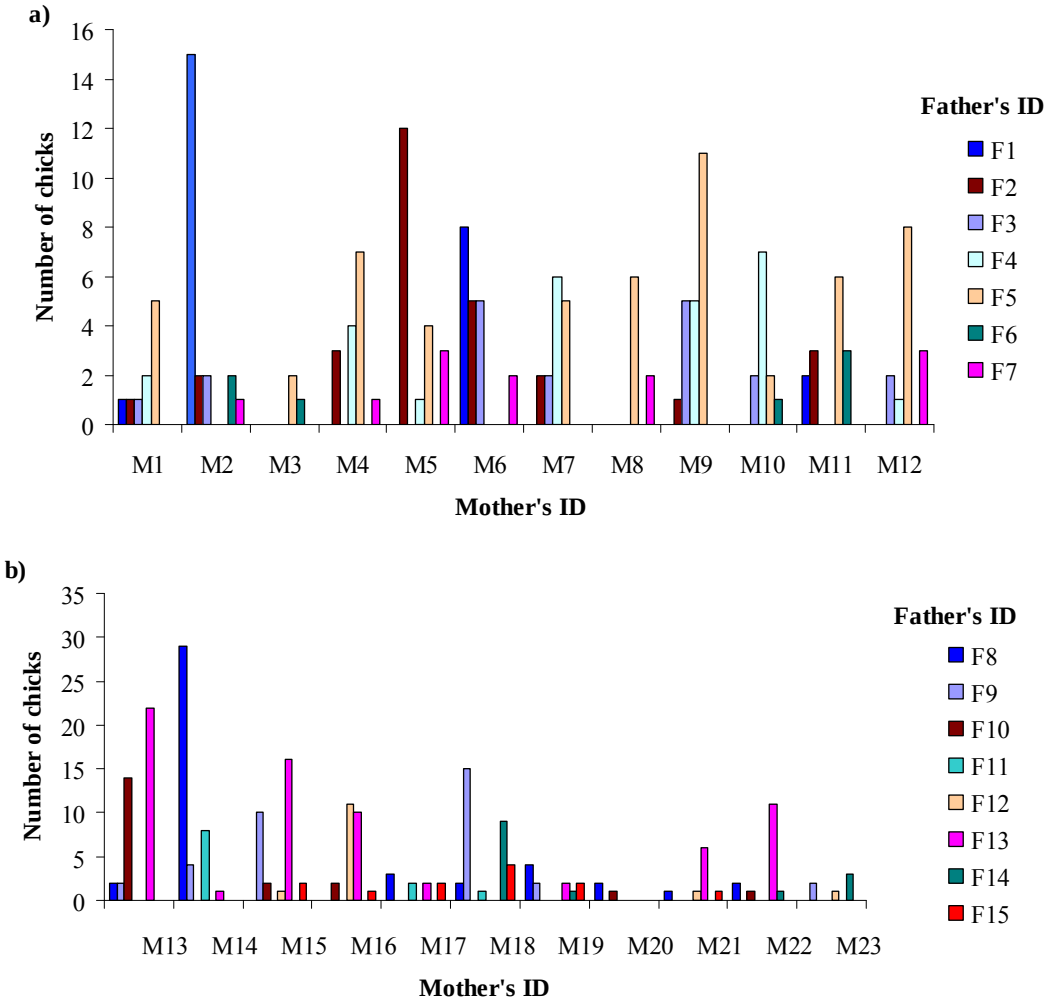

Fig. 1. The number of chicks sired by specific combinations of ostrich parents in camp 1 (a) and camp 2 (b). Female's ID: female identification number; male's ID: male identification number.

Males sired on average 26.4 offspring (SD = 18.20) with 78 % (camp 1) and 77 % (camp 2) of chicks sired by only four males in each case. Females produced on average

17.23 offspring (SD = 10.71), with half of the females producing 65.1 % (camp 1) and 76 % (camp 2) of chicks in each case.

3.2. Egg mass, chick mass and survival

Egg mass varied from 1.07 kg to 1.7 kg (mean = 1.35, SD = 0.12, N = 398); chick mass at hatching from 0.6 kg to 1.09 kg (mean = 0.82 kg, SD = 0.09, N = 398); and chick mass at 1 month from 0.87 kg to 5.20 kg (mean = 2.81 kg, SD = 1.04, N = 99). We found that day-old chick mass was strongly predicted by egg mass (r_{251} = 0.837, p = 0.001, after we controlled for laying date). However, we did not find such a correlation with chicks aged 1 month (r_{64} = 0.049, p = 0.343). No difference was found between the sexes in egg mass ($F_{1,397}$ = 0.689, p = 0.407), hatchling mass ($F_{1,397}$ = 1.446, p = 0.230), or mass at 1 month of age ($F_{1,98}$ = 1.667, p = 0.200).

Sixty-four per cent of hatchlings survived to 1 month of age. Survival was predicted by egg mass (chi-square test of association: χ^2_5= 3.925, r = 0.54, p = 0.048) and more significantly with hatchling mass (chi-square test of association: χ^2_4= 6.613, r = 0.69, p = 0.03), with larger hatchlings having higher survival rates. Furthermore, we did not detect any seasonal effect on chick survival to 1 month of age (chi-square test of association: χ^2_4= 0.278, p = 0.598).

3.3. The relationship between paternal traits and egg and chick mass

The PCA on colour measurements revealed that three principal components explained between 95.8% and 98.9% of the total variance in the five traits measured (Table 2). The first principal component (PC1) summarized between 70.5% and 94.8% of the spectral variation in these traits, whereas the second and third principal components (PC2 and PC3) accounted for 3.3-17.5%, and 0.8-7.8% respectively.

Table 2. Principal component analysis for the colour measurements of bill, neck, black feathers, white feathers and legs of the 15 male ostriches.

	Principal components (% of variation explained)			
Trait	PC1	PC2	PC3	Total
Bill	87.4	6.1	3.4	96.9
Neck	89.8	6.3	2.3	98.5
Black Feathers	70.5	17.5	7.8	95.8
White Feathers	94.8	3.3	0.8	98.9
Legs	90.0	5.5	3.0	98.5

In reflectance spectra of natural objects, PC1 is usually relatively flat, in which case it describes achromatic variation or "achromatic brightness" (Endler & Théry 1996). PC2 and PC3 therefore describe variation in spectral shape and are indirectly related to hue and saturation (Endler 1990; Bennett et al., 1994; Cuthill et al., 1999).

Egg mass variation was affected by the following paternal traits: PC1 of black feathers ($F_{1,14} = 1.290$, $R^2 = 0.10$, $p = 0.008$), and PC2 of white feathers ($F_{1,14} = 1.919$, $R^2 = 0.55$, $p = 0.001$), black feathers ($F_{1,14} = 1.460$, $R^2 = 0.20$, $p = 0.005$) and neck ($F_{1,14} = 1.370$, $R^2 = 0.14$, $p = 0.024$; Table 3, Fig. 2). Egg mass was positively affected by PC1 (Fig. 2a) and PC2 (Fig. 2b) scores of black feathers, and positively affected by PC2 scores of white feathers (Fig. 2c) and neck (Fig. 2cd). Hatchling mass and chick mass at 1 month of age were both positively affected by PC2 of white feathers ($F_{1,14} = 3.955$, $R^2 = 0.58$, $p = 0.001$ and $F_{1,14} = 3.157$, $R^2 = 0.42$ $p = 0.012$ respectively; Table 3). Neither male nor female age, nor weight influenced egg mass or day-old mass ($P > 0.05$). Furthermore, we found a seasonal effect on both egg mass ($F_{1,126} = 1.424$, $R^2 = 0.10$, $p = 0.014$) and chick mass ($F_{1,126} = 1.537$, $R^2 = 0.14$, $p = 0.004$), with heavier eggs (and consequently heavier chicks) being produced later in the breeding season (Table 3). No such effect was found for month-old chicks ($P > 0.05$).

Table 3: Factors influencing egg mass, hatchling mass, and chick mass at one month old (General Linear Mixed Model GLMM) in ostriches (23 females laid 398 eggs with 15 males). Significant relationship (P < 0.05) are indicated in bold.

	Egg mass			Hatchling mass			One month old mass		
Variable	df	F	p	df	F	p	df	F	p
PC1 scores for									
black feathers	1,14	1.290	**0.008**	1,14	0.038	0.845	1,14	0.553	0.469
white feathers	1,14	0.027	0.715	1,14	0.050	0.823	1,14	0.012	0.921
legs	1,14	0.135	0.715	1,14	0.797	0.373	1,14	0.010	0.959
neck	1,14	0.042	0.838	1,14	3.056	0.082	1,14	0.018	0.897
bill	1,14	0.499	0.484	1,14	3.665	0.067	1,14	0.141	0.719
PC2 scores for									
black feathers	1,14	1.460	**0.005**	1,14	3,21	0.084	1,14	0.002	0.965
white feathers	1,14	1.919	**0.001**	1,14	3,955	**0.001**	1,14	3.157	**0.012**
legs	1,14	0.077	0.783	1,14	0.051	0.822	1,14	0.014	0.913
neck	1,14	1.370	**0.024**	1,14	0.057	0.811	1,14	0.551	0.467
bill	1,14	0.716	0.402	1,14	0.324	0.570	1,14	0.003	0.917
PC3 scores for									
black feathers	1,14	0.883	0.775	1,14	0.094	0.759	1,14	0.206	0.658
white feathers	1,14	0.020	0.888	1,14	0.322	0.571	1,14	0.011	0.917
legs	1,14	0.573	0.453	1,14	0.001	0.972	1,14	0.598	0.449
neck	1,14	0.087	0.769	1,14	0.008	0.927	1,14	0.982	0.337
bill	1,14	0.677	0.414	1,14	0.789	0.376	1,14	0.013	0.910
Female mass	1,22	1.665	0.204	1,22	0.006	0.943	1,22	1.609	0.422
Female age	1,22	0.764	0.389	1,22	0.777	0.404	1,22	0.006	0.952
Male mass	1,14	0.373	0.544	1,14	0.035	0.853	1,14	0.002	0.967
Male age	1,14	0.955	0.334	1,14	0.09	0.764	1,14	0.013	0.910
Laying date	1,125	1.424	**0.014**	1,125	1,537	**0.004**	1,38	0.248	0.898
Laying order	1,40	1.655	0.089	1,40	1.374	0.083	1,18	1.483	0.201
Clutch size	1,13	0.255	0.981	1,13	0.269	0.981	1,12	3.627	0.072
Sex	1,1	1.657	0.200	1,1	0.176	0.676	1,1	2.237	0.151
Camp	1,1	0.200	0.655	1,1	0.440	0.503	1,1	1.939	0.186
Female within camp	1,21	1.041	0.155	1,21	1.403	0.070	1,21	0.498	0.738

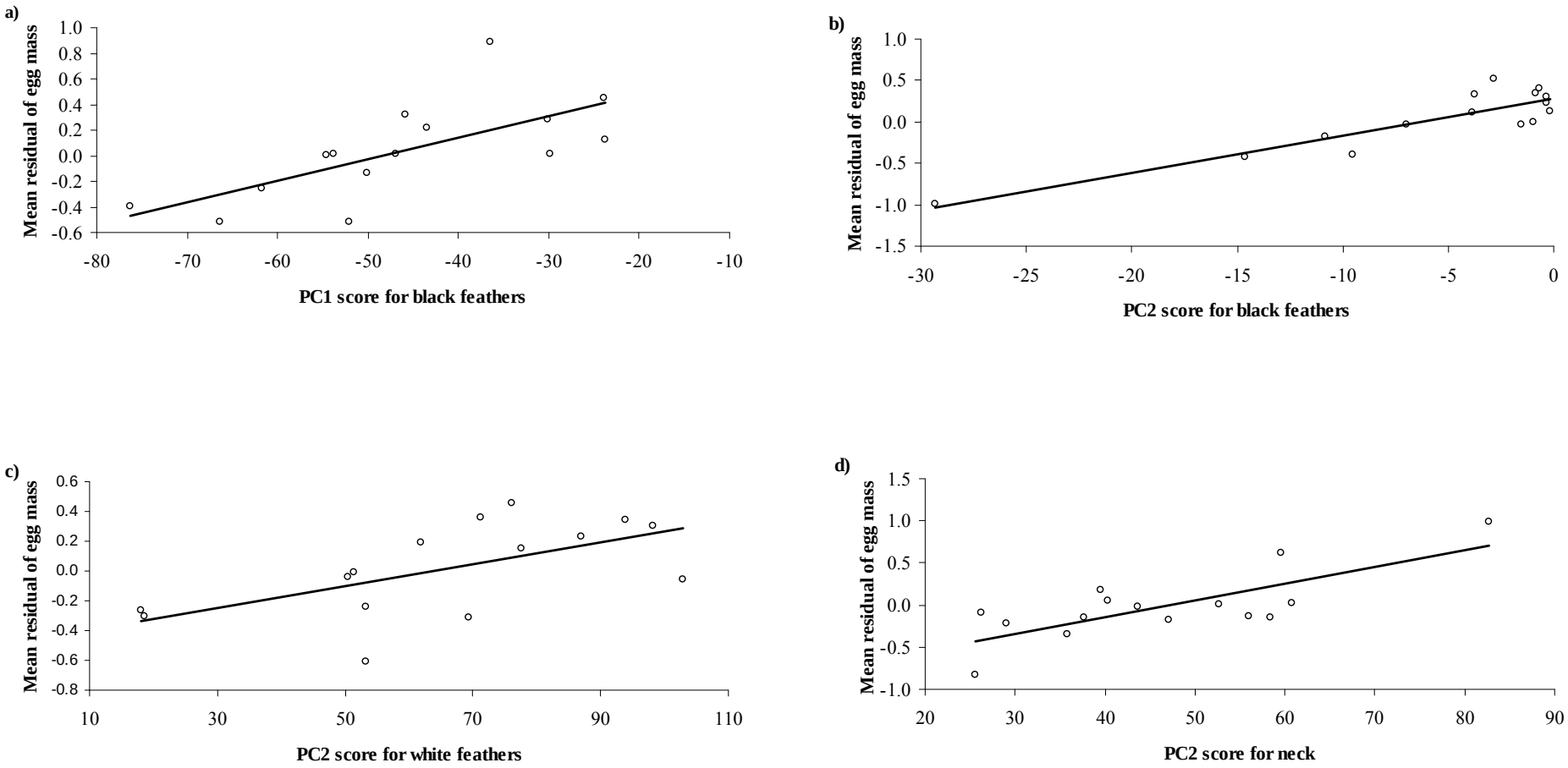

Fig.2. Mean residual of egg mass in relation to the principal component coefficients derived from the reflectance spectra from 15 male ostriches: (a) PC1 from black feathers; (b) PC2 from black feathers; (c) PC2 from white feathers; and (d) PC2 from neck. Mean residuals for each male are derived from a GLMM controlling for multiple matings among females (see methods for details).

Reflectance spectra for both white feathers and neck all revealed a maximum reflectance at intermediate wavelengths (440-580nm), absent in the reflectance spectra of black feathers (Fig. 3).

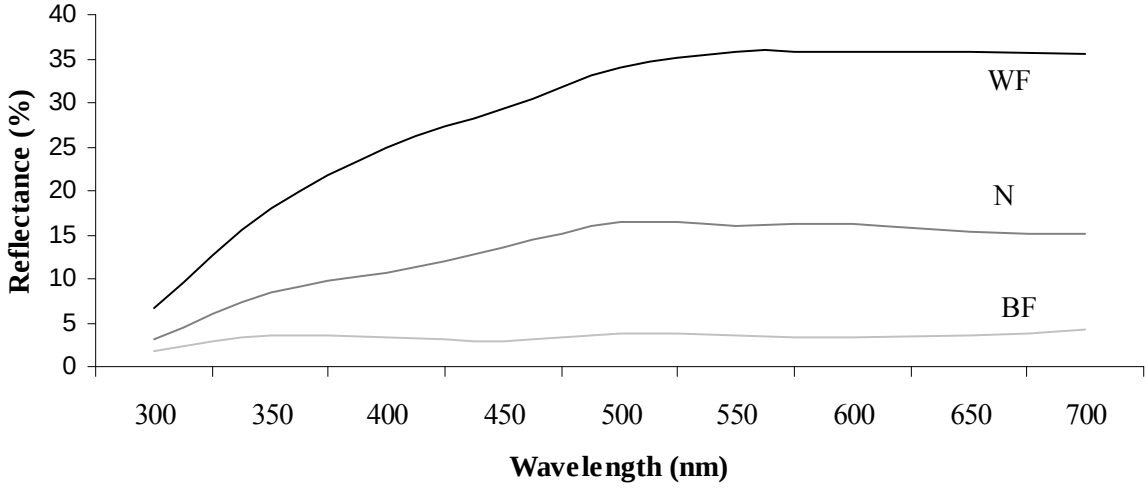

Fig.3. Spectral reflectance of black feathers (BF), white feathers (WF) and neck (N) of 15 male ostriches.

PC1 for black feathers was relatively flat across the spectrum whereas PC2 for this trait was high in the first part of the UV range (300-340nm), low in the second part of the UV range (340-460nm, with a peak at 380nm), and high again at longer wavelengths (500-680nm). PC2 for white feathers was low in the shorter (300-340nm) and longer wavelengths (580-700nm) and showed a peak reflectance at intermediate wavelengths (440-580nm); and PC2 for the neck was relatively low at short wavelengths (300-440nm), but showed the same peak reflectance between 450nm and 580nm observed for PC2 in white feathers (Fig. 4).

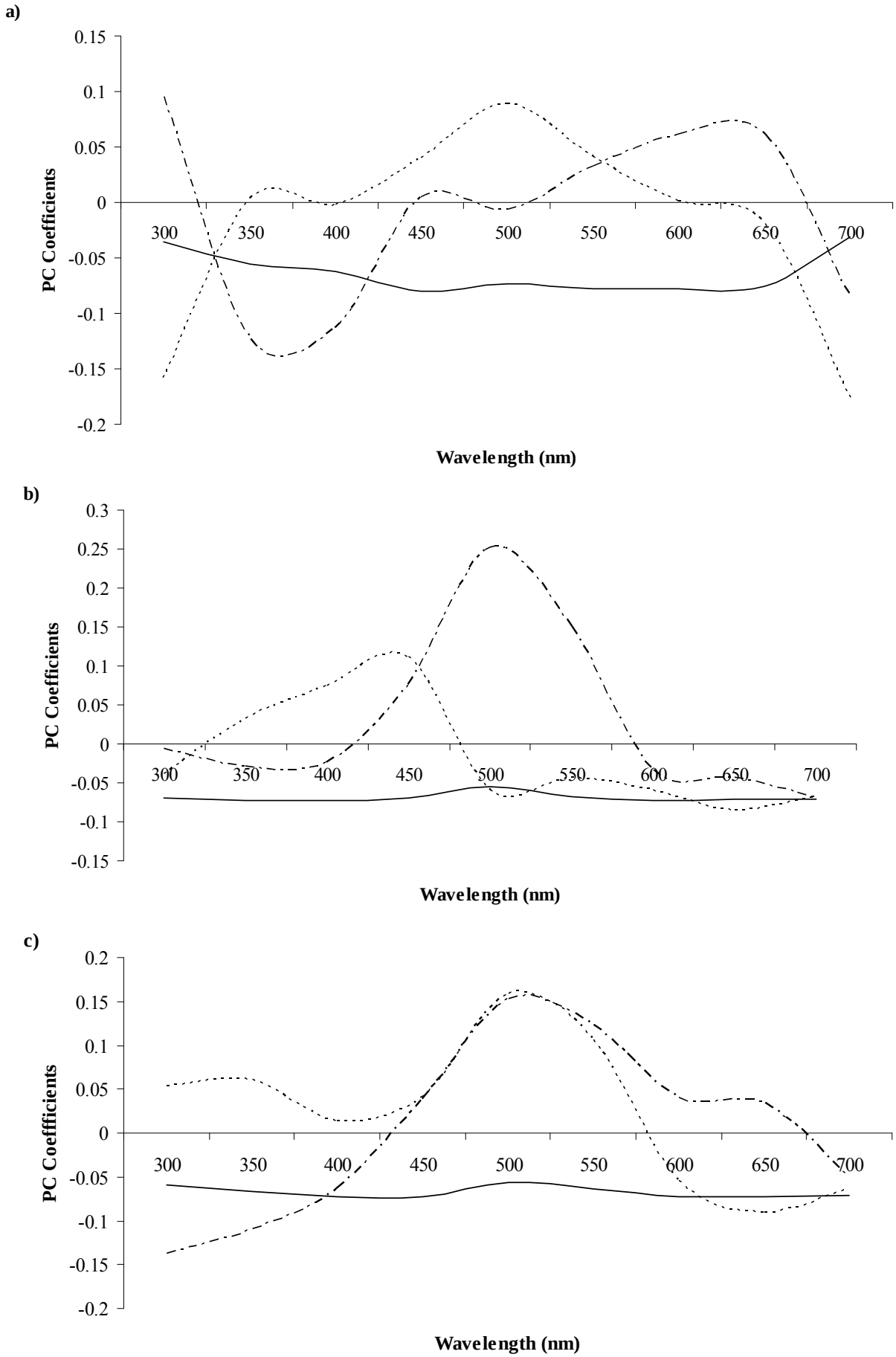

Fig. 4. Principal component coefficients derived from the reflectance spectra for (a) black feathers; (b) white feathers; and (c) neck of 15 male ostriches. Solid lines indicate PC1; dashed lines: PC2; dotted lines:PC3

4. Discussion

Our findings demonstrate that female ostriches, which mate with several males, invest differently at the egg stage with respect to specific combinations of male traits. The reproductive skew observed in our study is consistent with that observed in the wild (Bertram 1992; Kimwele & Graves 2003). The ostrich communal nesting system is unique in that clutches are laid by several females in the same nest, but only the major female and the territorial male incubate the eggs. However, Kimwele & Graves (2003) established that every female in the group of nests they investigated was both a major and a minor female on different male territories. Therefore, females usually mate preferentially with one specific male on his territory, and then with neighbouring males at a lower rate.

Our results showed that females laid heavier eggs when mated to males with higher values for PC2 of the neck and white feathers, as well as for PC1 and PC2 of black feathers. Furthermore, hatchling mass and chick mass at 1 month of age were also positively affected by PC2 of white feathers. An analysis of the reflectance spectra for these traits revealed interesting particularities. First, both types of feathers and the neck distinctly showed a maximum reflectance at intermediate wavelengths (440-580nm; Fig. 4). Second, PCA showed this same peak for the white feathers and the neck, but also a peak at 380nm for the black feathers, the "violet" part of the human visible spectrum. Wright & Bowmaker (2001) demonstrated that ostriches are most sensitive to intermediate wavelengths, with their four spectral cone classes having a maximum absorbance between 405 and 570nm (the "violet" to "green" part of the light spectrum). During the courtship display ('kantling' behaviour), the male typically sits on his legs, while his wings are held forward and his neck swings from side to side (Bertram 1992). The black feathers, white feathers and neck are thereby directly exposed to the female, amplifying their conspicuousness. Colour preferences of female ostriches may have evolved from foraging preferences: if a female is attracted to this range when searching for food, then males could

have plausibly exploited these predispositions in their courtship signals. Bubier et al. (1996) demonstrated that the "green" colours (i.e 500nm-578nm) induced more pecking responses in ostrich chicks, and ostriches in the wild eat preferentially from green foliage (Cooper & Palmer 1994).

Additionally, the contrast between white and black feathers could act as a signal of the male's condition to the female. Females often choose males with the most conspicuous traits (Andersson 1994). These traits could reduce the time it takes to locate a male (Darwin 1871), but could also indicate male quality (Trivers 1972, Zahavi 1975). Even though white patches might be cheaper to produce than highly pigmented ornaments (Torok et al., 2003), the costs associated with producing this trait could arise from its maintenance. White feathers are known to be more prone to breakage (Kose & Møller 1999), and are also more conspicuous to predators. Therefore, a male's ability to display and maintain such a contrast (and especially in the context of kantling behaviour) could provide the female with valuable information on the male's condition, and, consequently, act as a signal which influences the degree of her investment in eggs. However, as we did not make observations on courtship, we do not know whether the intensity and duration of this courtship behaviour could also influence the size of the eggs laid by females, nor the extent to which major males might perform more elaborate displays.

Recent studies have emphasized the fitness consequences of laying eggs of different sizes. Maternal investment, particularly nutrients and energy, allocated to eggs can profoundly influence the development of embryos and survival of hatchlings. Offspring fitness through hatching success, growth and survival has generally been assumed to be related to egg size (Clutton-Brock 1991). Females in a number of bird species lay larger eggs when mated to attractive males (Cunningham & Russell 2000; Rutstein et al. 2004), or invest more in egg resources such as yolk immunoglobulin (Saino et al., 2002) or testosterone (Gil et al., 1999). For instance, chicks hatched from eggs with high amounts of

testosterone beg for food more intensively and therefore grow faster than other chicks (Schwabl 1993; Lipar et al., 1999). However, body mass at hatching might depend mainly on the amount of energy contained in eggs, whereas at later stages body mass also depends on other factors, such as parental feeding rate or environmental quality (Smith & Bruun 1998). In ostriches, egg mass affects the survival of chicks: young from larger eggs are more likely to survive in the month following artificial hatching (Cloete et al. 2004). This has been substantiated by our study, in which both egg mass and hatchling mass had a positive effect on chick survival to 1 month of age.

Furthermore, we found that females tend to lay heavier eggs at the end of the breeding season, suggesting that females might allocate more resources to offspring hatching later in the season. This is of a particular interest, as chicks hatched at the end of the breeding season typically encounter harsher conditions as they have to grow during the winter season. The ability of mothers to transmit antibodies to their offspring has been documented in birds (reviewed in Grindstaff et al. 2003). Even though maternal effects generally have their greatest impact early in development and then decrease as offspring mature (Price 1998; Wolf & Brodie 1998), maternal antibodies may continue to affect offspring phenotype by influencing growth and developmental rates as well as the strength and diversity of the immune response (Boulinier & Staszewski 2008; Hasselquist & Nilsson 2009). This could explain why we did not detect any seasonal effect on chick survival to 1 month of age. However, whether female ostriches invest differently in yolk immunoglobulin or testosterone during the course of the breeding season or on the basis of male traits remains unknown, but could explain additional chick size variation within clutches observed in the farming environment.

In conclusion, the colouration of wing feathers and neck and the brightness of the black feathers of males appeared to influence the size of the egg laid by females mated to them. To our knowledge, this is the first study providing evidence that female ostriches

discriminate between males and adjust their degree of investment at the egg stage according to specific male traits. These results are of potential interest to the ostrich farming industry, which is plagued by high chick mortality. A better understanding of mating decisions and maternal investment could provide ostrich farmers with cues as to how to select individuals producing fit offspring.

CHAPTER THREE Male coloration reveals different components of immunocompetence in ostriches *Struthio camelus*

(M. Bonato, M.R. Evans, D. Hasselquist & M.I. Cherry)

(Animal Behaviour, in press)

ABSTRACT

It has been suggested that secondary sexual ornamentation signals male ability to resist infections, as only high-quality individuals are able to invest both in high immune defence and elaborate ornament expression. Such ornaments could thus serve as indicators of male quality and could be used by females in choosing mates. Ostriches are sexually dimorphic with regard to coloration of their feathers, bill, neck and legs, and have a promiscuous mating system, with a high degree of reproductive skew, particularly in males. We investigated the relationship between the coloration of the feathers, bill, neck and legs of 15 male ostriches maintained in a breeding flock; and the cell-mediated (measured using a phytohemagglutinin injection) and humoral components of their immune systems, as well as their heterophil:lymphocyte (H:L) ratio. We found that male responses to PHA injection and humoral responses to tetanus were predicted by leg coloration; humoral responses to diphtheria were predicted by white feather colouration; and the heterophil/lymphocyte ratio was related to bill coloration. These traits, which relate to male immune capacity, are exposed during male-male interactions and courtship display, so we suggest that these visual cues could provide valuable information on male quality to females (as well as rival males), forming the basis of mate choice in this species.

1. Introduction

Females of many species prefer to mate with males displaying the most elaborate ornamentation. Under the assumption that the expression of these traits is condition dependent, only males in prime condition will be able to develop the most exaggerated ornamentation (Andersson 1994), thereby revealing either direct or indirect genetic benefits that a female could obtain by mating with a given male. For instance, ornaments may reflect general condition (Goransson et al. 1990), the ability to forage for food (Slagsvold & Lifjeld 1988; Senar et al. 2002) or the ability to cope with parasites (Hamilton & Zuk 1982; Møller & Saino 1994). In birds, females frequently use sexually dimorphic characteristics to discriminate between males during mate choice (Andersson 1994; Saino et al. 2002). Furthermore, several studies have shown that sexual dimorphic traits such as feather characteristics (length of tail feathers: Møller & Petrie 2002; plumage coloration: Gonzalez et al. 1999; Doucet et al. 2004), beak coloration (Faivre et al. 2003) and spurs (Ohlsson et al. 2002) also reflect the male's ability to raise an immune response against novel antigens.

Immune function often shows strong condition dependence with only individuals in good condition (as illustrated by the expression of ornamentation) being able to produce strong immune responses. Norris & Evans (2000) defined immunocompetence as the ability of a host to prevent or control infection by pathogens and parasites, and variation in immunocompetence is assumed to represent general individual disease resistance. Consequently, females basing their mate choice decisions on ornamentation could acquire males with better resistance to parasites, resulting in either direct benefits in species with paternal care and/or indirect genetic benefits when offspring inherit genes for superior immunocompetence (Folstad & Karter 1992; Andersson 1994; Westneat & Birkhead 1998). In accordance with this, Bonneaud et al. (2005) showed that a specific MHC allele was associated with higher responses to two different T-cell-dependent antigens:

phytohaemagglutinin (PHA which is cell-mediated) and sheep red blood cells (which are mediated humorally).

Ostriches are promiscuous, and in the wild both males and females have multiple partners (Bertram 1992; Kimwele & Graves 2003). The ostrich communal nesting system is unique in that the major female allows minor females to lay in her nest, even though they provide no parental care. Only the major female and major male provide parental care in the form of incubation and guarding the offspring until independence. Furthermore, a remarkable feature of cohorts is that chicks differ greatly in size, and these size differences are likely to have a genetic basis arising from parental genotypic differences. Ostriches are sexually dimorphic; females have a dull-brown plumage while males have a black plumage with some white feathers, as well as coloured bill and legs (Deeming 1999). Both males and females use a repertoire of visual displays, in several of which the wings play a major part. For instance, wings are used during aggressive encounters with predators or opponents, in which ostriches raise both wings high above the body or flick them alternately up and down beside the body. Most importantly, wings are involved in the courtship display (or 'kantling' behaviour), whereby the male typically sits on his legs, while his wings are held forward, directly exposed to the females, and his neck swings from side to side (Bertram 1992). The kantling display is also used during antagonistic interactions between males, and is usually performed by a male who is driving a competitor away. Furthermore, during the breeding season the bare shins and the beak of ostrich males change from light pink to crimson red (Lambrechts 2004). Females were found to be able to discriminate between males and to invest differently at the egg stage with respect to the coloration of male traits involved in the kantling display (see Chapter two). Although the extent to which male traits reflect individual condition remains unknown, chick size variation could potentially be explained by females choosing and/or

investing in males of a higher quality (i.e with elaborate ornaments and/or higher immunocompetence), thereby enhancing offspring fitness.

Our aim in this study was to examine whether male traits (specifically coloration) reflect a male's ability to raise an immune response. Because of the complexity of the immune system, stimulation of more than one component of the immune system is required (Sheldon & Verhulst 1996; Norris & Evans 2000; Viney et al. 2005) to elucidate potential trade-offs between traits and immunocompetence. We assessed cell-mediated and humoral immunocompetence with challenge tests that have been extensively used in immunoecology studies. The advantage of these techniques is that individuals are exposed to a standardized challenge to their immune system and the response of the immune system is quantified in a standardized way. In addition, we monitored the heterophil: lymphocyte (H:L) ratio at the time of sampling, which provides a crude estimate of an individual's current immune status (Davis et al. 2008). Avian vision differs from that of humans in several ways: birds are sensitive to the UV part of the spectrum (320-400 nm) to which humans are blind (Bowmaker 1980; Bennett & Cuthill 1994) and have four cone types, rather than three as found in humans, implying that birds have the potential for tetrachromatic vision (Chen & Goldsmith 1986; Jane & Bowmaker 1988; Bowmaker et al., 1997; Wright & Bowmaker 2001). Therefore, we used UV-visible range spectrophotometry to measure accurately the colour of the bill, neck, feathers and legs of 15 males maintained within a breeding flock. In particular, the white feathers, and pink bill and legs, are particularly conspicuous during the breeding season, and we suggest that male ostriches use multiple signals to advertise their quality to both male competitors and females, thereby forming the basis of sexual selection in this species.

2. Materials and Methods

2.1. Study population

We carried out the study on 15 South African black ostrich males (*S. camelus var. domesticus*), maintained at a research farm in Oudtshoorn, South Africa, from August 2005 to March 2006. The breeding flock consisted of two groups, each in an 8 hectare camp, containing a ratio of male:female individuals of 7:12 and 8:11 respectively. Males and females were all of breeding age (range 2-5 years). Diets of the birds were formulated according to the nutrient requirements of the birds at the specific stage of growth (Brand & Gous 2006) and water were provided twice a week. Males selected for this experiment were roughly of the same height, and were weighed before the experiment using an electronic balance (Rudd, Pomona, South Africa). We recorded colour measurements on the birds in November 2005, in the middle of the breeding season, when the bare shins and the bill of ostrich males develop a typical colour, ranging from light pink to crimson red (Lambrechts 2004). The cell-mediated (together with blood samples to estimate the H:L ratio) and the humoral immune assays were performed a month apart in December 2005 and January 2006 to minimize interaction between the different experiments. Individuals were carefully restrained by hand during the colour measurements and the injections. Three trained technicians held the bird while another injected the PHA or the diphtheria and tetanus vaccine (see below). The same method was used for the blood sampling. The procedures lasted between 5 and 10 min and we verified that the bird resumed normal behaviour a few minutes afterwards. We also verified that the injections did not cause visible wounds or infections during the following days. We observed no negative effects of the injections in our study. Ethical clearance for this work was granted by the Stellenbosch University ethics committee.

2.2. Colour measurements

Reflectance spectra between 300 and 700 nm were recorded using an Ocean Optics USB 2000 spectrophotometer and a PX-2 xenon lamp (Ocean Optics, Dunedin, Florida, USA) on 5 different traits (bill, neck skin, black feathers, white feathers and legs) on each male. As each trait appeared uniform in colour, it was measured 10 times in randomly allocated places. Reflection was recorded using a probe held normal to the surface, collecting light from a spot of 6 mm in diameter. A white reference (Spectralon 99% white standard) and a dark reference for calibration were taken before measuring each individual trait.

2.3. Immune assays

We estimated two components of immunocompetence: the T-cell-mediated immune response and the B-cell humoral response. In addition, we recorded the heterophil: lymphocyte (H:L) ratio at the same time as we conducted the T-cell mediated immune response assay, to provide a crude estimate of the bird's current immune status.

Cell mediated immunity was challenged by using a phytohemagglutinin injection (PHA). Although this is a standard method of assessing cell-mediated immunity in poultry (Cheng & Lamont 1988), a recent study on house sparrows *Passer domesticus* has shown that PHA swelling is more complex than previously thought: it is correlated with cell mediated components of the immune system, but not exclusively so, as some of the swelling is attributable to other aspects of immune function, both innate and adaptive (Martin et al 2006). Our results should be interpreted in this context. Males were inoculated subdermally with 0.4 mg of a PHA solution (Sigma, L-8754) dissolved in 0.04 ml of Phosphate Buffered Saline (PBS) in the right wing web, and with 0.04 ml of PBS in the left wing, as a control test. We measured the swelling of the wing webs on three occasions; before the injection, 6 h and 24 h later. On each occasion we used a digital

calliper and measured the swelling of the wing web three times and used the average of these measures. Repeatabilities of measurements (Lessells & Boag 1987) were 0.79 and 0.83 for the right and left wing respectively ($p < 0.001$). The PBS control injection was administrated despite the recommendation of Smits et al. (1999) as we observed slight swellings at the point of injection (6h: mean = 1.04, SD = 0.36: 24h: mean = 1.33, SD = 0.39). The wing-web swelling was calculated as the difference in thickness of the PHA-injected versus the PBS-injected wing, which indicates the strength of the response to the PHA injection.

To measure the B-cell-mediated humoral response, we elicited an antibody response in the birds by injecting a solution of 0.5 ml of a diphtheria-tetanus vaccine (DTVAX) in their neck. As the concentration of a dose of this vaccine (0.5 ml) is calculated for an adult human, we used the same concentration for our adult ostriches, which had a mean mass of 115.60 kg; SD = 12.33. We collected 100µL of blood was from the right external jugular vein before the injection, and 10, 14, 21 and 30 days post injection. After centrifugation (4000 rpm for 10 min), plasma was stored at -70 °C. To assess the level of antibodies in the plasma, we then conducted an enzyme-linked immunosorbent assay, ELISA (Hasselquist et al. 1999; Råberg et al. 2003, Hanssen et al. 2008). Briefly, 96-well microtiter plates (Costar, Cambridge, MA, U.S.A.) were coated with either diphtheria or tetanus antigens at 4 °C for 24 h. After washing plates and blocking wells with 3% milk powder diluted in 0.01 M PBS/Tween 20, we added diluted plasma samples (see below) to the wells and allowed them to incubate overnight at 4 °C. Plates were then washed, and a rabbit-anti-ostrich Ig antiserum obtained from Prof Dirk Bellstedt (Department of Biochemistry, University of Stellenbosch, Stellenbosch, South Africa) (diluted 1:800) was added to each well. Following 1h of incubation at 37 °C and a wash, peroxidase-labeled goat-anti-rabbit serum (1:2000 dilution; Sigma A6154) was added to the wells. After a secondary incubation (45 min at 37 °C) and a final wash, 2,2-azino-bis-3-

ethylbenzthialzoline-6-sulfanic acid (ABTS; Sigma, A1888) and peroxidase were added to the wells, and the plates were then immediately transferred to a molecular devices V_{max} kinetic reaction ELISA reader. The plates were read every 30 s for 12 min using a 405 nm wavelength filter. Antibody concentrations were calculated according to the slope of substrate conversion over time in units 10^{-3} x optical densities (OD) per minute (m_{OD} / min), with a higher slope indicating a higher titer of anti diphtheria or anti tetanus antibodies in the sample. Each plate also included baseline samples collected from birds before injections (negative controls). Plasma samples diluted to 1:400 (for diphtheria antibodies) and 1:800 (for tetanus antibodies) were used in all analyses. All samples were run in duplicate (mean coefficient of variation between duplicate samples = 5.5%), and the average of the two readings was our measure of antibody levels in the plasma. Blanks with only buffer were also included on each plate. Final measures of antibody levels were expressed as the difference between baseline and post immunization antibody titres of individuals and were log transformed to obtain a more normally distributed data set and facilitate the use of parametric statistics.

To establish the H:L ratio, we collected blood smears by venipuncture from the wing vein of the birds just before the injection of PHA in the wing web. Blood smears were air dried, fixed in 100% methanol the same day and stained with 5% Giemsa solution. We counted a total of 100 white blood cells on each slide by moving the microscope stage from one field to another and calculated the H:L ratio. High H:L ratios are traditionally considered to indicate stress in poultry (Gross & Siegel 1983).

2.4. Statistical analysis

A principal component analysis (PCA) was performed on the reflectance spectra for all five traits for each male, and this reduced a number of highly correlated variables (reflectance at 2.4 nm intervals) to a small number of independent variables. The PCA on

colour measurements revealed that three principal components explained between 95.8% and 98.9% of the total variance in the five traits measured (Table 1 see chapter two). The first principal component (PC1) summarized between 70.5% and 94.8% of the spectral variation in these traits, whereas the second and third principal components (PC2 and PC3) accounted for between 3.3% and 17.5%; and between 0.8% and 7.8%, respectively. In reflectance spectra of natural objects, PC1 is usually relatively flat, in which case it describes achromatic variation or 'achromatic brightness' (Endler & Théry 1996) while PC2 and PC3 therefore describe variation in spectral shape and are indirectly related to hue and saturation (Endler 1990; Bennett et al. 1997; Cuthill et al. 1999).

Responses to PHA injection, antibody titers of the humoral responses, and H:L ratios were log transformed to achieve normally distributed residuals. We used a paired t-test and a repeated measure ANOVA to detect any differences in the strength of the responses across time for the responses to PHA injection and humoral responses respectively, and we selected the strongest immune responses for the subsequent analysis.

A generalized linear model (GLM) was constructed for each of the immune assays, with camp as a fixed factor, and using male body mass and age as well as principal component scores for the spectrometric measures of each male trait measured as explanatory variables. All variables were initially included, and then dropped until the model contained only significant terms. As four GLMS were constructed, Bonferronni adjustments were used to control for type I errors (Wright 1992) lowering the significance threshold to 0.0125. Statistical analyses were performed using SPSS 16 (SPSS Inc., Chicago, IL, U.S.A.)

3. Results

3.1. Immune assays

The responses to PHA injection were highly variable between individuals and higher 24 h after the injection, although not statistically different from the measurement recorded 6 h post-injection (6 h: mean = 2.54mm, SD = 2.14; 24 h: mean = 3.92mm, SD = 3.64, paired t test: t = -1.791, df = 14, p = 0.095). The primary antibody responses for both diphtheria and tetanus were highest 21 days after the injection (repeated measures ANOVA: diphtheria: $F_{1,3} = 44.24$, p = 0.0002 ; tetanus: $F_{1,3} = 28.73$, p = 0.0001; Fig. 1) and were only weakly correlated with each other ($r_{14} = 0.398$, p = 0.071).

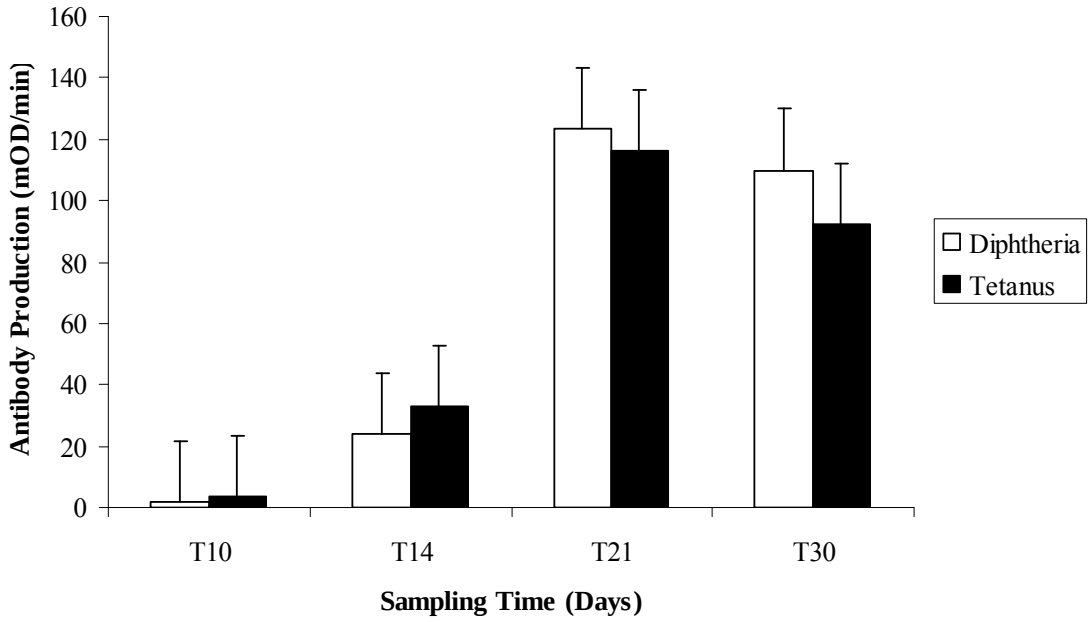

Fig 1. Antibody production to a diphtheria-tetanus vaccine in 15 male ostriches

Finally, the H:L ratio was also highly variable between individuals (mean = 0.42, SD = 0.22). We did not detect any effect of camp on the cell-mediated response ($F_{1,1} = 0.336$, p = 0.752), the humoral response (diphtheria: $F_{1,1} = 0.249$, p = 0.626; tetanus: $F_{1,1} = 0.280$, p = 0.572) or the H:L ratio ($F_{1,1} = 0.614$, p = 0.447). For subsequent analyses, we used the strongest response to the PHA injection and the humoral response (24 h post injection, and

21 days postinjection ,respectively). When analysing intercorrelations between these immune assays, we found none of them were correlated with each other ($p > 0.05$).

3.2. Immune function and colour measurements

The immune responses were not predicted by male age or body mass ($P > 0.05$), but by the principal component values of bill, white feathers and legs (Table 2 and Fig. 2).

Table 2: Factors influencing the cell-mediated response (PHA response), the humoral response (diphtheria and tetanus antibody responses) and the Heterophil-Lymphocyte ratio (H/L) in 15 male ostriches

	PHA response			Diphtheria response		Tetanus response		H/L	
Variable	df	F	p	F	p	F	p	F	p
PC1 scores for									
black feathers	1,14	0.570	0.505	1.762	0.316	1.613	0.332	0.240	0.645
white feathers	1,14	0.063	0.818	1.847	0.245	0.004	0.957	0.681	0.447
legs	1,14	1.768	0.195	1.903	0.272	1.342	0.366	0.005	0.945
neck	1,14	2.068	0.069	0.052	0.842	1.030	0.385	0.010	0.926
bill	1,14	0.739	0.439	2.430	0.092	0.946	0.433	0.113	0.750
PC2 scores for									
black feathers	1,14	1.074	0.110	1.932	0.228	0.405	0.590	0.386	0.561
white feathers	1,14	1.090	0.092	**2.466**	**0.010**	0.713	0.487	0.913	0.383
legs	1,14	**3.392**	**0.044**	0.448	0.572	1.580	0.336	0.239	0.645
neck	1,14	0.391	0.576	0.506	0.518	1.198	0.354	0.569	0.484
bill	1,14	1.087	0.374	2.340	0.104	0.593	0.522	**6.974**	**0.010**
PC3 scores for									
black feathers	1,14	0.323	0.609	0.421	0.583	1.631	0.330	1.759	0.255
white feathers	1,14	0.824	0.406	0.005	0.951	0.720	0.552	0.996	0.375
legs	1,14	0.608	0.471	1.957	0.210	**4.164**	**0.002**	1.462	0.293
neck	1,14	0.003	0.961	1.953	0.212	2.991	0.182	0.177	0.691
bill	1,14	0.011	0.922	2.325	0.105	0.793	0.467	2.103	0.221
Male mass	1,14	0.867	0.388	1.997	0.257	0.295	0.642	2.458	0.192
Male age	1,14	0.404	0.548	0.144	0.741	1.166	0.393	0.711	0.548
Camp	1,1	0.336	0.752	0.249	0.626	0.28	0.572	0.614	0.447

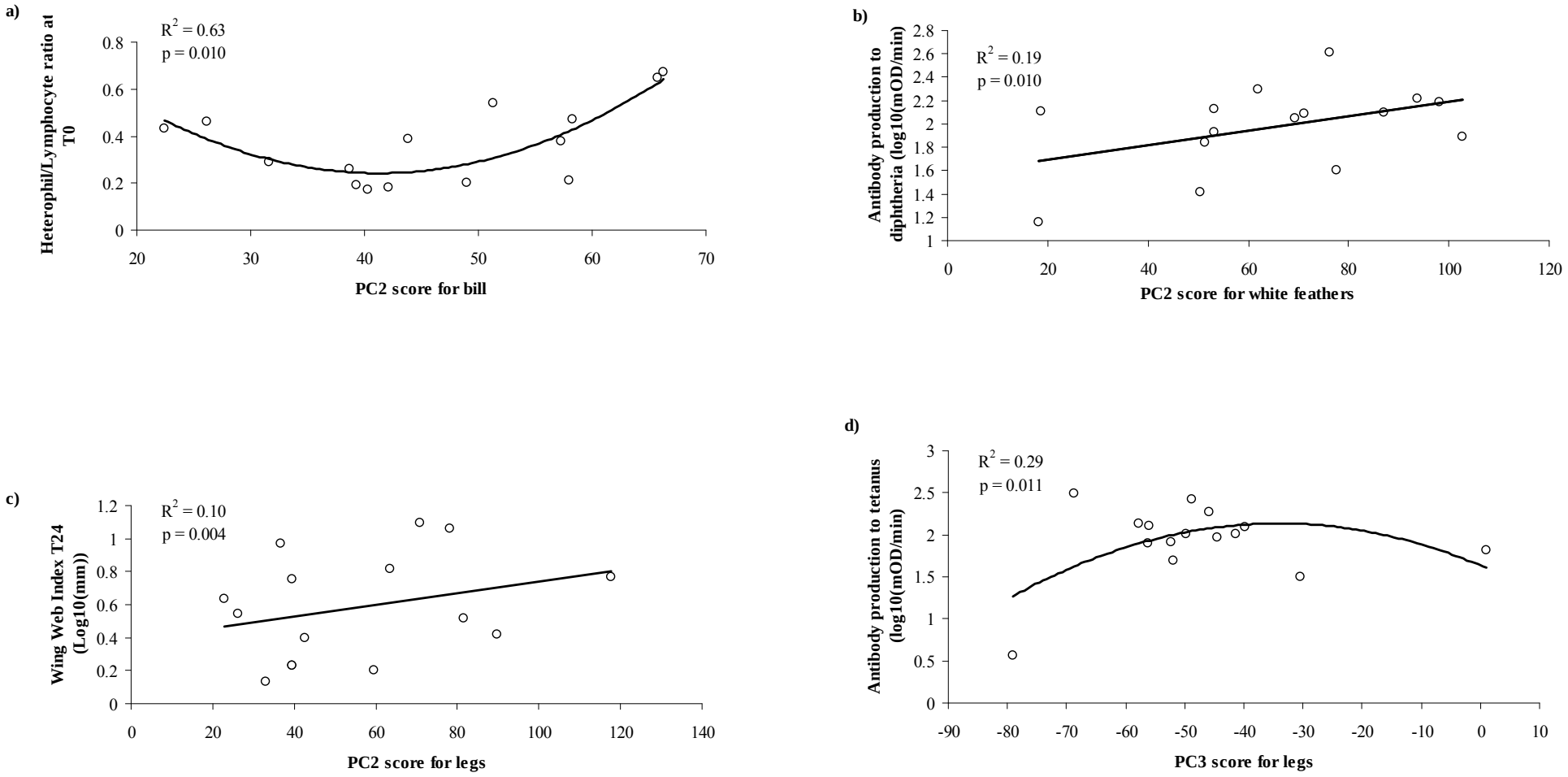

Fig 2. Components of the immune system in relation to the principal component coefficients derived from the reflectance spectra for (a) the bill; (b) the white feathers; (c) and (d) the legs of 15 male ostriches.

The H:L ratio showed a quadratic relationship with PC2 of the bill ($F_{1,14}$ = 6.974, p = 0.010) and responses to the PHA injection showed a positive relationship with PC2 of the legs ($F_{1,14}$ = 3.392, p = 0.004). For the humoral assay, the diphtheria response showed a positive relationship with PC2 of the white feathers ($F_{1,14}$ = 2.466, p = 0.010), whereas the tetanus response showed a quadratic relationship with PC3 of the legs ($F_{1,14}$ = 4.164, p = 0.002).

The reflectance spectra for these traits were similar to those reported for other bird species (Fig. 3). The white feathers spectrum rose gradually from 300 to 500 nm, above

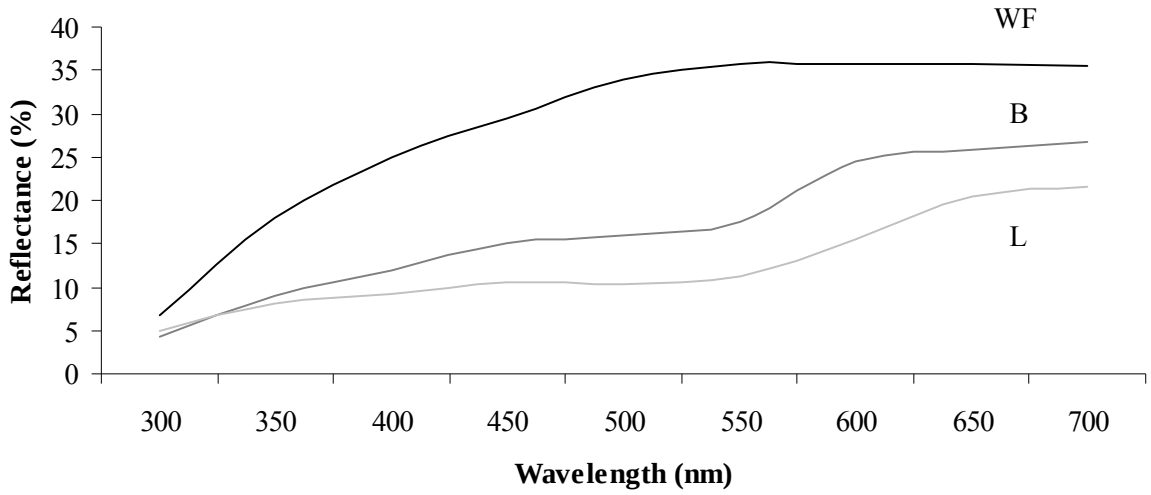

Fig 3. Spectral reflectance of bill (B), white feathers (WF) and legs (L) of the 15 male ostriches.

which it was uniformly high and flat (see Mennill et al. 2003; Shawkey & Hill 2006), while the reflectance spectra of the bill and the legs also rose gradually, but flatten out to display maximum reflectance above 600 and 650 nm respectively (see Pryke et al. 2002; Bolund et al. 2007). PC1 for all these traits was relatively flat across the spectra, and therefore represents 'achromatic brightness' (Endler & Théry 1996; Fig. 4). PC2 and PC3 were not spectrally flat, and thus represent chromatic variation (hue and saturation) of these traits. PC2 of the bill was found to be high and negative at shorter and longer wavelengths, therefore representing a contrast between UV and "redness". PC2 of the legs showed the same pattern, although with a more pronounced peak at intermediate

wavelengths. PC3 for both traits was also high and positive at shorter wavelengths and high and negative at longer wavelengths. By contrast, PC2 for white feathers showed peak reflectance at intermediate wavelengths, representing the "green" part of the spectrum.

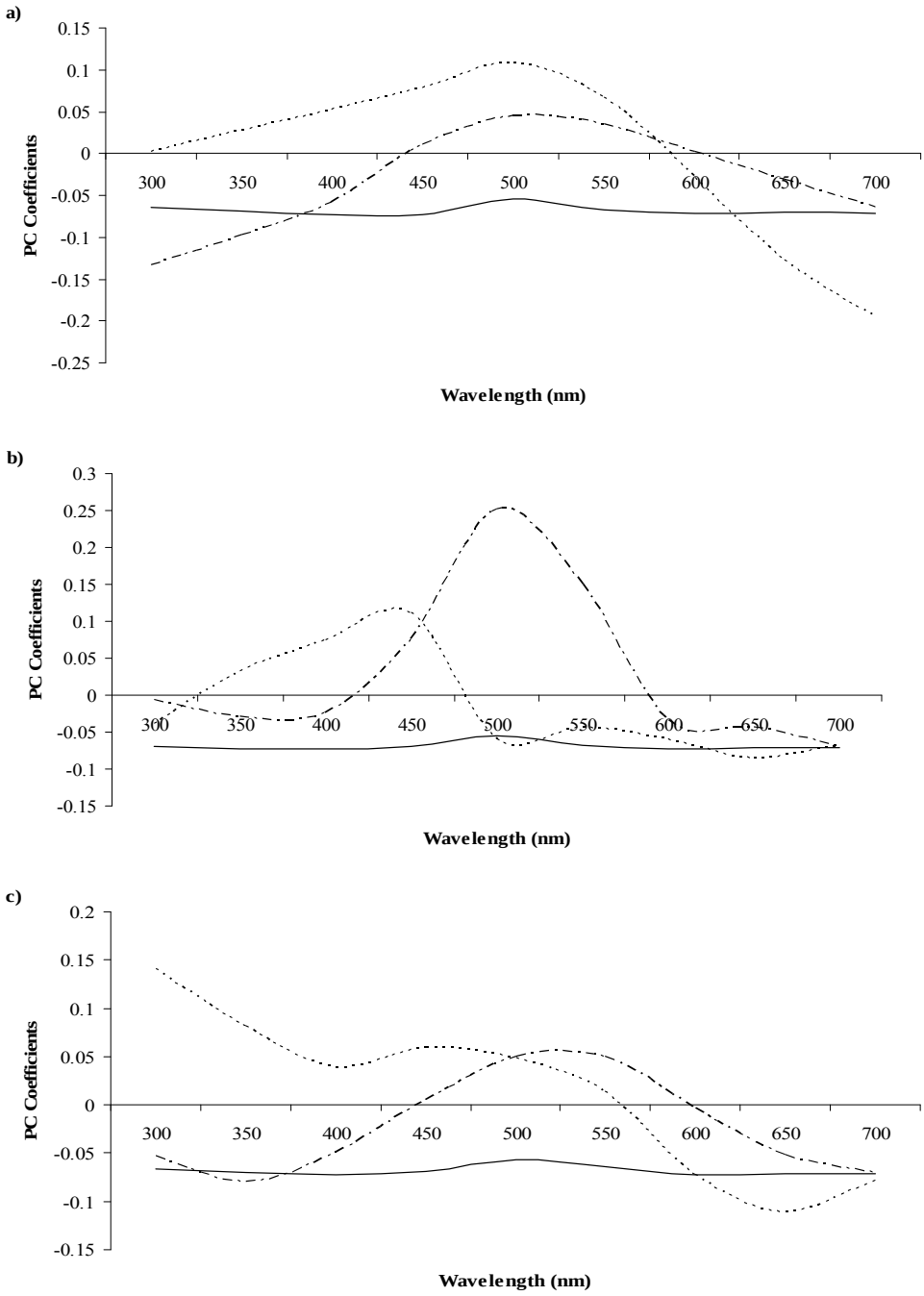

Fig 4. Principal component coefficients derived from the reflectance spectra for 3 body regions of 15 male ostriches: a) bill; b) white feathers; c) legs. Solid lines indicate PC1; dashed lines: PC2; dotted lines: PC3. Descriptions are provided only for principal components which predicted components of the immune system. a) PC2 predicted the heterophil/lymphocyte ratio. PC2 is high and negative in shorter (300nm - 400 nm) and longer wavelengths (575nm - 700nm), and slightly high and positive at intermediate wavelength (400nm - 575nm). b) PC2 predicted the humoral response to diphtheria. PC2 is high and negative in shorter (300nm - 340nm) and longer (575nm - 700nm) wavelengths and shows a peak reflectance at intermediate wavelengths (440nm - 575nm). c) PC2 predicted the cell-mediated response, while PC3 predicted the humoral response to tetanus. PC2 is high and negative in shorter wavelengths (300nm - 425nm) with a peak at 350nm, and in longer wavelengths (575nm - 700nm) and slightly high and positive in intermediate wavelengths (425nm - 575nm). PC3 is high and positive in shorter and intermediate wavelength (300nm - 525nm) and high and negative in longer wavelength (525nm and 700nm), with a peak at 650nm.

4. Discussion

Our findings revealed that different male traits reflect different components of the immune system. We found that males had: (i) a higher response to PHA injection with higher value of PC2 of the legs; (ii) a higher humoral response to diphtheria with higher value of PC2 of the white feathers and higher humoral response to tetanus for intermediate value of PC3 of the legs; and (iii) a lower H:L ratio with intermediate value of PC2 of the bill. These findings are consistent with the multiple signalling hypothesis (Møller and Pomiankowski 1993), which posits that multiple signals in sexual selection, reflect different aspects of individual quality. For instance, in the pheasant, *Phasianus colchicus*, females shows a preference both for spur length (which reflects condition and viability: Goransson et al. 1990) and for male display activity, which is correlated with parasite load (Johnstone 1995).

For the traits we measured, PC2 (the component explaining most of the colour variation) of the white feathers showed a peak reflectance between 400 and 575 nm, although less striking for the bill and the legs, as compared to the white feathers. Wright & Bowmaker (2001) demonstrated that ostriches are most sensitive to intermediate wavelengths, with their four spectral cone classes having a maximum absorbance between 405 nm and 570 nm. As these traits reflect in the area of maximum absorbance of the bird's visual spectrum, they could act as amplifiers whereby high-quality males are able to advertise their condition optimally. As male ostriches use a variety of displays to communicate and interact with females and/or other males (Bertram 1992), these traits could serve both to attract potential mates (via the courtship display where white feathers are exposed) and to deter other males or predators from engaging in antagonistic interactions.

Our results showed that males that are able to maintain trait colouration are able to raise an immune response. We found that responses to PHA injection were higher in males

with higher values for PC2 of legs. This positive relationship between the responses to PHA injection and leg coloration may be strategically important for male ostriches, especially during the breeding season because of competition and conflicts over territories and/or females. As legs are mostly used to strike opponents and small predators (Bertram 1992), they are more prone to injuries. Thus, the cell-mediated immunity may be mobilized to heal these injuries and consequent infections, and may be strategically particularly important for male ostriches during the breeding season. In accordance with this, the bare shins of ostrich males change in colour from light pink to crimson red as males become territorial, and continuing into the breeding season (Bertram 1992; Lambrechts 2004).

The analysis of the humoral response revealed an interesting feature, in that responsiveness to the two antigens reflected different traits. The humoral response to diphtheria was higher in males with higher PC2 values for white feathers, and the humoral response to tetanus was higher with intermediate PC3 values for the legs.

The positive relationship between the coloration of the white feathers and the primary response to diphtheria is of particular interest as it suggests that white feathers are condition dependent. Female ostriches lay heavier eggs in response to male traits, specifically involved in the courtship display (see Chapter two), with PC2 of the white feathers predicting egg mass more strongly than any other trait. No studies to date have investigated whether the coloration of ostrich white feathers could increase predation risk, but males and females specifically use white feathers in attempting to distract predators from chicks (Bertram 1992). A male's ability to display and maintain such a contrast between its black and white feathers could provide the female with valuable information on the male's condition, in particular its ability to raise an immune response, acting as a signal which influences the degree of her investment in eggs. Similarly, in female eiders, *Somateria mollissima,* the width of the white wing bars reflects previous immune

challenges, and could therefore potentially be used as an honest signal of individual quality (Hanssen et al. 2008).

The finding of peak primary response to tetanus at intermediate levels of PC3 in leg coloration suggests that this component of defence has not only fitness benefits but also costs (Viney et al. 2005), and the expression of this trait may reflect variation in how individuals trade off these costs and benefits. Studies on blackbirds, *Turdus merula* (Faivre et al. 2003), and red junglefowl, *Gallus gallus spadiceus* (Zuk & Johnsen 1998), have demonstrated that males pay a cost for the expression of a secondary sexual character by decreasing investment in one of the components of the immune system. In blackbirds, males with more orange bills were less immunocompetent when the humoral response was tested than when the cell-mediated response was tested (Faivre et al 2003). In our study, since the response to PHA injection also reflected the coloration of the legs, the maintenance of this specific component of the immune system might have profound effects on the maintenance of the humoral responses.

A precondition of condition-dependent models of immune trade-offs is that multiple components of the immune system should be inter correlated (Westneat & Birkhead 1998). Recent studies have found contrasting results; in some avian species different components of immunity showed correlated responses (Westneat et al. 2003; Ekblom et al. 2005) whereas in others they did not (Zuk & Johnsen 1998; Faivre et al. 2003). We only found a weak positive relationship between humoral responses to diphtheria and tetanus, while there were no relationships between these and either responses to PHA injection or H:L ratio, which in turn were unrelated to each other. However, we measured only primary immune responses, as the disturbance caused to the breeding flocks did not allow additional measurements. Variation may exist in the quality of immunological memory which determines the strength of the secondary immune response: as the secondary

response is stronger; variation between individuals could be greater and have more impact on their health.

Furthermore, the quadratic relationship between H:L ratio and bill coloration suggests that only individuals with intermediate PC2 values have a low H:L ratio. The H: L ratio is widely used as a stress estimator in poultry (Gross & Siegel 1983; Maxwell 1993) and is known to increase in response to various stress factors, such as infections or disturbance. Although this signal could inform females and/or competitors of the general condition of an individual, the use of the H:L ratio should be interpreted with care, as it could indicate either an immuno competent individual or an individual currently fighting an infection (Sheldon & Verhulst 1996; Norris & Evans 2000).

Immunocompetence appears to be affected by the general condition of the individual, which in turn could be reflected in male size, the expression of ornamentation, or territory quality (Blount et al. 2003; Norris & Evans 2000; Møller & Petrie 2002). These could all provide insights on male quality to females and could potentially inform her mating decisions. Studies on the effect of testosterone on the expression of sexual ornamentation, immunity and mating strategies have led to contrasting results across species (Roberts et al. 2004). In male ostriches, the level of testosterone increases with the level of aggression between males related to territorial behaviour during the breeding season (Degen et al. 2004). As male aggression is characterized by forward kicking and kantling behaviour displayed to other males (Bertram 1992), and as the shins of ostrich males change in colour during that time (Lambrecht 2004), this emphasizes the crucial role of both legs and white feathers. Further studies should therefore be conducted to determine whether testosterone could drive the degree of the immune response observed, as well as the expression of these specific male traits.

Coloration in birds is essentially derived from two types of pigmentation, melanins and carotenoids. Melanins are responsible for most black, brown and brick-red coloration,

are cheap to produce and can be synthesized by the organism (Maynard Smith & Harper 1988). The major component of melanins is the amino acid tyrosine, which is also an important precursor in immunological processes (Owens & Wilson 1999). Carotenoids, by contrast, are responsible for most bright yellow, orange and red coloration and are exclusively acquired through the diet (Brush 1990). They also play an important role in many immunological and metabolic pathways, as their expression can be affected by parasitism (Lozano 1994). In particular, manipulation of melanin (McGraw 2003) and carotenoid (Blount et al. 2003) concentration in other bird species has demonstrated the relationship between these pigments, mate quality and consequently sexual attractiveness. For instance, modulation of carotenoid supplies in zebra finches, *Taeniopygia guttata*, showed that females chose to mate with carotenoid-rich males displaying redder bills and a higher cell-mediated response (Blount et al. 2003). The limitation of our study is that we were unable to manipulate the concentration of the pigments responsible for trait coloration, something which could usefully be investigated in further studies.

In conclusion, the coloration of the bill, white feathers and legs appears to reflect different components of the immune response of ostrich males. High-quality males appear to be able to maintain these signals but also to maintain both cell- and humorally-mediated components of their immune system. These findings are consistent with the multiple signal hypothesis suggesting that multiple ornaments signal different aspects of general quality. Our results explain patterns observed in maternal investment in ostriches, and could be used by the ostrich farming industry in selecting breeding males.

CHAPTER FOUR Growth rate and hatching date in ostrich chicks reflect humoral but not cell-mediated immunocompetence

(M. Bonato, M.R. Evans, D. Hasselquist, S.W.P. Cloete & M.I. Cherry)

(Behavioral Ecology and Sociobiology, submitted)

ABSTRACT

A trade-off between immune response and life-history traits, in particular growth rate, has been documented in various bird species. Ostriches are fast-growing birds and a typical feature of cohorts is that they differ greatly in size. By using techniques widely used in immunoecology, we investigated the relationship between hatching date and growth rate of chicks, and both cell-mediated (measured using a phytohemagglutinin injection) and humoral immune responses in ostrich chicks maintained on a breeding farm. Chicks with higher growth rates had intermediate responses to both diphtheria and tetanus toxoids. By contrast, growth rates and responses to PHA injection were unrelated. We found that chick growth rate variation may be explained beyond a certain threshold by a trade-off between the humoral response and growth. Both responses to PHA injection and humoral responses in chicks were found to decrease with chick hatching date, suggesting a higher parasite pressure during winter. These results could explain patterns observed in mating decisions and maternal investment in ostriches, and have implications for ostrich farming.

1. Introduction

The vertebrate immune system has evolved as a defence mechanism against parasites and pathogens, and hence plays a crucial role in host survival and fitness (Goldsby et al. 2000). Individuals are affected to varying degrees by the negative impact of parasites, because of inter-individual variations in genetic and non-genetic factors affecting general phenotypic condition (Nordling et al. 1998; Gonzalez et al. 1999). Goossens et al (1997) reported that the main effect of disease was the suppression of growth in farmed goats, inevitably leading to reduced fitness. The control of parasites in chickens led to a major improvement in feed efficiency, weight gain, reproductive performance and survival (Stephenson 1994), underlying the crucial role of the immune system.

Life-history theory predicts that natural selection should favour the evolution of physiological mechanisms that ensure an optimal allocation of limited resources to competing activities (Stearns 1992). Although Klasing (1998) and Hasselquist and Nilsson (personal communication) have argued that the energetic costs of immune function might be minimal compared to other physiological processes such as tissue growth, there is increasing empirical and experimental evidence that a trade-off between immune responses and other life-history traits exist (Lochmiller and Deerenberg 2000), in particular survival (Møller and Saino 2004; Hanssen et al. 2008), and growth rate (Norris and Evans 2000; Hasselquist and Nilsson personal communication). For instance, immune responses to non-pathogenic antigens have been demonstrated to reduce growth in domestic poultry (Klasing et al. 1987) and in Japanese quails (Fair et al. 1999). Energy and nutrients are required for offspring development, and the decline in growth observed during the immune system stimulation indicates that the strategies of resource allocation shift towards survival and away from non essential processes such as growth (Lochmiller and Deerenberg 2000).

In ostrich chicks kept in farmed conditions mortality rates are highly variable and can reach 50% before 3 months of age (Verwoerd et al. 1999). Furthermore, ostriches are

fast-growing birds and a remarkable feature of cohorts is that they differ greatly in size (Deeming and Ayres 1994; Deeming et al. 1993). Factors influencing growth rates in ostrich chicks are believed to include the protein content in the diet (Deeming 1996), group size (Deeming and Ayres 1994; Mushi et al. 1998) and disease (Deeming and Ayres 1994). In the wild, ostriches are promiscuous, with males and females having multiple partners (Bertram 1992; Kimwele and Graves 2003. The ostrich communal nesting system is unique in that the major female allows minor females to lay in her nest even though they provide no parental care. Up to 18 females may lay in a nest, but only the major female and major male incubate the eggs and guard the offspring until independence (Bertram 1992; Kimwele and Graves 2003). One to six minor females usually lay between 20 and 40 additional eggs, with some clutches containing up to 67 eggs (Sauer and Sauer 1966; Bertram 1992). As more eggs are laid in the nest than can be incubated (a maximum of 20 eggs), the major female usually ejects surplus eggs from the incubated central clutch. Typically, she arranges the eggs into a central, incubated clutch and a ring of peripheral, unincubated eggs that will never develop. Kimwele and Graves (2003) demonstrated that she usually contributes a disproportionate number of fertile eggs to the central incubated clutch. Hatching takes place over a period of 2-3 days and families of chicks are combined into creches, overseen by the major male and female. The ostrich communal nesting system is thus a particularly interesting one to work on, as species with a promiscuous rather than socially monogamous mating system may have evolved mechanisms for devoting more energy to immune surveillance and response, given the greater likelihood for disease transmission associated with multiple partners.

The aim of this study was to examine whether variation in levels of immune defence was related to offspring growth rates. Because of the complexity of the immune system, stimulation of more than one of its components is required (Sheldon and Verhulst 1996; Norris and Evans 2000; Viney et al. 2005) to elucidate potential trade-offs between

immunocompetence and chick growth rates. We assessed cell-mediated and humoral immunocompetence with challenge tests that have been extensively used in immunoecology studies. The advantage of these techniques is that individuals are exposed to a standardized challenge to their immune system, and the immune response is then quantified in a directly comparable way in all individuals.

2. Materials and Methods

2.1. Sampling population

The study was carried out on South African black ostriches (*S. camelus var. domesticus*), maintained at a research farm in Oudtshoorn, South Africa, from August 2005 to March 2006. The breeding flock consisted of 2 groups in 8 hectare camps containing male: female ratios of 7:12 and 8:11 respectively. Eggs were collected on a daily basis, weighed and stored in electronic incubators until hatching. Chicks were then sexed through cloacal examination (Gandini and Keffen 1985), tagged on the right wing web with a 2 cm brass ear tag used in ostrich farming (Hasco, Dayton, USA), and weighed with an electronic balance (Mercer) once a month from the day of hatching to 360 days of age. All chicks were allowed to dry off for 8 to 10 hours after hatching, and subsequently transferred to an extensive chick rearing facility where they were kept in groups of 100-110 chicks. The rearing barn was divided into several stalls where chicks were distributed according to their age. During their first two weeks, chicks were maintained at a constant temperature of 25°C. Thereafter, the temperature was decreased by 1°C per week, until chicks were 2 months old when heating was stopped. Light was provided from 8am to 5pm. Food and fresh water were supplied ad libitum. At 3 months old, chicks were tagged with a 7 cm neck tag (Hasco, Dayton, USA) and were transferred to outdoor camps. Individuals were carefully restrained by hand during the measurements and the injections. Two trained technicians held the bird while another injected the PHA or the diphtheria and

tetanus vaccine (see below). The same method was used for the blood sampling. The procedures lasted between 5 and 10 minutes and we verified that the bird resumed normal behaviour a few minutes afterwards. We also verified that the injections and the blood sampling did not cause visible wounds, skin irritations or infections during the following days. We did not observed any negative effects of the injections or the blood sampling in our study. Ethical clearance for this work was granted by the Stellenbosch University ethics committee.

2.2. Weight data and estimates of growth rates

Monthly body mass records (n = 447) were available for chicks that hatched in the experiment. Individual body mass measures ranged from 0.9 kg at 30 days of age (mean = 2.80 kg, SE = 0.11) to 96 kg at 360 days of age (mean = 68.93 kg, SE = 2.38). Because of the wide range in absolute values, weight data were transformed to square roots or cube roots to stabilize the variance across ages. Being derived from repeated records on the same chicks, these data were considered as longitudinal. Growth trends for each chick were modelled by using cubic splines (Verbyla et al. 1999). The splines considered initially consisted of three components, namely: a fixed linear component, random deviations from linearity following a smooth trend, and random deviations from linearity not conforming to a smooth trend. Linear and non-linear components of the splines were treated as interactive variables with paddock of origin, to obtain indications of potential differences in these trends between paddocks. The initial analyses involved fitting various combinations of fixed paddock effects, random spline components and interactions between them to obtain an operational model. The data were analysed by using ASREML software (Gilmour et al. 2006). Repeated records pertaining to all animals were accommodated by the addition of each animal identity as a single random effect in the operational model. Pedigree information obtained for all individual chicks was included in

the analysis, to obtain estimates for live weight for all parents contributing progeny in both colonies. Differentiation between direct additive and permanent environmental animal effects was not attempted, as the data did not include sufficient information to do so with any degree of certainty. Values for live weight that were derived from this repeatability model analysis for individual chicks and their parents were related to measures of immunocompetence.

2.3. Immune responses

We estimated two measures of immunocompetence: the T-cell mediated immune response and the B-cell humoral response. We first assessed the T-cell-mediated immune responses on 4 month old chicks, and then the B-cell humoral immune response on the same chicks a month later in order to avoid any interaction between the two tests.

Cell mediated immunity was challenged by using a phytohemagglutinin injection (PHA). Although this is a standard method of assessing cell-mediated immunity in poultry (Cheng and Lamont 1988), a recent study on house sparrows *Passer domesticus* has shown that PHA swelling is more complex than previously thought: it is correlated with cell mediated components of the immune system, but not exclusively so, as some of the swelling is attributable to other aspects of immune function, both innate and adaptive (Martin et al 2006). Our results should be interpreted in this context. Four month old chicks were inoculated sub-dermally with 0.4 mg of a PHA solution (Sigma, L-8754) dissolved in 0.04ml of Phosphate Buffered Saline (PBS) in the right wing web, and with 0.04 ml of PBS in the left wing, as a control test. We measured the swelling of the wing webs on three occasions: before the injection, 6 hours and 24 hours later. On each occasion we used a digital calliper and measured the swelling of the wing web three times and used the average of these measures. Repeatabilities of measurements (Lessells and Boag 1987) were 0.82 and 0.85 for the right and left wing respectively ($p < 0.001$). The PBS control

injection was administrated despite the recommendation of Smits et al. (1999) as we observed slight swellings at the point of injection (6h: mean = 0.94, SD = 0.21: 24h: mean = 0.97, SD = 0.29). The wing-web swelling was calculated as the difference in thickness of the PHA-injected versus the PBS-injected wing, which indicates the strength of the response to the PHA injection.

To measure the B-cell mediated humoral response, we elicited an antibody response in the birds by injecting a solution of 0.5 ml of a Diphtheria-Tetanus vaccine (DTVAX) in their neck when they reached 5 month of age. 80 µL of blood was collected from the right external jugular vein before the injection, and 10, 14, 21 and 30 days post-injection. After centrifugation (4000 rpm for 10min), plasma was stored at -70°C. To assess the level of antibodies in the plasma, we then conducted an Enzyme-Linked Immunosorbent Assay, ELISA (Hasselquist et al. 1999; Råberg et al. 2003, Hanssen et al. 2008). Briefly, 96-well microtiter plates (Costar, Cambridge, Mass) were coated with either diphtheria or tetanus antigens at 4°C for 24h. After washing plates and blocking wells with 3% milk powder diluted in 0.01 M PBS/Tween 20, diluted plasma samples (see below) were added to the wells and allowed to incubate overnight at 4°C. Plates were then washed, and a rabbit-anti-ostrich Ig antiserum obtained from Prof Dirk Bellstedt (Department of Biochemistry, University of Stellenbosch, Stellenbosch, South Africa) (diluted 1:800) was added to each well. Following 1h of incubation at 37°C and a wash, peroxidase-labeled goat-anti-rabbit serum (1:2000 dilution; Sigma A6154) was added to the wells. After a secondary incubation (45 min at 37°C) and a final wash, 2,2-azino-bis-3-ethylbenzthialzoline-6-sulfanic acid (ABTS; Sigma, A1888) and peroxidase were added to the wells, and plates were then immediately transferred to a Molecular Devices V_{max} kinetic reaction ELISA reader. Plates were read every 30s for 12min using a 405nm wavelength filter. Antibody concentrations were calculated according to the slope of substrate conversion over time in units 10^{-3} x Optical Densities (OD) per minute (m_{OD} / min), with a higher slope indicating

a higher titer of anti-diphtheria or anti-tetanus antibodies in the sample. Each plate also included baseline samples collected from birds before injections (negative controls). Plasma samples diluted to 1:400 (for diphtheria antibodies) and 1:800 (for tetanus antibodies) were used in all analyses. All samples were run in duplicate (mean coefficient of variation between duplicate samples = 5.5%), and the average of the two readings was our measure of antibody levels in the plasma. Blanks with only buffer were also included on each plate. Final measures of antibody levels were expressed as the difference between baseline and post-immunization antibody titres of individuals and were log transformed to obtain a normally distributed data set and facilitate the use of parametric statistics.

2.4. Sample size and statistics

Because of a high chick mortality during the first 5 months of age (75%) we were able to challenge the cell-mediated response on only 104 chicks (54 males and 50 females), and the humoral response to the vaccine injection on only 43 chicks (18 males and 25 females). This high mortality rate cannot be attributed to our experiment, as the overall mortality of the chicks maintained on the farm was estimated at 70%, and is thought to be linked to unusually cold weather and intense parasite loads during this particular season (S. Engelbrecht, personal communication). Responses to PHA injection and antibody titers of the humoral responses were log-transformed to achieve normally distributed residuals. We used a paired t-test and a repeated measurement ANOVA to detect any sex differences, as well as differences in response strength across time to PHA injection and diphtheria and tetanus toxoids respectively, and we selected the strongest response in each case for subsequent analysis. An ANOVA was then constructed for each of the immune responses to underline any sex differences in immunocompetence. Finally, to examine whether immunocompetence could affect chick growth rates, we constructed an ANOVA for each immune response, with estimates of chick growth rate as the dependent variable, sex and

the immune response as fixed factors, and hatching date as a random factor. All statistical analyses were performed using SPSS 16 (SPSS Inc., Chicago, IL, U.S.A.)

3. Results

The response to PHA injection was highly variable between individuals and higher 24 hours after the injection, although not statistically different from the measurement recorded 6 hours post-injection (6h: mean = 1.49mm, SD = 1.45; 24h = 1.58, SD = 1.33, paired t-test: t = - 4.09, df = 103, p = 0.162; Fig 1a). The primary antibody responses for both diphtheria and tetanus were highest 21 days after the injection (diphtheria: $F_{1,3}$ = 11.391 p = 0.0001; Fig 1b; tetanus: $F_{1,3}$ = 8.847, p = 0.0001; Fig 1c). Furthermore, we did not detect any sex differences across time for the response to the PHA injection ($F_{1,1}$ = 0.842, p = 0.361) nor for the responses to diphtheria ($F_{1,3}$ = 0.678, p = 0.587) and tetanus ($F_{1,3}$ = 1.659, p = 0.244).

No significant correlation was found between the PHA response and responses to either diphtheria or tetanus ($p > 0.05$). However, we found a moderate positive correlation between the strength PHA injection response 6 h and 24 h post injection (r = 0.233, p = 0.009, N = 103), as well as a positive correlation between the humoral responses to diphtheria and tetanus (r = 0.574, p = 0.001, N = 43). For subsequent analyses, we used the strongest response to PHA injection (24 h post-injection) and the humoral response (21 days post-injection). We did not find any effect of sex on the strength of the responses for the three immune assays (response to PHA injection: $F_{1,103}$ = 0.04, p = 0.848; diphtheria response: $F_{1,42}$ = 1.53, p = 0.230; tetanus response: $F_{1,42}$ = 0.42, p = 0.526; Fig 1.).

However, we found a negative effect of hatching date on the response to PHA injection ($F_{1,103}$ = 4.82 , p = 0.011; Fig 2a), diphtheria and tetanus responses ($F_{1,42}$ = 16.87, p = 0.001; and $F_{1,42}$ = 34.75, p = 0.001, respectively; Fig 2b). Furthermore, chicks sampled for the diphtheria- tetanus injection in late autumn / winter had significantly lower pre-

injection antibody titres compared to chicks sampled in summer / early autumn (diphtheria: $F_{1,32} = 28, 573$, $p = 0.001$, $N = 33$; tetanus: $F_{1,32} = 55.36$, $p = 0.001$).

The analysis of chick growth rates did not reveal any sex differences ($F_{1,1} = 2.40$, $p = 0.124$).

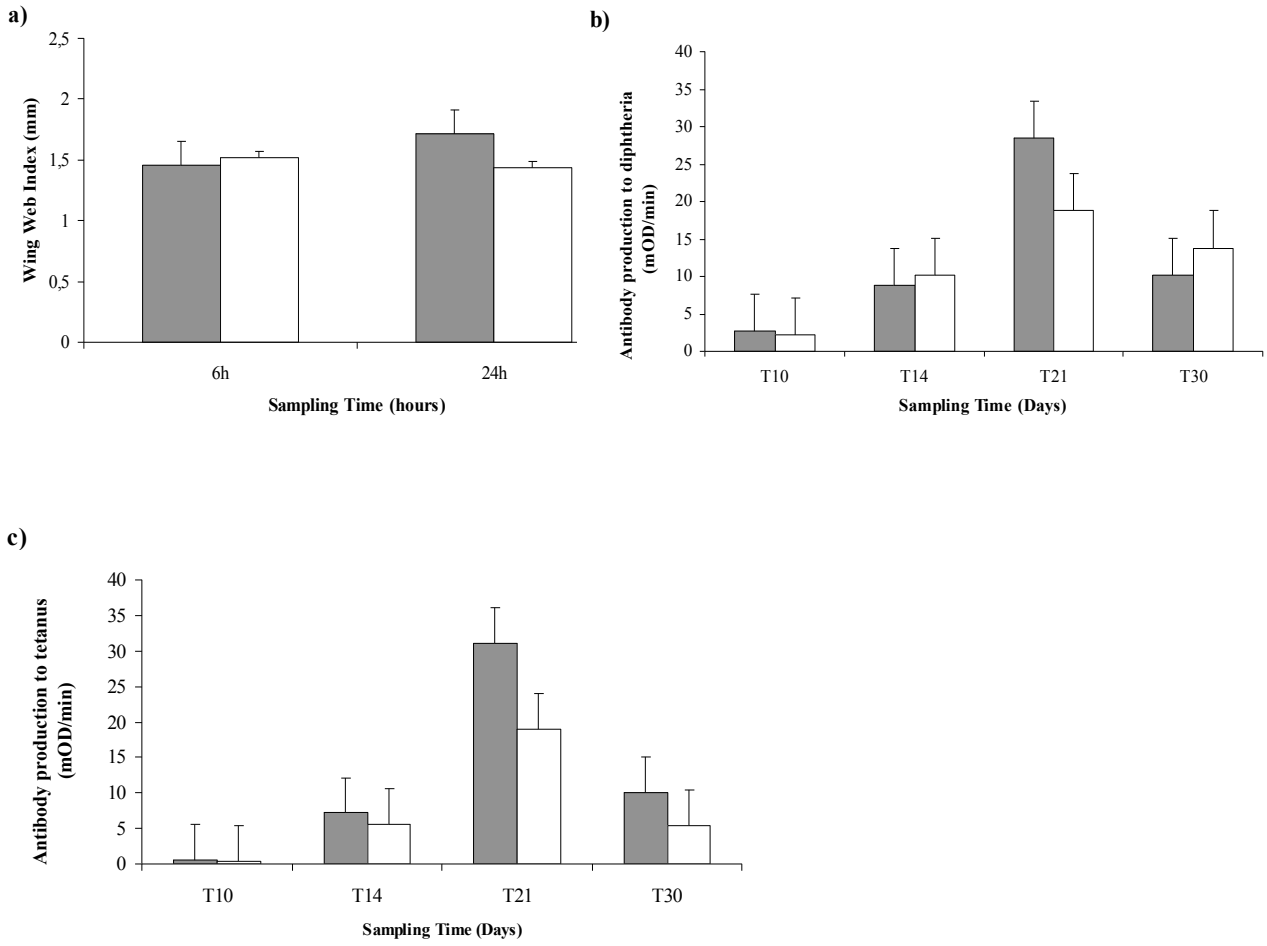

Fig 1. (a) PHA responses in 104 ostrich chicks (54 males and 50 females); humoral responses against (b) diphtheria and (c) tetanus in 43 ostrich chicks (18 males and 25 females) using a diphtheria-tetanus vaccine injection. Grey bars indicate males; white bars: females. (a) swelling measured 6h and 24h post injection; (b) and (c) antibody production measured 10 (T10), 14 (T14), 21 (T21) and 30(T30) days after a first injection with the vaccine.

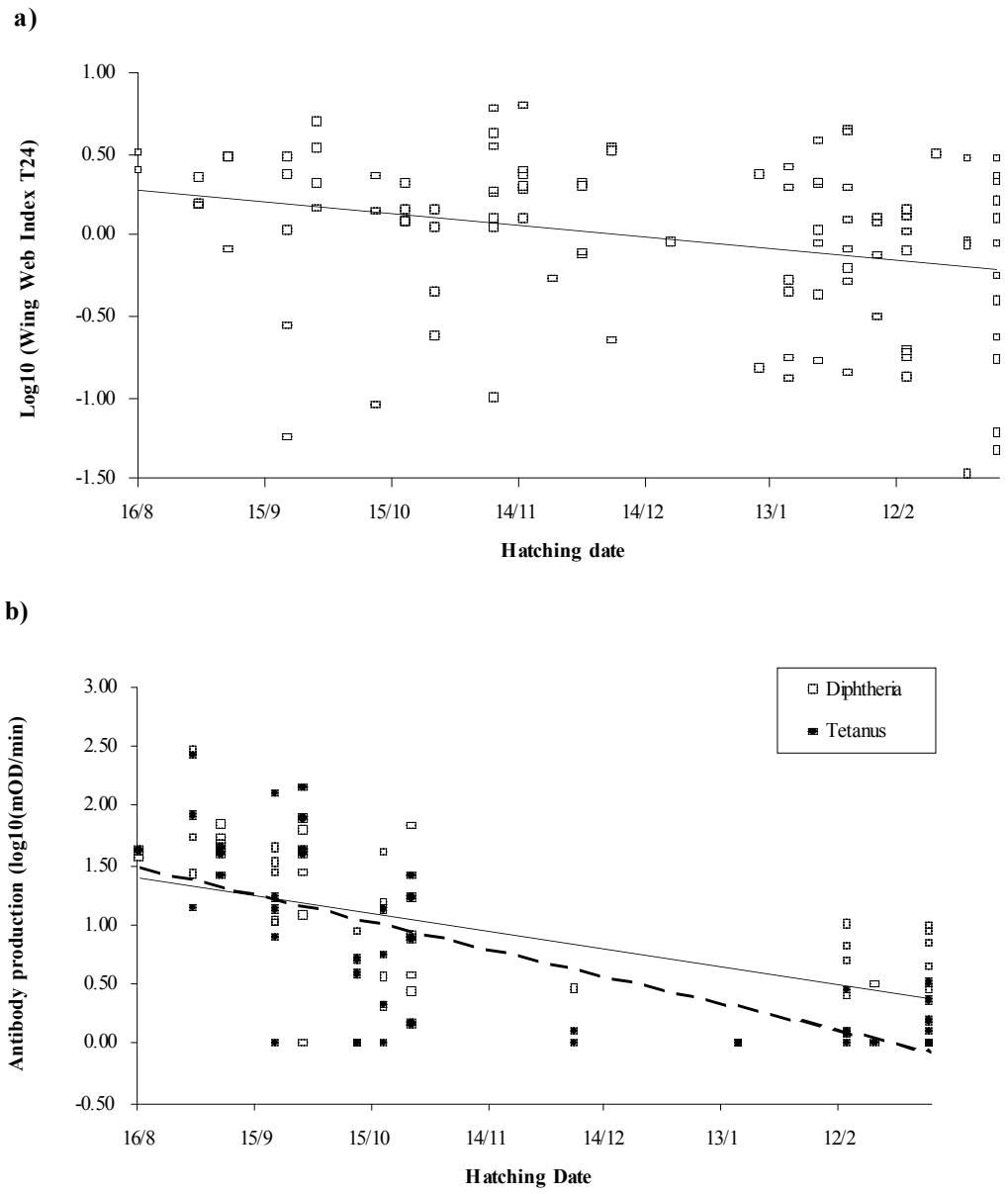

Fig 2. The relationship between immune responses and hatching date: a) Response to PHA injection; b) Humoral response to diphtheria-tetanus vaccine: solid line indicates the relationship with diphtheria toxoid; the dashed line with tetanus toxoid.

When analyzing potential relationships between the ability to raise an immune response and the variation observed in growth rates after controlling for hatching date, we found that the response to PHA injection was unrelated to growth rate estimates ($F_{1,74} = 1.475$, $p = 0.657$). However, we found that chicks with higher growth rate estimates had intermediate responses for both diphtheria and tetanus ($F_{1,42} = 6.79$, $p = 0.018$; and $F_{1,42} = 5.25$, $p = 0.009$ respectively; Fig. 3).

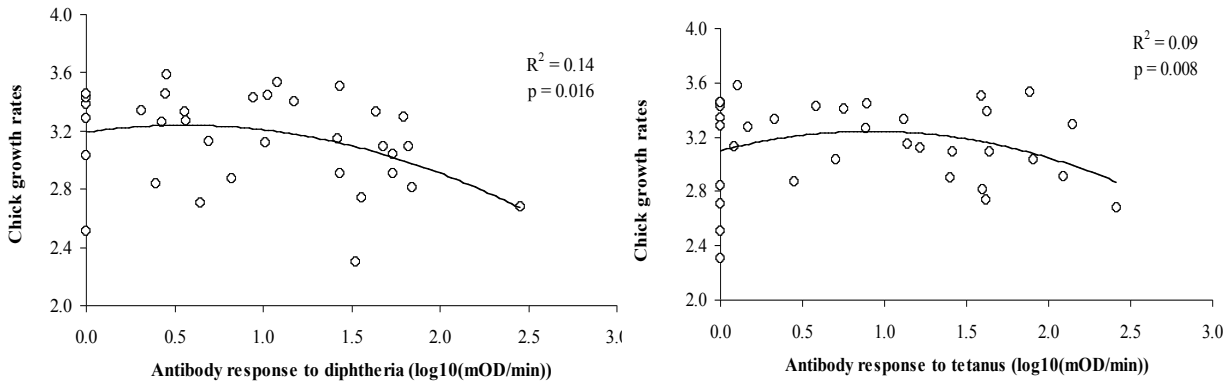

Fig 3. The relationship between the humoral response to a diphtheria-tetanus vaccine injection and growth rates of 43 ostrich chicks. Growth rates were derived from estimates of live weight for individual chicks and their parents using an animal model (for details see Material and Methods section).

Furthermore, we did not detect any effect of the interaction between sex and immune responses for both the responses to PHA injection ($F_{1,1} = 0.780$, $p = 0.543$) and humoral responses (diphtheria: $F_{1,1} = 0.380$, $p = 0.774$; tetanus: $F_{1,1} = 1.243$, $p = 0.307$).

4. Discussion

Our findings revealed that only humoral responses showed a significant relationship with chick growth. Chicks with intermediate responses for both primary responses to diphtheria and tetanus antigens had higher growth estimates, but we did not find any effect of the response to PHA injection on chick growth estimates.

Viney at al (2005) have pointed out that as optimal immune responses are context specific, they are not necessarily those of the greatest magnitude. The patterns shown in Fig. 3 of growth rates as a function of the primary responses of diphtheria and tetanus suggest that this component of defence may be under stabilizing selection because it has both fitness benefits and costs, and that variation in the humoral response relates to variation in how individuals trade off these costs and benefits. Råberg, and Sterjnman

(2003) investigated survival rates of offspring as a function of humoral immune defence. They demonstrated that in blue tits, *Parus caeruleus*, the primary response to diphtheria was also subject to stabilizing selection (whereas the secondary response to tetanus was subject to positive directional selection). They did not find any significant selection on either primary response to tetanus or secondary response to diphtheria. By contrast, we measured only primary immune responses to both antigens, as the disturbance caused to the chicks did not allow additional measurements.

Non-pathogenic antigen challenges performed in other bird species have confirmed a trade-off between growth and immunity (Klasing et al. 1987; Fair et al. 1999; Whitaker and Fair 2002; Hasselquist and Nilsson pers.comm.). Beyond a threshold level, variation in chick growth rates observed in ostriches could be explained by a trade-off between energetic investment in the humoral response as opposed to growth. In addition, very high immune responses may result in immunopathology (Råberg et al. 1998) with potentially severe consequences for birds, including reduced growth (Hanssen et al. 2008).

By contrast, PHA responses did not show any relationship with chick growth. Studies on blue tits, *Parus caeruleus* (Brommer 2004), and magpies, *Pica pica* (Soler et al. 2003), also demonstrated that there was no relationship between PHA responses and growth rates. Furthermore, previous studies show that PHA responses are often more affected by body condition or rearing environment (Birkhead et al. 1999; Lifjeld et al. 2002; Kilpimaa et al. 2005) than antibody responses (Råberg et al. 2003). However, most of these studies have investigated the relationship between growth and cell-mediated responses when food availability and/or food quality varies. Supplementary feeding of methionine, an amino acid known to directly affect T-cell production (Grimble and Grimble 1998) in blue tit and magpie nestlings led to an increase in cell-mediated response, but reduced growth rates compared to control nestlings (Soler et al. 2003; Brommer 2004). However, Alonso-Alvarez and Tella (2001) highlighted that the relationship between changes in dietary

proteins in food intake and cell-mediated response may not be linear. They found that captive gulls appear to reach a threshold above which the increase in food intake did not enhance the cell-mediated response. In our study, as chicks were provided ad libitum with food, a potential trade-off between responses to PHA injection and growth estimates might have been masked. The cell-mediated response might play a significant role during the first few weeks of age, as chicks are prone to injuries through interactions with counterparts in the form of aggressive pecking (Lambert et al. 1995).

The finding that both PHA and humoral responses were lower in chicks hatching at the end of the breeding season might suggest that parents invest less effort in producing later eggs and offspring compared with earlier breeding attempts, which in turn might lead to reduced immunocompetence in offspring which hatch later in the season (Gasparini et al. 2001). Life-history theory predicts that reproductive investment should decrease during the season because the residual reproductive value of offspring commonly decreases (Stearns 1992). However, this is unlikely to have occurred in our study as females were found to lay heavier eggs at the end of the breeding season (see Chapter two).

So it is more likely that in ostriches females anticipate harsher environmental conditions for offspring hatched later in the breeding season by allocating more resources to them, as compared to offspring hatched earlier in the season. Maternal investment in eggs, particularly of nutrients and energy, can have a profound influence on the development of embryos and survival of hatchlings. The ability of mothers to transmit antibodies to their offspring has been documented in birds (for a review Grindstaff et al. 2003). Even though maternal effects generally have their greatest impact early in development and then decrease as offspring mature (Price 1998; Wolf and Brodie 1998), maternal antibodies may continue to affect offspring phenotype by influencing growth and developmental rates, as well as the strength and diversity of the immune response (Boulinier and Staszewski 2008; Hasselquist and Nilsson 2008). Whether female ostriches

invest differently in yolk immunoglobulin during the course of the breeding season remains unknown.

Another possible explanation for the trends observed related to hatching date could indicate that mothers and their early-hatching chicks (sampled during spring and summer) may have been exposed to more parasites earlier in the breeding season, when more copulations between individuals take place than later in the season, and hence had enhanced immune function ("breeding season - high exposure hypothesis": Hasselquist 2007). By contrast, late-hatching chicks (sampled during autumn and winter) may have lower immune responses (Hasselquist 2007), with a concomitant increase in risk of disease exposure when they are growing (Botes 2004). Additionally, as ostrich chicks older than 3 months were kept in outdoor camps, late-hatching chicks sampled during the winter season might have been more exposed to infections, which might account both for the lower pre-injection antibody titres observed, as well as the decrease in their immune responses.

In conclusion, this study suggests that chick growth rate variation may be explained, at least in part, by a trade-off between humoral immune response and growth. Chicks with higher growth rates had intermediate response to both diphtheria and tetanus toxoid antigens, suggesting that this component of defence is under stabilizing selection. These results could explain patterns observed in mating decisions and maternal investment in ostriches, and could be of value to the ostrich farming industry, which is plagued by high chick mortality.

CHAPTER FIVE Male ostrich (*Struthio camelus*) feather colour signals humoral immunocompetence and influences offspring growth rate

(M. Bonato, M.R. Evans, D. Hasselquist, S.W.P. Cloete & M.I. Cherry)

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ABSTRACT

The immunocompetence handicap hypothesis predicts that only high quality individuals are able to invest both in high immunocompetence and elaborate ornament expression. Such ornaments serve as indicators of male quality (or 'good genes') and could be informative for female mating decisions. The ostrich mating system is promiscuous and a remarkable feature of cohorts is that offspring may differ greatly in size. As disease may influence growth rates, we examined whether variation in levels of disease resistance (i.e., immunocompetence) in both parents, as well as secondary sexual traits of male parents, were related to chick growth. The coloration of the feathers, bill, neck and legs of male ostriches, maintained in a breeding flock, was measured using UV-visible spectrophotometry. We measured cell-mediated responses as the skin swelling induced by a phytohaemagglutinin injection; and humoral immune function by recording the antibody response to a diphtheria-tetanus vaccine. Chicks were weighed monthly to calculate growth rates; and parentage analysis was performed using microsatellite markers. We found that chick growth rate and chick humoral response were predicted by their father's response to the diphtheria vaccine and their mother's response to the tetanus vaccine, suggesting that there is a heritable component of the humoral immune system. Responses to PHA injection in chicks were not related to parental responses. The colour of white feathers in males was a strong predictor of offspring growth rates. As this trait is exposed during the courtship display, we suggest that this visual cue could serve as a signal to females of male humoral immunocompetence, whereby females might increase the fitness of their offspring.

1. Introduction

The handicap principle of sexual selection (Zahavi 1977) predicts the evolution of honest sexual signals which express condition dependency, thereby reflecting male genetic quality (Andersson 1994). Males of high genetic quality will therefore be able to express the most exaggerated sexual traits or displays, whereas males of poor genetic quality will be unable to incur the associated costs (Grafen 1990). Females prefer to mate with the most elaborately ornamented males as many secondary sexual characters demonstrate strong condition dependence, and can be produced only by males in prime condition (Andersson 1994; Iwasa & Pomiankowski 1994). Furthermore, several studies have shown that sexual dimorphic traits such as feather characteristics (length of tail feathers: Møller & Petrie 2002; plumage coloration: Gonzalez et al. 1999, Doucet et al. 2004), beak coloration (Faivre et al. 2003) and spurs (Ohlsson et al. 2002) reflect a male's ability to raise an immune response against novel antigens.

Immune function often demonstrates strong condition-dependence, with only individuals in good condition being able to produce strong immune responses. The immunocompetence handicap hypothesis (Folstad & Karter 1992) suggests that the expression of secondary sexual traits might reflect an individual's immune capacity, with only high quality individuals being able to produce extravagant secondary traits because of the trade-offs between androgens and immune capacity (Norris & Evans 2000). Consequently, females basing their mate choice decisions on ornamentation could acquire males with better resistance to parasites, resulting in either direct benefits in species with paternal care; and/or indirect genetic benefits when offspring inherit genes for superior immunocompetence (Folstad and Karter 1992; Andersson 1994; Westneat & Birkhead 1998; von Schantz et al. 1999). In accordance with this, Bonneaud et al. (2005) showed that a specific MHC allele in the house sparrow was associated with higher responses to

two different T-cell dependent antigens: phytohaemagglutinin (which is cell-mediated) and sheep red blood cells (which are mediated humorally).

In ostrich chicks kept in farmed conditions, mortality rates are highly variable and can reach 50% before 3 months of age (Verwoerd et al. 1999). Furthermore, ostriches are fast-growing birds and a remarkable feature of cohorts is that they differ greatly in size (Deeming & Ayres 1994; Deeming et al. 1993). Factors influencing growth rates in ostrich chicks are believed to include the protein concentration in the diet (Deeming 1996), group size (Deeming & Ayres 1994; Mushi et al. 1998) and disease (Deeming & Ayres 1994). In the wild, ostriches are promiscuous, with males and females having multiple partners (Bertram 1992; Kimwele & Graves 2003). They are sexually dimorphic: females have a dull-brown plumage while males have black plumage with some contrasting white feathers, as well as coloured bill and legs (Deeming 1999). Recently, females were found to be able to discriminate between males and to invest differently at the egg stage with respect to male traits (see Chapter two), and that both cell- and humoral-mediated components of their immune system are reflected by these traits (see Chapter three).

The aim of this study was to examine whether variation in parental levels of immune defence was related to offspring growth rates and immune responses, and whether male traits (specifically coloration) reflect the male's ability to raise an immune response. Because of the complexity of the immune system, stimulation of more than one component of the immune system is required to investigate such relationships adequately (Sheldon & Verhulst 1996; Norris & Evans 2000; Viney et al. 2005). Therefore, we assessed cell-mediated and humoral immunocompetence with challenge tests that have been extensively used in immunoecology studies (e.g., Hasselquist et al. 1999; Martin et al. 2006). Furthermore, avian vision differs from that of humans in several ways: birds are sensitive to the UV part of the spectrum (UV: 320-400 nm) to which human are blind (Bowmaker 1980; Bennett & Cuthill 1994); and have four cone types, rather than three as found in

humans, implying that birds have the potential for tetrachromatic vision (Chen & Goldsmith 1986; Jane & Bowmaker 1988; Bowmaker et al. 1997; Wright & Bowmaker 2001). Therefore, we used UV-visible range spectrophotometry to measure the colour of the beak, neck, feathers and legs of 15 males maintained within a breeding flock.

2. Materials and Methods

2.1. Sampling population

The study was carried out on South African black ostriches (*S. camelus var. domesticus*), maintained at a research farm in Oudtshoorn, South Africa, from August 2005 to March 2006. The breeding flock consisted of 2 groups in 8 hectare camps containing male: female individuals of 7:12 and 8:11 respectively. Eggs were collected on a daily basis, weighed and stored in electronic incubators until hatching. Chicks were then sexed through cloacal examination (Gandini & Keffen 1985), tagged and weighed with an electronic balance (Mercer) once a month, from the day of hatching to 360 days of age. All chicks were allowed to dry off for 8 to 10 hours after hatching, and subsequently transferred to an extensive chick rearing facility where they were kept in groups of 100-110 chicks. The rearing barn was divided into several stalls where chicks were distributed according to their age. During their first two weeks, chicks were maintained at a constant temperature of 25°C. Thereafter, the temperature was decreased by 1°C per week, until 2 months old when heating was stopped. Light was provided from 8am to 5pm. Food and fresh water were supplied *ad libitum*. From an age of three months, chicks were transferred to outdoor camps.

2.2. Parentage determination

As we could not assess parentage with certainty in our camp, 50ul of blood was collected from the jugular vein of both the adult birds and the day-old chicks and stored in Vacutainer™ tubes kept at 4°C until parentage analysis could be conducted.

DNA was extracted from blood samples using a standard protocol with overnight digestion with proteinase K and phenol/chloroform extraction. The amount of DNA was estimated and dilutions were made to approximately 1ng / µL.

Genotyping was determined for 6 primers selected from literature: CAU1, CAU7, CAU14, CAU17, CAU64, CAU76 (Tang et al. 2003). All Polymerase Chain Reactions (PCR) were realized in a total reaction volume of 10 µL containing 1 µL of the DNA solution, 1µM of each primer , 0.25 units of YB-*Taq* Polymerase in the manufacturer's buffer, 0.2nm of each dNTP and 1.5-3mM $MgCl_2$ (YorBio, UK). The reaction profile was 95°C for 5 min, then 95°C for 30s, then T_a for 30s, then 72°C for 30s for 35 cycles, and finally 72°C for 10 min, where T_a is the annealing temperature, adapted to obtain optimal reactions. All PCRs were performed in a Px2 Thermal Cycler (Thermo Electron Corporation, MA, USA). PCR products were diluted with 50µl of ddH20 and multiplexed into two sets: set 1 CAU1, CAU76 and CAU14; and set 2 CAU7, CAU17 and CAU64. For each set, a mixture of 1ul of the products and 9µl of loading buffer containing Genescan™ 500 LIZ™ size standard and formamide (volumes of 0.15 and 8.85 respectively) (Applied Biosystem, Foster City, CA, USA) was made and denatured for 5min at 95°C. Plates were then run in 3130xl Genetic Analyser (Applied Biosystem, Foster City, CA, USA). If the reaction failed, PCRs were re-run with modification of $MgCl_2$ concentration or annealing temperature.

Genemapper 4.0 (Applied Biosystem, Foster City, CA, USA) was used for allele scoring and the parentage assignment was conducted via CERVUS 3.0 (Kalinowski et al. 2007). This programme performs an allele frequency analysis by using exclusion and

likelihood-based approach. Because neither parent was known, CERVUS recommends a two-step analysis with the first step running the group of parents with fewer candidates (fathers in this case), and the second step to running the analysis with the mothers using the results of the first step. Both camps were analysed separately for the parentage assignment, as some adults in camp 1 and in camp 2 were related.

2.3. Weight data and estimates of growth rates

Monthly weight records were available for chicks (n = 447) that hatched in the experiment. Individual weights ranged from 0.9 kg at 30 days of age to 96 kg (mean = 2.80 kg, SE = 0.11) at 360 days of age (mean = 68.93 kg, SE = 2.38). Because of the wide range in absolute values, weight data were transformed to square roots and cube roots to stabilize the variance across ages. Being derived from repeated records on the same chicks, these data were considered as longitudinal. Growth trends for each chick were modelled by using cubic splines (Verbyla et al. 1999). The splines considered initially consisted of three components: a fixed linear component, random deviations from linearity following a smooth trend, and random deviations from linearity not conforming to a smooth trend. Linear and non-linear components of the splines were treated as interactive variables with paddock of origin, to obtain indications of potential differences in these trends between paddocks. The initial analyses involved fitting various combinations of fixed paddock effects, random spline components and interactions between them to obtain an operational model. The data were analysed by using ASREML software (Gilmour et al. 2006). Repeated records pertaining to all animals were accommodated by the addition of each animal as a single random effect in the operational model. Pedigree information obtained for all individual chicks was included in the analysis, to obtain estimates for live weight for all parents contributing progeny in both colonies. Differentiation between direct additive and permanent environmental animal effects was not attempted, as the data did not include

sufficient information to do so with any degree of certainty. Values for live weight that were derived from this repeatability model analysis for individual chicks and their parents were related to measures of immunocompetence.

2.4. Immune responses

We estimated two measures of immunocompetence: the T-cell mediated immune response and the B-cell humoral response. We first assessed the T-cell-mediated immune responses on adults and 4 month old chicks; and then the B-cell humoral immune response a month later, in order to minimise interaction between the two tests. For this experiment, we used measurements of a sub-sample of chicks (74 chicks for the cell-mediated response; 34 chicks for the humoral response) for which we had parentage determination.

Cell mediated immunity was challenged by using a phytohemagglutinin injection (PHA). Although this is a standard method of assessing cell-mediated immunity in poultry (Cheng & Lamont 1988), a recent study on house sparrows *Passer domesticus* has shown that PHA swelling is more complex than previously thought: it is correlated with cell mediated components of the immune system, but not exclusively so, as some of the swelling is attributable to other aspects of immune function, both innate and adaptive (Martin et al 2006). Our results should be interpreted in this context. Birds were inoculated sub-dermally with 0.4 mg of a PHA solution (Sigma, L-8754) dissolved in 0.04ml of Phosphate Buffered Saline (PBS) in the right wing web, and with 0.04 ml of PBS in the left wing, as a control test. We measured the swelling of the wing webs on three occasions; before the injection, 6 h and 24 h later. On each occasion we used a digital calliper and measured the swelling of the wing web three times and used the average of these measures. Repeatabilities of measurements (Lessells & Boag 1987) were 0.79 and 0.82 for the adults and 0.83 and 0.80 for the chicks, for the right and left wing respectively ($p < 0.001$). The PBS control injection was administrated despite the recommendation of

Smits et al. (1999) as we observed slight swellings at the point of injection. The wing-web swelling was calculated as the difference in thickness of the PHA-injected versus the PBS-injected wing, which indicates the strength of the response to the PHA injection.

To measure the B-cell mediated humoral response, we elicited an antibody response in the birds by injecting a solution of 0.5 ml of a Diphtheria-Tetanus vaccine (DTVAX) in their neck. Blood samples were collected before the injection, and 10, 14, 21 and 30 days post-injection. After centrifugation (4000 rpm for 10min), plasma was stored at -70°C. To assess the level of antibodies in the plasma, we then conducted an Enzyme-Linked Immunosorbent Assay, ELISA (Hasselquist et al. 1999; Råberg et al. 2003, Hanssen et al. 2008). Briefly, 96-well microtiter plates (Costar, Cambridge, Mass) were coated with either diphtheria or tetanus antigens at 4°C for 24h. After washing plates and blocking wells with 3% milk powder diluted in 0.01 M PBS/Tween 20, diluted plasma samples (see below) were added to the wells and allowed to incubate overnight at 4°C. Plates were then washed, and a rabbit-anti-ostrich Ig antiserum obtained from Prof Dirk Bellstedt (Department of Biochemistry, University of Stellenbosch, Stellenbosch, South Africa) (diluted 1:800) was added to each well. Following 1h of incubation at 37°C and a wash, peroxidase-labeled goat-anti-rabbit serum (1:2000 dilution; Sigma A6154) was added to the wells. After a secondary incubation (45 min at 37°C) and a final wash, 2,2-azino-bis-3-ethylbenzthialzoline-6-sulfanic acid (ABTS; Sigma, A1888) and peroxidise were added to the wells, and plates were then immediately transferred to a Molecular Devices V_{max} kinetic reaction ELISA reader. Plates were read every 30s for 12min using a 405nm wavelength filter. Antibody concentrations were calculated according to the slope of substrate conversion over time in units 10^{-3} x Optical Densities (OD) per minute (m_{OD} / min), with a higher slope indicating a higher titer of anti-diphtheria or anti-tetanus antibodies in the sample. Each plate also included baseline samples collected from birds before injections (negative controls). Plasma samples diluted to 1:400 (for diphtheria

antibodies) and 1:800 (for tetanus antibodies) were used in all analyses. All samples were run in duplicate (mean coefficient of variation between duplicate samples = 5.5%), and the average of the two readings was our measure of antibody levels in the plasma. Blanks with only buffer were also included on each plate. Final measures of antibody levels were expressed as the difference between baseline and post-immunization antibody titres of individuals and were log transformed to obtain a normally distributed data set and facilitate the use of parametric statistics.

2.5. Colour measurements

Reflectance spectra between 300 and 700nm were recorded using an Ocean Optics USB 2000 spectrophotometer and a PX-2 xenon lamp (Florida, USA) on 5 different traits (bill, neck skin, black feathers, white feathers and legs) on each male. As each trait appeared uniform in colour, it was measured 10 times in randomly allocated places. Reflection was recorded using a probe held normal to the surface, collecting light from a spot of 6mm in diameter. A white reference (Spectralon 99% white standard) and a dark reference for calibration were taken before measuring each individual trait.

2.6. Sample sizes and statistics

A total of 38 adults and 396 chicks were sampled. From the 38 adults genotyped, 7 to 15 alleles per locus were detected with an observed heterozygosity of 0.567 to 0.926 (Table 1, see Chapter two). The total exclusion probabilities for first and second parents in camp 1 were 0.991 and 0.994 respectively; and in camp 2 they were 0.993 and 0.996. Paternity and maternity were both assessed at the 95% confidence interval (95 and 83 assignments respectively in camp 1; 161 and 134 assignments in camp 2) and at the 80% confidence interval (81 and 93 in camp 1; 61 and 88 in camp 2). However, because of a high chick mortality (62% during the first 3 months of age), we were able to calculate the

growth rates of only 131 chicks (64 males and 67 females). We then examined any possible sex difference in chick growth rates by performing an ANOVA.

Responses to PHA injection and antibody titres of the humoral responses of parents were log-transformed to achieve normally distributed residuals. We used a paired t-test and a repeated measurement ANOVA to detect any differences in the strength of the responses across time for the cell-mediated and humoral responses respectively. An ANOVA was then constructed for each of the immune responses to investigate any sex differences in immunocompetence.

A Principal Component Analysis (PCA) was performed on the reflectance spectra for all 5 traits for each male, and reduced a number of highly correlated variables (reflectance at 2.4nm intervals) to a small number of independent variables. In reflectance spectra of natural objects, the first principal component (PC1) is usually relatively flat, in which case it describes achromatic variation or "achromatic brightness" (Endler & Théry 1996). The second and third principal component (PC2 and PC3) therefore describe variation in spectral shape and are indirectly related to hue and saturation (Endler 1990; Bennett et al. 1997; Cuthill et al. 1999).

To examine heritability of immune responses, we calculated an aggregate of parental antibody responses using a PCA for both response to PHA injection, and the diphtheria / tetanus treatments. In the latter case, four analyses were performed: using the response to diphtheria of both parents; the response to tetanus of both parents; the maternal response to tetanus and the paternal response to diphtheria; and the maternal response to diphtheria and the paternal response to tetanus. In addition, we calculated an aggregate for offspring responses to the diphtheria-tetanus vaccine. We used the first principal component obtained from each of these PCAs as a measure of parental responses to the PHA injections and humoral immune functions respectively, as well as a measure of offspring humoral response, in subsequent analyses. This first principal component explained 91.3 %

of the variation in the case of parental response to the PHA injection; 81.5% of the variation in the case of parental humoral response; and 83.4 % in the case of offspring humoral response. Parent-offspring relationships between both PHA responses and humoral responses were then estimated using general linear models, with the parental aggregate response as the independent variable; offspring response as the dependent variable; and hatching date as a fixed factor, to control for seasonal effect.

To examine the relationship between parental immunocompetence and chick growth rates, we used a General Linear Model (GLM) for each of the immune responses, with growth rate as the dependent variable, the aggregate of parental PHA responses and parental humoral responses as independent variables, and only hatching date as a fixed factor, as sex did not have any effect on chick growth rates ($p > 0.05$). We then performed a similar GLM to investigate the relationship between chick growth rates and the colour of their sire's feathers, bill, neck and legs, with chick growth rate as the dependent variable; and spectrophotometric measurements of these paternal traits entered as independent variables. All the independent variables were initially included in the model, and then dropped until the model contained only significant terms. All statistical analyses were performed using SPSS 16.

3. Results

The response to PHA injection was highly variable between individuals and higher 24 hours after the injection, although not statistically significant from the measurement recorded 6 hours post-injection (6h: mean = 2.75mm, SD = 2.26; 24h: mean = 3.38mm, SD = 3.28, paired t-test: $t = -0.20$, $df = 37$, $p = 0.85$). We did not find any effect of sex on either response (6h: $F_{1,37} = 0.44$, $p = 0.51$; 24h: $F_{1,37} = 0.268$, $p = 0.61$). The primary antibody responses for both diphtheria and tetanus were highest 21 days after the injection (diphtheria: $F_{1,3} = 216.31$ $p = 0.0001$; tetanus: $F_{1,3} = 166.69$ $p = 0.0001$). Furthermore, we

found an effect of sex, where females had higher responses than males but only to the diphtheria toxoid 21 days and 30 days after the injection (21 days: t = 2.47, p = 0.018, df = 36; 30 days: t = 2.31, p = 0.027, df = 36: Fig 1).

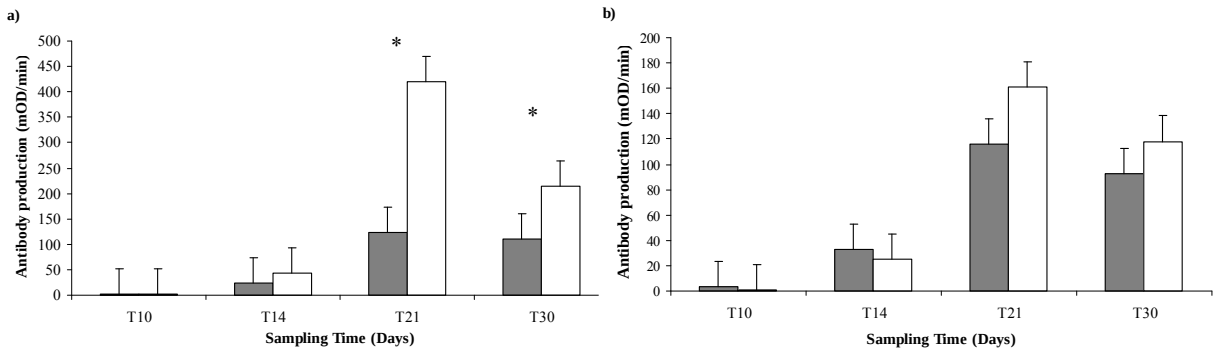

Fig 1. Humoral responses to (a) diphtheria and (b) tetanus in 38 adult ostriches (15 males and 23 females) using a diphtheria-tetanus vaccine injection. Grey bars indicate males; white bars females. Tetanus (a) and diphtheria (b) antibody production was measured 10 (T10), 14 (T14), 21 (T21) and 30(T30) days after a first injection with the vaccine. The asterisks indicate sex differences in the antibody response (P < .05).

When inter-correlations between the two immune assays were analyzed, none of them were found to be significant (p > 0.05). However, we found a positive correlation between the strength of PHA response 6 h and 24 h post injection (r = 0.62, p = 0.001, N = 38), as well as between the responses to diphtheria and tetanus after controlling for sex (r = 0.61, p = 0.001, N = 38). For subsequent analysis, we used the strongest responses to the PHA response (24 hours post-injection) and the humoral response (21 days post-injection).

We found similar results for the chicks, which were found to have a higher cell-mediated response 24 h post-injection, and 21 days post injection for the diphtheria-tetanus vaccine. No effect of sex was detected for either immune assay, allowing us to combine the data across sexes. As both PHA responses and humoral responses in chicks were found to decrease with chick hatching date, we controlled for this parameter in subsequent analyses.

We did not find any relationship between parental and offspring PHA responses ($F_{1,75}$ = 0.732, p = 0.746), or between parental and offspring responses to diphtheria ($F_{1,33}$ =

1.057, p = 0.546) or tetanus ($F_{1,33}$ = 5.097, p = 0.102). Nor was there any relationship between offspring aggregate response to diphtheria and tetanus, and the combination of their mother's response to diphtheria and their father's response to tetanus ($F_{1,33}$ = 1.143, p = 0.347). However, we found that the combination of the father's response to diphtheria and the mother's response to tetanus had a positive effect on the chick's aggregate response to diphtheria and tetanus ($F_{1,33}$ = 4.14, p = 0.042; Fig 2). Heritability, estimated as the coefficient from a linear regression of chick aggregate response to diphtheria and tetanus on the combination of paternal responses to diphtheria and the maternal responses to tetanus (Falconer & Mackay 1996) was 0.47 +/- 0.14 ($F_{1,33}$ = 3.83, p = 0.034).

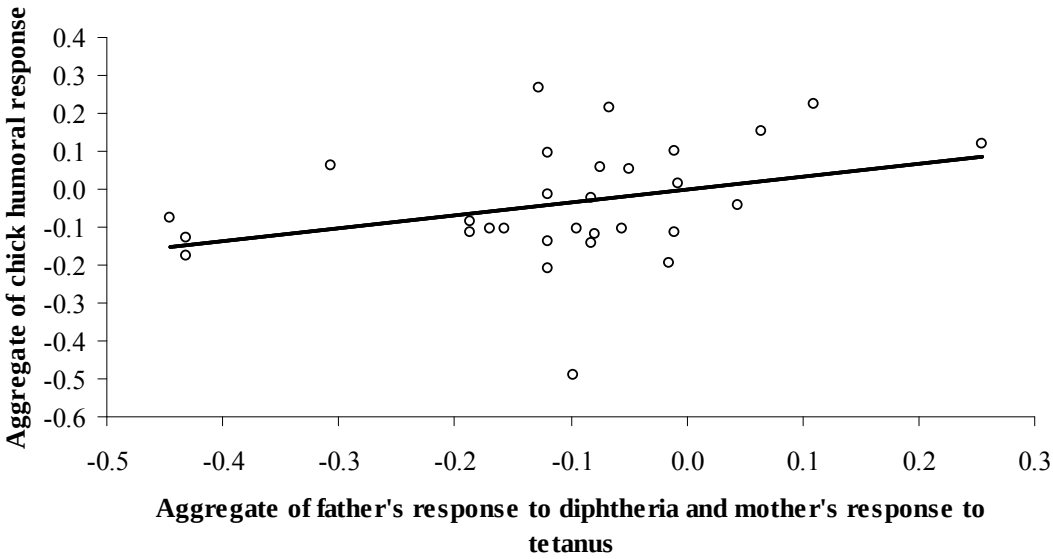

Fig. 2. The relationship between parental and offpring humoral immune responses (to a diphtheria-tetanus vaccine) in ostriches. The aggregate of parental responses was estimated using the first principal component of a PCA performed on the combination of the father's response to diphtheria (N = 15) and the mother's response to tetanus (N = 23). Offspring responses were estimated using the first principal component from a PCA of their antibody responses to diphtheria and tetanus (N = 34). Offspring from pairs with a combination of a male with a high response to diphtheria and a female with a high response to tetanus, had higher aggregate responses to diphtheria and tetanus (F1,33 = 4.14, p = 0.042).

When analyzing potential relationships between the parental ability to raise an immune response and chick growth rates, we found that the responses to PHA injection of parents did not influence growth rate estimates ($F_{1,\ 129} = 1.148$, $p = 0.12$). However, we found that chicks with higher growth estimates had a father with a higher immune response to diphtheria and a mother with a higher response to tetanus (father's response to diphtheria: $F_{1,129} = 1.709$, $p = 0.015$, Fig. 3a; mother's response to tetanus: $F_{1,129} = 2.417$, $p = 0.001$, Fig. 3b). Females with higher responses to tetanus were found to mate with males with higher responses to diphtheria ($F_{1,29} = 2.801$, $p = 0.028$, controlling for multiple mating with the same father; Fig 3c). Furthermore, Fig. 3d indicates that the combination of a chick's father's response to diphtheria and mother's response to tetanus had a positive effect on offspring growth rate, with the highest growth rates being experienced by individuals whose fathers had high responses to diphtheria and whose mothers had high responses to tetanus (GLM: $F_{1,129} = 3.509$, $p = 0.030$).

The PCA on colour measurements revealed that three principal components explained between 95.8% and 98.9% of the total variance in the five different traits measured (Table 2 see chapter two). PC1 summarized between 70.5% and 94.8% of the spectral variation in these traits, whereas PC2 and PC3 accounted for between 3.3% and 17.5%; and between 0.8% and 7.8% respectively. Only PC2, the component explaining most of the variation in the coloration of a male's white feathers, predicted chick growth rates ($F_{1,\ 130} = 1.63$, $p = 0.028$; Fig 4).

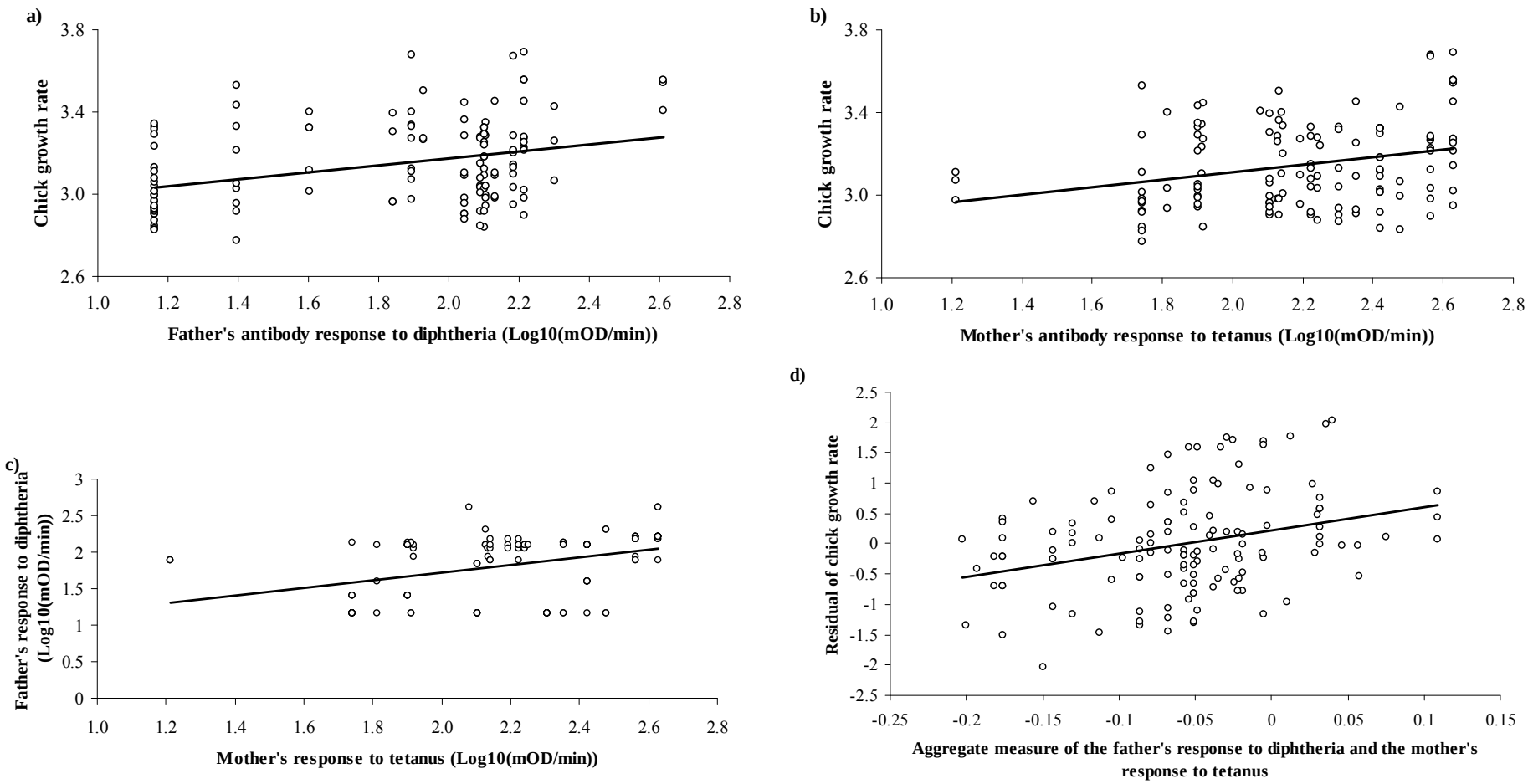

Fig 3. The relationship between aggregate parental humoral responses to a diphtheria-tetanus vaccine injection and growth rates of 131 ostrich chicks: (a) father's response to diphtheria and chick growth rates ($F_{1,130}$ = 1.709, p = 0.015); (b) mother's response to tetanus and chick growth rates ($F_{1,130}$ = 2.417, p = 0.001); (c) Mother's response to tetanus and father's response to diphtheria ($F_{1,29}$ = 2.801, p = 0.028); (d) Aggregate of parental responses and chick growth rates ($F_{1,129}$ = 3.509, p = 0.030). This aggregate was estimated using the first principal component of a PCA performed on the father's response to diphtheria and the mother's response to tetanus. Growth rates were derived from estimates of live weight for individual chicks and their parents (15 males and 23 females) using an animal model (for details see Methods section).

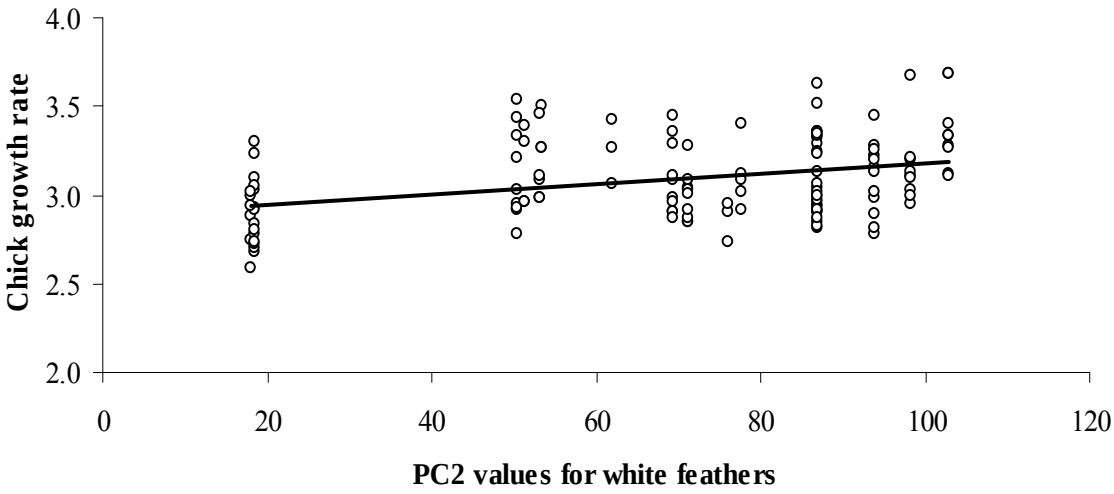

Fig 4. Chick growth rate is predicted by the colouration (reflected by PC2) of white feathers of their sires ($F_{1, 130} = 1.63$, $p = 0.028$).

4. Discussion

Our findings revealed that chick growth rates are related to the humoral response of both parents, but through antibody responses to different antigens. Chick growth rates were positively correlated to their father's response to the diphtheria toxoid and their mother's response to the tetanus toxoid. Furthermore, parental dyads combining a father with a high response to diphtheria and a mother with a high response to tetanus produced offspring with higher growth rates, and offspring with a higher humoral immunocompetence. By contrast, the relationship between chick growth rate and parental responses to PHA injection were not significant, and no relationship was found between chick and parental responses to PHA injection.

First, the finding of a relationship between different parental antibody responses and chick growth rates suggests that females seek the best paternal genes in terms of disease resistance to increase their offspring fitness. Recent studies on birds have provided evidence that females can obtain both direct and indirect benefits by choosing males with more elaborate traits (Hill 1991; Hasselquist et al. 1996; Foerster et al. 2003). The Immunocompetence Handicap Hypothesis (ICHH: Folstad & Karter 1992) suggests that the expression of secondary sexual traits might reflect an individual's immune capacity

where only high quality individuals will be able to produce extravagant secondary traits because of the trade-offs between androgens and immune capacity. Consequently, females basing their mate choice decisions on secondary sexual traits could acquire males with better resistance to parasites, resulting in either direct benefits in species with paternal care; and/or indirect genetic benefits when offspring inherit genes for superior immunocompetence (Folstad & Karter 1992; Andersson 1994; von Schantz et al. 1996, 1999; Westneat & Birkhead 1998). In accordance with this, our results showed that the colouration of the white feathers of males (as reflected by PC2 for this trait) predicts chick growth rate: chicks with higher growth rates had fathers with higher values for PC2. In addition, a positive relationship between the colouration of a male's white feathers and his response to diphtheria has been previously demonstrated in ostriches (see Chapter three). Therefore, by choosing a father of a better quality (as reflected by the colouration of his white feathers), offspring may inherit their father's humoral immune function, and consequently grow faster. In accordance with this, we found a positive relationship between the combination of parental humoral response (father's response to diphtheria and mother's response to tetanus) and chick humoral response, suggesting the heritability of this component of defence. Heritability of immune responses has also been reported to diphtheria and tetanus toxoids in blue tits (Råberg et al. 2003) and to sheep red blood cells in barn owls (Roulin et al. 2000). In blue tits, the heritability of response to diphtheria was 0.21 +/- 0.51; and that of responses to tetanus 1.21 +/- 0.40. In both cases, responses were found to profoundly affect the probability of survival to the next breeding season (Råberg & Stjernamn 2003). In ostriches, we were unable to assess survival rates in relation to these humoral responses.

Second, the previously reported observation that females invest differently according to the quality of their mate potentially reinforces our explanation for the relationship between the colouration of male white feathers and chick growth rates. Ostriches laid

heavier eggs in response to male traits, specifically those involved in the courtship display (see Chapter two). Amongst these traits, the colouration of white feathers of males predicted the mass of eggs laid by females mated to them. Even though white patches might be cheaper to produce compared to highly pigmented ornaments (Torok et al. 2003), costs associated with this trait probably arise from maintenance, as ornament expression is more sensitive to disease than that of other morphological traits (Zuk et al. 1990; Møller 1994). That white feather colouration may reflect previous antigen/disease exposure and humoral immunocompetence has also been found in female eiders (Hanssen et al. 2008). In addition, in Chapter three we found that the reflectance spectrum of the white feathers revealed a peak reflectance in the region of maximum absorbance of the bird's visual spectrum (between 405nm and 570nm: Wright and Bowmaker 2001), potentially amplifying the conspicuousness of this trait. Therefore a male's ability to display these contrasting white feathers could provide the female with valuable information on his condition (such as its ability to raise an immune response), and consequently, act as a signal which influences the degree of her investment in eggs. This is of particular interest as maternal antibodies transmitted via the egg's yolk may affect the offspring phenotype, by influencing the strength and diversity of the immune response (Lemke & Lange 1999; Hasselquist & Nilsson 2009), as well as growth and development rates (Gustafsson et al. 1994; Hasselquist and Nilsson 2009). As male white feathers appear to signal good genes that increase offspring fitness, they could be the subject of female mate preference as well as underlie the differential maternal investment observed.

Third, we found that males had lower diphtheria responses compared to females. The ICHH predicts that androgens (such as testosterone) suppress immune function through direct interactions with receptors of immune cells or through indirect routes such as elevation of stress hormone levels or increased costly behavioural activities (Owen-Ashley et al. 2004). However, studies on the effect of testosterone on the expression of sexual

ornamentation, immunity and sexual strategies have showed contrasting results across species (Roberts et al. 2004). In male ostriches, the level of testosterone increases with the level of aggression between males, which is displayed in territorial behaviour during the breeding season (Degen et al. 2004). Male aggression is characterized by forward kicking and kantling behaviour, where white feathers are directly exposed to opponents (Bertram 1992), thus emphasizing the crucial role of the white feathers. Alternatively, Hasselquist (2007) has raised the "mating season stress hypothesis" by which stress hormones (such as corticosterone) act to suppress immune function. According to this hypothesis, the sex with the highest variance in reproductive success (often males, as in the case of ostriches : Kimwele & Graves 2003; Chapter two) may suffer suppressed immune function during the breeding season, due to courtship displays, interactions with competitors and the development of secondary sexual characters (Zuk 1994; Norris & Evans 2000). Further studies comparing measurements of antibody responses, corticosterone and/or testosterone levels during and outside of the breeding season could potentially provide more insight into the driving forces behind immune responses, as well as the expression of male traits involved in the courtship display of ostriches.

We did not any relationship between parental responses to PHA injection and chick growth rates, and no relationship between offspring and parental responses. However, previous studies suggest that cell-mediated responses are often affected more by condition or rearing environment, both in chicks and in adult birds (Birkhead et al. 1999; Lifjeld et al. 2002; Kilpimaa et al. 2005) compared with antibody responses (Råberg et al. 2003). In our study, as both chick and adults were provided with *ad libitum* food, potential variations in cell-mediated responses between individuals (and particularly in males) may have been masked. Therefore, experiments varying food quality and/or quantity could potentially highlight genetic differences in the ability to raise cell-mediated responses.

Finally, parents tended to mate assortatively in relation to their ability to raise a humoral response, suggesting that males may also exert choice of females. Several studies seem to concur with this hypothesis, especially when search costs for females are low, and where females may vary in reproductive quality (Burley 1977; Johnstone et al. 1996; Kokko & Monaghan 2001). For instance, studies on male mate choice in insects have found preferences for traits that reflect female fecundity, such as body size or body mass (for a review see Bonduriansky 2001). In birds, much emphasis has been recently made on the role of female ornamentation specifically in the context of mate choice (Amundsen 2000a; Amundsen 2000b), where males were found to prefer ornamented females (Amundsen et al. 1997; Cornwallis & Birkhead 2006), and allocate sperm accordingly to particular breeding attempts (Cornwallis & Birkhead 2007). Unfortunately, we do not have any measurements on female ornamentation in our study. But as ostriches are highly promiscuous, sperm competition is likely to occur, and could potentially explain the pattern observed in terms of mating preferences in relation to immunocompetence.

Mate choice is often evoked as a means to maintain variability in the major histocompatibility complex (MHC) (for a review: Tregenza & Wedell 2000), a set of genes which triggers the immune response and immunological self-non-self recognition (Roitt 1997). Ostrich chicks with higher growth rates have been found to have intermediate responses to both diphtheria and tetanus toxoids (see Chapter four), suggesting a cost associated with the maintenance of this component of defence. In this context, MHC disassortative mating preferences could increase the resistance of the progeny to disease by increasing their heterozygosity at MHC loci (Potts & Wakeland 1990; for a review Penn 2002) and consequently enhance offspring fitness. Richardson et al (2005) found that female Seychelles warblers increase offspring MHC diversity through extra pair copulations. Although we have no knowledge of ostrich mating preferences in relation to

their MHC genes, the fact that their mating system is highly promiscuous suggests that a similar mechanism could operate in this species.

In conclusion, this study shows that the colour of white feathers in males has a strong effect on offspring growth rate and could therefore serve as a signal to females of male humoral immunocompetence. Chick growth rates were positively affected by the humoral response of both parents, but through different antibody responses: chick growth rates were positively correlated to their father's response to the diphtheria toxoid and their mother's response to the tetanus toxoid. Furthermore, the colouration of white feathers predicted chick growth rates, as well as a male's ability to raise an antibody response to the diphtheria toxoid. This suggests that this trait is condition dependent, and therefore an honest signal of individual quality. Furthermore, chick humoral responses were found to be related to their mother's response to tetanus and to their father's response to diphtheria, indicating that this component of the immune system is heritable. These results provide valuable insights into mating decisions and maternal investment in ostriches, and could be of value to the ostrich farming industry, which is plagued by high chick mortality.

CHAPTER SIX GENERAL CONCLUSIONS

This study has highlighted that in ostriches, male traits associated with visual displays (and in particular their colouration) can influence the degree of maternal investment at the egg stage (females laid heavier eggs when mating with more desirable males), as well as chick growth rate. These traits appear to advertize the ability of a male to raise an immune response. Patterns of immune defence in ostrich chicks suggest a trade-off between humoral immune responses and growth rate. In addition, chick humoral responses were found to be related to the humoral response of both parents, indicating that this component of the immune system is heritable. Within the context of ostrich farming, these results could partially explain size variations observed in cohorts of chicks, as well as high mortality rates during their first three months of age.

This study emphasizes the influence of immune defence in the evolution of life-history traits, as signals relating to immunocompetence appear to be important in female mate choice. Both sexes in promiscuous species may devote more energy to immune surveillance than in monogamous species, given the greater likelihood of disease transmission, as well as other risks (i.e. injuries) associated with mating with multiple partners.

1. Female investment and male coloration

Life-history theory predicts that females should modify their investment in a particular breeding attempt according to the likelihood of its success, as the investment of females in reproduction is typically higher than that of males. By measuring the colouration of male traits using UV-visible range spectrophotometry, I have established that variation in egg mass observed in ostriches can be directly linked to differential maternal investment relating to male attractiveness. More specifically, females tend to lay eggs of different weight in relation to the colour of the neck, white and black body feathers; and the brightness of black feathers. All these traits are involved in the courtship

display, suggesting that female ostriches assess males and invest accordingly. Moreover, there are obvious fitness benefits from producing heavier eggs as these produced heavier chicks that had higher survival to one month of age. There are also seasonal effects on egg mass, with heavier eggs being laid at the end of the breeding season. This suggests that females might anticipate harsher environmental conditions for late-hatching offspring which have to grow during the winter, and thus allocate them more resources than to offspring hatched earlier in the season.

2. Male coloration and immune function

It has been suggested that secondary sexual characters signal male ability to resist infections, as only high quality individuals are able to invest both in high immune defence and elaborate ornament expression. In investigating potential relationships between the coloration of male traits and immune responses, male responses to PHA injection and humoral responses to tetanus were related to leg coloration; humoral responses to diphtheria were related to white feather colouration; and the H:L ratio was related to bill colouration. In accordance with the multiple signal hypothesis (Møller & Pomiankowski 1993), this suggests that males use multiple signals to advertise their overall quality. In particular, the positive relationship between response to PHA injection and leg colouration, as well as between the diphtheria response and white feathers, suggest that males which are able to display more elaborate secondary sexual characters, can also continue to maintain an immune response. Such characteristics could have a profound effect on both male-male interactions and female mating decisions. Males use their legs for striking opponents, especially during the establishment of territories (Bertram 1992), and could use their colouration to assess the condition of rivals; and white feathers are amongst the traits that influence the degree of maternal investment in eggs. Furthermore, all these traits reflect in the area of maximum absorbance of the ostrich visual spectrum, and could thus potentially

act as amplifiers whereby high quality males would be able to advertise their condition optimally.

The finding that the coloration of the legs was stronger both for intermediate values of the humoral response to tetanus; and for higher responses to PHA injection indicates that there might be a cost associated with the development of this trait. Data are consistent with the view that males decrease investment in one of the components of the immune system (in this case the humoral response) to be able to fully express this trait. This suggests that this component of the immune system has not only fitness benefits but also costs, and that the expression of the leg colouration may reflect variation in how individuals trade off these costs and benefits.

3. Relationship between chick growth rate and immune function

A trade-off between immune response and life-history traits, in particular growth rate, has been documented in various bird species. Ostriches are fast-growing birds and a typical feature of cohorts is that they differ greatly in size. Chicks with higher growth rates were found to have intermediate responses to stimulation of their humoral immune system with diphtheria and tetanus vaccines, suggesting again that not only fitness benefits, but also costs are associated with mounting an immune response. Therefore, variation in humoral responses and growth rates relates to how individual chicks trade off these costs and benefits. Such a trade-off between growth and immunity is in accordance with observations of other bird species challenged with non-pathogenic antigens (Fair et al. 1999; Whitaker & Fair 2002; Hasselquist & Nilsson subm.). By contrast studies on blue tits *Parus caeruleus* (Brommer 2004) and magpies, *Pica pica* (Soler et al. 2003), suggested that there was no relationship between the response to PHA injection and growth rates. However, previous studies show that cell-mediated responses are often more affected by

body condition or rearing environment (Birkhead et al. 1999; Lifjeld et al. 2002; Kilpimaa et al. 2005) than antibody responses (Råberg et al. 2003).

Both responses to PHA injection and humoral responses in chicks decreased throughout the breeding season, suggesting a higher pressure from pathogens during winter (May to September in the southern hemisphere). This did not result from a decrease in reproductive investment during the season, as predicted by life-history theory (Stearns 1992), as the opposite effect was observed.

4. Male signals, mate choice and offspring fitness

The immunocompetence handicap hypothesis predicts that only high quality individuals are able to invest both in high immunocompetence and elaborate ornament expression. Such ornaments serve as indicators of male quality (or 'good genes') and could inform female mating decisions. As disease may influence growth rates, I examined whether chick growth was related to variation in levels of disease resistance (i.e. immunocompetence) in both parents, as well as secondary sexual traits of male parents. Interestingly, chick growth rates were found to be related to the humoral responses of both parents, but through different antibody responses. Parental dyads combining a father with a high response to diphtheria and a mother with a high response to tetanus produced offspring with higher growth rates. This suggests that mothers may seek the best paternal genes in terms of disease resistance to increase their offspring fitness. As the coloration of their fathers' white feathers predicted chick growth rates, as well as a father's ability to raise an antibody response, this visual cue could serve as a signal to females of male humoral immunocompetence, therefore forming the basis of mate choice. By choosing a father of a better quality (as reflected by the coloration of his white feathers), offspring may inherit their father's level of humoral immune function, and consequently grow faster. In accordance with this, we found a positive relationship between the same parental dyads

described previously (the father's response to diphtheria and the mother's response to tetanus) and chick humoral response, indicating that this component of defence is heritable. Heritability of immune responses has also been recorded for diphtheria and tetanus toxoids in blue tits (Råberg et al. 2003) and to sheep red blood cells in barn owls (Roulin et al. 2000).

Male adult ostriches were found to have lower diphtheria responses than females. This suggests that their immune function might be suppressed either by androgens (such as testosterone) as predicted by the ICHH (Foldstad & Karter 1992), or by stress hormones (such as corticosterone) as predicted by the "mating season stress hypothesis" (Hasselquist 2007).

I did not find any relationship between parental responses to PHA injection and chick growth rates, and between offspring and parental PHA responses. However, as previously discussed, potential variation in cell-mediated responses between individuals may have been masked as both chick and adults were provided with *ad libitum* food.

5. Future work

This study shows that there is still ample scope for further investigation of factors that influence mate choice in ostriches. Key areas that require further investigation are the influence of environmental heterogeneity (food availability) on the relationship between sexual selected traits, immunity and fitness; mutual mate choice; and the genetic basis to immunity and its influence on mating decisions.

First, it would be interesting to vary food availability and/or food quality in ostriches, to investigate in particular whether a trade-off between the cell-mediated response and growth rates exists in poor nutritional environments.

Second, an investigation of the relationship between the heritability of humoral response and chick survival could partially explain the high chick mortality observed in

ostrich farming, and could enable ostrich farmers to reduce such high mortality rates within the first three month after hatching.

Third, it would be interesting to investigate whether female ostriches invest more in yolk immunoglobulin, in response to male colouration; and/or in relation to variations in environmental conditions as demonstrated in other bird species (mallard, *Anas platyrhynchos*: Cunningham & Russell 2000; zebra finch, *Taeniopygia guttata*: Rutstein et al. 2004).

Fourth, observations on the intensity and magnitude of the kantling behaviour of males could determine the extent to which displays influence female mate choice, as well as whether males developing more colourful ornaments and higher immune responses are in addition able to perform elaborate courtship displays. The finding that adult males had lower antibody responses to diphtheria than females suggests that males might experience "mating season stress". The "mating season stress hypothesis" postulates that stress hormones (such as corticosterone) act to suppress immune function (Hasselquist 2007). Therefore, males may suffer suppressed immune function during the breeding season, due to courtship displays, interactions with competitors and the development of secondary sexual characters (Zuk 1994; Norris & Evans 2000). Further studies comparing measurements of antibody responses, corticosterone and/or testosterone levels, both during and outside the breeding season could potentially provide more insight into the driving forces behind immune responses, as well as the expression of male traits involved in the courtship display of ostriches.

Fifth, as ostrich parents appear to mate assortatively in relation to their ability to raise a humoral response, males may also engage in mate choice of females. In birds, recent work has illustrated the role of female ornamentation specifically in the context of mate choice (Amundsen 2000a; Amundsen 2000b), where males were found to prefer ornamented females (Amundsen et al. 1997; Cornwallis & Birkhead 2006), and allocate

sperm to particular breeding attempts (Cornwallis & Birkhead 2007). Mating preferences of males could be assessed both before copulation (by conducting behavioural observations) and after copulation (by measuring sperm allocation) in relation to female relative attractiveness as determined by their social status (major or minor female), plumage traits and/or body condition.

Finally, I suggest that females may seek the best combination of maternal and paternal genes in terms of disease resistance to increase their offspring fitness. Mate choice is often evoked as a means to maintain major histocompatibility complex (MHC) variability, a set of genes which triggers the immune response and immunological self-non-self recognition (Roitt 1997). MHC-based mate preferences have been recently demonstrated in female Seychelles warblers (*Acrocephalus sechellensis*), which increase offspring MHC diversity through extra pair copulations (Richardson et al. 2005). Ostrich chicks with higher growth rates have been found to have intermediate responses to both diphtheria and tetanus toxoids (see Chapter four) suggesting a cost associated with the maintenance of the humoral response. In this context, MHC disassortative mating preferences could increase the resistance of the progeny to infectious diseases by increasing their heterozygosity at MHC loci (Potts & Wakeland 1990; for a review see Penn 2002) and consequently enhance offspring fitness through higher growth rates. Although we have no knowledge of ostrich mating preferences in relation to their MHC genes, the fact that their mating system is highly promiscuous suggests that a similar mechanism could operate in this species, making it worthy of investigation.

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