CAROTENOIDS AND THE COSTS OF REPRODUCTION: STUDIES IN THE LESSER BLACK-BACKED GULL

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I declare that the work recorded in this thesis is entirely my own unless otherwise stated and that it is of my own composition. No part of this work has been submitted for any other degree.

Jonathan Blount
April 2002

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SUMMARY

1. Life history theory predicts that a reproduction is costly, and a physiological trade-off exists between investment in current and future reproduction; in one respect this could correspond to a trade-off between the allocation of resource(s) to reproduction and immune defence. However, the identity of the limiting resource(s) remains a contentious issue in evolutionary ecology. In birds, it seems possible that carotenoid pigments could be limiting; birds cannot synthesise carotenoids de novo (they must obtain them in their diet). Studies of domesticated hens have shown that carotenoids can enhance immune function, antioxidant activity, and also sperm quality, and carotenoids are also transferred from maternal circulation into egg yolk. This thesis consists of experiments designed to test whether a trade-off between reproduction and immune function is modulated by carotenoid supply, using free-living lesser black-backed gulls, *Larus fuscus*, as a study species.

2. The effects of carotenoid supply on maternal phenotype, and the implications for egg production capacity and egg phenotype were studied. It is shown that dietary carotenoid supplementation during the pre-laying period translated into increased maternal body levels of carotenoids and antioxidant activity, and lower plasma levels of immunoglobulins (Ig). In turn, carotenoid-fed females produced eggs containing high carotenoid but low Ig concentrations (i.e. passive immunity), whereas the opposite pattern was observed in controls. It is hypothesised that high yolk carotenoid levels might compensate for low levels of passive immunity in determining chick performance.

3. The role of physiological discrimination among ingested carotenoids (differential uptake, transport, deposition, or metabolic conversions) in determining yolk carotenoid profiles is examined in a carotenoid supplementation experiment. We supplementally fed pre-laying gulls with a cocktail of four carotenoids, or a carotenoid-free (control) supplement, then compared the yolk carotenoid profile and susceptibility to lipid peroxidation in eggs that they laid. In comparison with controls, there was an increase in the

yolk concentrations of seven carotenoids, and also unidentified carotenoids in eggs produced by carotenoid-supplemented females. However, the relative proportions of five classes of carotenoid did not differ significantly, and consequently the percentage profile of yolk carotenoids was positively correlated, between feeding treatments, possibly indicating metabolic transformations and differential transfer of carotenoids from maternal diet to yolk. Potential energetic costs to females, and benefits in terms of antioxidant capacity imparted to offspring, are discussed.

4. The role of carotenoid supply as a resource underlying a trade-off between chick-rearing capacity (i.e. work rate) and immune function is investigated in female gulls using a factorial experiment (carotenoid supplementation crossed with inoculation; the inoculum comprised a non-replicating antigen – sheep red blood cells (SRBC)). Experimental nests were given a control clutch of eggs produced by non-manipulated birds to incubate and rear. It is shown that females that had received an immune challenge and the control (carotenoid-free) supplement produced relatively light foster fledglings in comparison with females of the other treatments, consistent with the explanation that there was a trade-off between immune function and chick-rearing capacity which was mediated by carotenoid supply.

5. The role of carotenoid supply as a resource underlying a trade-off between egg production capacity and immune function is investigated in female gulls using a factorial experiment (carotenoid supplementation crossed with SRBC inoculation). Following removal of first clutches, it is shown that carotenoid-fed females had an increased egg production capacity (re-laying rate, clutch size), and produced eggs of higher quality than controls (higher yolk carotenoid levels; larger immune responses and higher survival in chicks reared singly in foster nests). However, there were no effects of maternal SRBC inoculation on any of these measures of egg production or egg quality. It is suggested that females facing an immune challenge maintained their level of investment in egg production, even at the expense of their own condition (chick-rearing capacity; see point 4, above), suggesting that egg quality may ultimately be more

important than parental condition during the rearing period in determining reproductive success.

6. The fertility of males sometimes correlates with their ornamental display, but a mechanistic explanation to universally link these traits has been lacking. I hypothesise that both sperm quality (fertility; structural integrity of DNA), and the substrates responsible for male ornamentation, may be vulnerable to free radical attack, which can be mitigated by antioxidants. I hypothesise that a link between ornamentation and sperm quality could arise if antioxidants are in limited supply, and the showiest males may be preferred because they are most likely to be fertile, or to provide sperm with undamaged genotypes that could give rise to fit offspring. To test these ideas I supplementally fed male gulls at the nest with carotenoids during the pre-laying period, then measured male carotenoid-based integument coloration, and the number of sperm that successfully penetrated the inner perivitelline membrane in a standardised area of yolk in first-laid eggs. Compared to controls, carotenoid-fed males had higher coloration indices. Sperm count indices did not differ significantly between feeding treatments, possibly due to small sample sizes, but were correlated positively with male coloration indices. The possibility that sperm quality is limited by carotenoid supply, and alternative explanations for these results, are discussed.

Chapter 1

GENERAL INTRODUCTION

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INTRODUCTION

Physiological trade-offs and life history evolution

The primary focus of this thesis is on resource-limitation as a mechanism underlying costs of reproduction in birds. Life-history theory posits that reproduction is costly, and thus a physiological trade-off should exist between the allocation of resources to current and future reproduction (Williams 1966; Stearns 1992). This could correspond in one respect to a trade-off between current reproduction and immune function (Gustafsson *et al.* 1994; Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000). This is because effective immune function may increase an individual's prospects for survival and hence future reproduction, but maintaining the immune system may incur costs that manifest as reduced reproductive capacity. Several studies have reported a positive correlation between reproductive effort and susceptibility to parasitism (e.g. Festa-Bianchet 1989; Gustafsson *et al.* 1994; Richner *et al.* 1995; Allander 1997; reviewed by Møller 1997), and more recent work has confirmed that high investment in reproduction can result in reduced immune function or vice versa (Deerenberg *et al.* 1997; Nordling *et al.* 1998; Ilmonen *et al.* 2000; Råberg *et al.* 2000). A major challenge that remains is to identify the physiological resources and mechanisms that underlie such interactions between reproduction and immune function (Sheldon & Verhulst 1996; Råberg *et al.* 1998; Westneat & Birkhead 1998; Norris & Evans 2000).

The simplest explanation for trade-offs is that individuals have finite resources, which they must divide among competing demands (Stearns 1992). Birds have proven to be useful models for studying resource-limitation of reproduction, because each reproductive attempt can be considered in terms of distinct stages (e.g. egg production, chick-rearing) that can be manipulated independently to elucidate their

relative costs (e.g. egg removal; brood enlargement), and manipulations of parental condition and supplies of specific resources can be performed (e.g. inoculation; dietary supplementation). For example, egg-removal experiments have revealed that females can incur considerable costs solely through egg production, including increased parasitism (Oppliger *et al.* 1996), reduced chick-rearing capacity (Heaney & Monaghan 1995; Monaghan *et al.* 1998) reduced future return rates and fecundity (Nager *et al.* 2001). Other studies using a brood enlargement protocol have shown that, independently of egg production costs, increased chick-rearing effort can translate into reduced immune responses following experimental inoculation (Deerenberg *et al.* 1997; Nordling *et al.* 1998), and reduced over-winter survival (Daan *et al.* 1996). Similarly, it has been shown that experimental inoculation with a benign antigen can result in reduced parental investment in chick-rearing (Ilmonen *et al.* 2000; Råberg *et al.* 2000). These studies suggest a reciprocal, resource-based trade-off. Could there be a single type of 'resource' that is potentially limiting for different aspects of reproduction and also immune function?

Maternal effects

An influence of parental phenotype on phenotypic variation among his / her offspring, independent of the offspring's genotype, is termed a 'maternal effect'. For example, life history theory predicts that there should be a trade-off between the number and quality of offspring (Stearns 1992). Consistent with that prediction, experimental enlargement of avian clutch size (by egg removal) has recently been shown to result in reduced egg quality, measured in terms of declining egg lipid content, and chick survival, with increasing egg number (Nager *et al.* 2000). The quality of eggs produced can profoundly influence fitness-related traits in both parent

and resultant offspring, but the mechanisms responsible for egg quality maternal effects in birds and other taxa are poorly understood (reviews by Bernardo 1996; Mousseau & Fox 1998). Several experiments have shown that fitness-related traits in offspring (hatchability, growth, survival) correlate positively with egg size, independently of the quality of the rearing environment (Amundsen & Stokland 1990; Bolton 1991; Reid & Boersma 1990; Amundsen *et al.* 1996; Styrsky *et al.* 1999, 2000; reviewed by Williams 1994). It has been hypothesised that yolk concentrations of hormones (e.g. Schwabl *et al.* 1997), passive immunity (Heeb *et al.* 1998) and antioxidants (Royle *et al.* 1999) are important currencies for egg quality maternal effects in wild birds (reviews by Williams 1994; Bernardo 1996; Blount *et al.* 2000). For example, variation in passive immunity (immunoglobulins) deposited into egg yolk has been linked to maternal exposure to parasites (Heeb *et al.* 1998; Gasparini *et al.* 2001), and variation in testosterone deposition into yolk has been linked to photoperiod in the pre-laying period (Schwabl 1996). However, little is known of the mechanisms by which costs of reproduction faced by females translate into variation in egg quality.

Maternal effects can encompass effects of paternal phenotype on phenotypic variation in offspring, too. Some recent evidence from studies of humans suggests that there are environmental influences on sperm quality, in terms of the structural integrity of the DNA within the sperm nucleus (i.e. the genome), which has consequences for offspring phenotype (see below). However, this kind of maternal effect has not been studied in wild birds.

Limitation of which resource(s)?

It has traditionally been assumed that energy limitation underlies physiological trade-offs between life history activities (Stearns 1992). However, empirical evidence relating to energetic limitation of reproduction and immune function has been equivocal (reviewed by Råberg *et al.* 1998; Norris & Evans 2000). For example, dietary supplementation with 'energy-rich' foods (e.g. animal fat; sunflower seeds) during the pre-laying period has variously been reported to either increase or have no effect on egg production (e.g. Bolton *et al.* 1992, 1993; Nager *et al.* 1997). Similarly, studies of domestic hens using multiple measure of immune function have shown that some but not other aspects of immune function were limited by energy supply, with no clear consensus between studies (Glick *et al.* 1981, 1983; reviewed by Norris & Evans 2000). Although it is known from studies of humans and laboratory animals that immune sensitisation can demand large increases in energy turnover (Lochmiller & Deerenberg 2000), experimental inoculation of tits, *Parus* species, with non-replicating antigens has variously been reported to have no effect on basal metabolic rate (Svensson *et al.* 1998), or to reduce basal metabolic rate (Ots *et al.* 2001).

Recent studies have emphasised the possibility that supplies of specific nutrients, rather than energy alone, could be limiting for reproduction and immune function. Several studies have shown that supplementation with 'protein-rich' foods (baked hen eggs; white fish) resulted in enhanced egg production capacity (e.g. Hiom *et al.* 1991; Bolton *et al.* 1992, 1993; Ramsay & Houston 1997; Clifford & Anderson 2001; reviewed by Martin 1987). However, such studies that provided whole foods rather than specific nutrients cannot conclude with certainty which constituent was responsible for the effects observed. Only one study has shown that supplemental

feeding with a specific class of nutrients, synthetic amino acids, resulted in increased egg production capacity (Ramsay & Houston 1998). As for energy supplies (see above), studies of captive birds have failed to find any simple relationship between protein supply and immune function, the effects observed being contingent on the particular branch of immune function being measured and showing no consistency among studies (Glick *et al.* 1983; Tsiagbe *et al.* 1987; Lochmiller *et al.* 1993; Gonzalez *et al.* 1999).

One possible explanation for the lack of consensus among studies concerning interactions between nutritional state, reproduction and immune function is that energy supply *per se* is not the key factor. Instead, perhaps energy turnover is the mechanism underlying life history trade-offs, because a fast metabolic rate incurs the production of harmful free radicals (Svensson *et al.* 1998). Free radicals are highly reactive atoms or molecules that are generated from normal oxidative metabolism and from inhalation or ingestion of environmental pollutants. Free radicals have unpaired electrons, and, in seeking to pair with other electrons, can damage DNA, proteins and lipids (reviewed by Stahl & Sies 1999). An individuals' capacity to benefit from a high turnover of energy could therefore be constrained by its ability to neutralise the resultant free radicals, using antioxidants such as carotenoid pigments (Svensson *et al.* 1998).

Much scope exists for investigations of the role of specific micronutrients as resources underlying variation in egg quality (Bernardo 1996) and immune function in birds (Norris & Evans 2000). One group of compounds that is potentially very important in this context is carotenoids (Lozano 1994; von Schantz *et al.* 1999; Blount *et al.* 2000), but the role of carotenoids in shaping the costs of reproduction has been little studied.

Carotenoids: biochemistry and biological activity

Carotenoids are a group of more than 600 lipid-soluble compounds with pigmenting and antioxidant properties. Carotenoids are synthesised *de novo* only by certain bacteria, fungi and higher plants, where they are involved in the light-harvesting system and in antioxidant defence against photooxidation (Goodwin 1984; Stahl & Sies 1999). In contrast, all animals must obtain carotenoids in their diet, although a limited range of subsequent metabolic transformations of carotenoids are possible (Goodwin 1984; Brush 1990; Stahl & Sies 1999). The chemical structure of carotenoids comprises a backbone of conjugated double bonds, which may be cyclized at one or both ends of the molecule. Carotenoids that consist only of carbon and hydrogen atoms are called carotenes (e.g. α-carotene, β-carotene, lycopene; Figure 1.1a). However, most carotenoids include at least one oxygen function, and are collectively called xanthophylls (e.g. lutein, zeaxanthin, canthaxanthin; Figure 1.1b).

a) b)

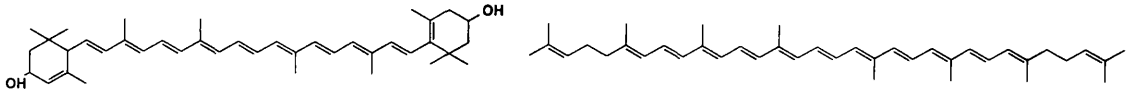

Figure 1.1: Chemical structures of carotenoids. a), Lycopene, a carotene. b), Lutein, a xanthophyll.

It has long been known that certain carotenoids possess provitamin A activity (Green & Mellanby 1930). Animals are not capable of synthesising vitamin A,

which is an essential nutrient for the development of vision, growth, immune function, skin and mucosa (reviewed by Lotan 1980). However, recent interest in the biological activity of carotenoids has focussed mainly on their role as antioxidants. As antioxidants, carotenoids can inactivate free radicals and thereby protect the structural integrity of DNA, proteins and lipids. The degree to which carotenoids exhibit antioxidant activity is related to their chemical structure, a larger number of conjugated double bonds conferring higher antioxidant capacity (Stahl & Sies 1999).

Carotenoids and immune function

Through antioxidant activity, carotenoids maintain membrane receptors that are essential for immune function. But carotenoids can also enhance immune function by specifically influencing the responses of cells involved in the generation of an immune response. Carotenoids have been shown to enhance T and B lymphocyte proliferation, stimulate effector T lymphocyte function and the production of cytokines and interleukins (reviewed by Bendich 1989; Chew 1993, 1996; Stahl & Sies 1999). Carotenoids can also indirectly influence immune function via several pathways. During the inflammatory stage of an immune response, macrophages and neutrophils produce free radicals as a weapon to destroy pathogens, but this mechanism can inadvertently damage host cells too (Chew 1996). Thus, effective antioxidant protection can also indirectly enhance the efficiency of immune responses by preventing over accumulation of free radicals in the vicinity of immune cells (Chew 1996). Studies using human and animal cells have identified a gene, connexin 43, the expression of which is dependent on carotenoids, that is responsible for intercellular signalling (Bertram 1999). Carotenoids can also repair (recycle)

each other, and other antioxidants including vitamins A, C and E, from the post-radical trapping state (Böhm *et al.* 1997; Mortensen & Skibstead 1997; Edge *et al.* 1998).

Carotenoid supply has been implicated in the efficiency of immune function and the etiology of many diseases in humans, laboratory mammals, domestic hens and fish species (reviewed by Bendich 1989; Chew 1993; Olson & Owens 1998; Stahl & Sies 1999; Møller *et al.* 2000). Evidence relating to birds comes from studies of domestic hens, where high dietary intake of carotenoids has been shown to result in reduced liver susceptibility to free radical attack (Woodall *et al.* 1996) and increased antibody titres following experimental challenge with Newcastle Disease Virus (McWhinney *et al.* 1989), and to prevent retarded growth following infection with *Escherichia coli* (Tengerdy *et al.* 1990).

Carotenoids and egg quality

Avian egg yolk is coloured yellowish-red by carotenoids. Our understanding of the biochemical basis and consequences of carotenoid deposition into yolk comes largely from studies of domestic hens. The yolk of avian eggs consists of lipid-rich droplets and dense proteinaceous granules that provide nutrients for the developing embryo (Speake *et al.* 1998; Vleck & Bucher 1998). Yolk derived fatty acids also serve specific functions in developing tissues; for example, to ensure optimal development and function of the brain and retina (Neuringer *et al.* 1988). Studies of several species of birds in captivity and the wild have shown that yolk lipids are highly unsaturated (Royle *et al.* 1999; Speake *et al.* 1999a,b; Surai *et al.* 2000; Surai *et al.* 2001a). Embryo tissues depend on this substrate of oxidizable, unsaturated fatty acids, but their abundance makes the tissues highly susceptible to free radical

attack (De Mann 1992). The risk of free radical attack is likely to be promoted by the high rates of oxidative metabolism displayed by various embryonic tissues (Noble & Cochi 1990), and is likely to increase as embryo development proceeds because of the accelerating rate of oxygen diffusion through the shell to fuel metabolism (Freeman & Vince 1974). Hatchlings may be especially vulnerable to oxidative stress, because the emergent chick becomes exposed to atmospheric concentrations of oxygen and undergoes a further dramatic increase in metabolic rate with the onset of pulmonary respiration and post-hatching growth (Freeman & Vince 1974; Vleck & Bucher 1998).

Carotenoid pigments are transferred from maternal circulation into yolk during oocyte maturation, and ultimately are distributed to the developing tissues of the embryo via the yolk sac membrane and the circulation (Surai & Speake 1998). Females fed carotenoid supplements deposit increased levels of carotenoids into their eggs, and consequently produce chicks with elevated tissue reserves of carotenoids (Haq & Bailey 1996; Surai & Speake 1998). Patterns of carotenoid mobilisation, transfer and deposition during embryogenesis and chick development suggest a particular role for carotenoids during hatching and the neonatal period. During the last few days of embryogenesis large amounts of carotenoids are transferred from the yolk to the embryo, where they are mainly stored in the liver (Surai & Speak 1998), and then rapidly and extensively mobilised into circulation during the neonatal period (Haq & Bailey 1996). Moreover, studies of domestic hens and wild bird species have shown that individual carotenoids are selectively transferred from the yolk to specific tissues in the chick, suggesting that they serve localised roles in offspring development (Surai *et al.* 2001b). In vitro studies of captive domestic hens have shown that lymphocyte proliferation in the spleen and bursa correlates

positively with yolk carotenoid levels (Haq *et al.* 1996), and tissue susceptibility to lipid peroxidation correlates negatively with yolk carotenoid levels (Lawler & O'Brien 1995; Surai & Speake 1998). Thus, studies of domesticated bird species in captivity have shown that maternally derived carotenoids can provide an important defence against oxidative stress in offspring, with consequences for the development of immune function.

Carotenoids and sperm quality

Studies of humans and domesticated animals have revealed that vertebrate spermatozoa are rich in highly polyunsaturated fatty acids, and display high rates of oxidative metabolism. These traits render sperm susceptible to free radical attack, which can result in reduced male fertility (e.g. Wishart 1984; Aitken *et al.* 1989; Liu *et al.* 1997). Apart from damaging sperm function, free radicals can also attack the DNA within the sperm nucleus translating into infertility or increased disease susceptibility in offspring (Ji *et al.* 1997; Roberts 1998).

Sperm viability is influenced by the antioxidant capacity in the gametes and surrounding seminal plasma. In domestic hens, increased dietary consumption of vitamins C or E has been shown to result in increased concentrations of these antioxidants in semen, reduced susceptibility to free radical attack (e.g. Surai *et al.* 1997), and increased fertility (e.g. Friedrichsen *et al.* 1980). Similarly, absence of a carotenoid (lutein) from the diet has been shown to result in reduced male fertility (Ferrand & Bohren 1948).

Carotenoids and oxidative stress

Oxidative stress occurs when free radical production exceeds the ability of antioxidants to inactivate them. If not under control, changes in membrane properties (fluidity, flexibility) and functions (intercellular signalling, enzymatic activities) arising through oxidative stress can result in impaired physiological systems, including brain function (Neuringer *et al.* 1988; Carney *et al.* 1991) and immune function (reviewed by Chew 1996). It has been suggested that oxidative stress is likely to be most prominent in individuals exhibiting high rates of oxidative metabolism arising through activities including physical exercise, rapid growth or immune system activation (Ji 1995; Vleck & Bucher 1998; von Schantz *et al.* 1999; Sen 2001). Consequently, investment in immune function or reproduction could in theory increase an individual's risk of oxidative stress (Lozano 1994; von Schantz *et al.* 1999). Similarly, with respect to females in particular, it seems plausible that carotenoid deposition into egg yolk could increase maternal susceptibility to oxidative stress if the supply of carotenoids is limited (Blount *et al.* 2000).

Could carotenoid supply be limiting in wild birds?

Carotenoids are commonly responsible for integument pigmentation in birds, often based on reds or yellows, although blues and greens can also be produced in carotenoid-protein complexes (Goodwin 1984; Latscha 1990). Many studies of birds and fish have shown that brighter carotenoid pigmentation is preferred during mate choice (female choice: e.g. Endler 1983; Hill 1990; Milinski & Bakker 1990; Zuk *et al.* 1990; Houde & Torio 1992; male choice: Burley & Coopersmith 1987; Hill 1993; Amundsen & Forsgren 2001; reviewed by Olson & Owens 1998; Møller *et al.* 2000). Since animals must ultimately obtain carotenoids in their diet (see above), it

therefore seems plausible that carotenoids could be limiting to wild animals. However, the mechanism by which carotenoid limitation could arise remains a contentious subject in evolutionary ecology (Olson & Owens 1998; Hill 1999).

It was originally proposed that carotenoid supply could be limiting due to environmental scarcity of these compounds; by this suggestion, individuals with brighter carotenoid pigmentation could reveal their superior foraging success (Endler 1980; Kodric-Brown 1985; Hill 1990). Subsequently, it was suggested that carotenoid limitation of ornament expression could be caused by parasites (Milinski & Bakker 1990; Houde & Torio 1992; Zuk 1992). It is known that infestation with coccidial gut parasites can impair the absorption of carotenoids in the intestinal mucosa (e.g. Ruff *et al.* 1974), and ectoparasites can inhibit the expression of carotenoids in integument (Houde & Torio 1992). Alternatively, it has most recently been suggested that carotenoid supply could be limiting because carotenoids are required for maintaining immune function and antioxidant defences (Lozano 1994). Carotenoids are widely distributed and probably abundant in the food of most animals (Goodwin 1984; Latscha 1990), but carotenoids could be limiting if the particular types of carotenoids required for physiological functions were not the same as the types of carotenoids supplied in the diet (Hill 1996). For example, carotenes are the most abundant class of carotenoid in nature, but birds preferentially accumulate xanthophylls in their tissues (Latscha 1990; Goodwin 1984). Since metabolic transformations of carotenoids are limited in scope, far less than completely efficient (Fox & Hopkins 1966; Fox *et al.* 1967), and expend energy (Brush 1990), this provides another possible route by which carotenoid supply could be limiting (Hill 1996).

Studies have shown that individuals with higher carotenoid intake develop more pigmented ornaments (e.g. Hill 1992; Grether *et al.* 1999), but it seems unlikely that foraging efficiency could explain all inter-individual variation in carotenoid pigmentation. There is also a considerable amount of evidence that the brightness of carotenoid pigmentation correlates with indices of parasitism (e.g. Milinski & Bakker 1990; Houde & Torio 1992; Skarstein & Folstad 1996; Zuk *et al.* 1990; Thompson *et al.* 1997), and immune function in birds and fish (e.g. Skarstein & Folstad 1996; Zuk *et al.* 1995, 1998; Saino *et al.* 1999; reviewed by Olson & Owens 1998; Møller *et al.* 2000). Parasite-induced activation of the immune system can reduce circulating carotenoid levels; studies of domestic hens have shown that carotenoids may become oxidised by free radicals produced during an inflammatory response (Allen 1997). There is a limited amount of evidence to suggest that that body carotenoid levels decline following immune system activation in wild birds. Saino *et al.* (2000) showed that carotenoid-based gape coloration declined in barn swallow chicks, *Hirundo rustica*, following injection with a benign antigen. Thus, evidence that carotenoid supply is limiting for antioxidant protection or immune function in wild birds is mostly indirect, coming from observational studies. I am not aware of any study that experimentally manipulated carotenoid supplies directly (e.g. through dietary supplementation) and measured the consequences for the size of an immune response or the ability to neutralise free radicals.

Rather than being in limited supply, could carotenoids be toxic to animals? By this suggestion, individuals with brighter carotenoid pigmentation could be advertising their superior ability to cope with the handicap of toxic carotenoids (Zahavi & Zahavi 1997). There is some in vitro evidence that carotenoids can exert prooxidant activity above threshold concentrations, and in conditions of high partial

oxygen pressure (Burton & Ingold 1984), and some in vivo evidence of increased risk of lung cancer in human smokers receiving carotenoid supplementation (Mayne 1996). However, potential physiologically harmful effects of carotenoids in wild animals have not been studied in any context (Olson & Owens 1998).

There is only relatively weak evidence to suggest that carotenoid supply could be limiting for egg production in wild birds. Studies have reported a decline in carotenoid-based integument pigmentation in wild female birds at the time of egg production (Burley *et al.* 1992; Negro *et al.* 1998). In domestic hens, such a decline occurs only in conditions of low dietary carotenoid supply, and reflects a mobilisation of endogenous stores of carotenoids (integument, body fat) for deposition into yolk (Klasing 1998). Also, yolk carotenoid concentrations have been shown to decline over the laying sequence in normal-sized clutches of three eggs in lesser black-backed gulls, *Larus fuscus* (Royle *et al.* 1999). In gulls, third-laid eggs give rise to chicks with lower survival prospects than eggs laid earlier in the sequence (Parsons 1975), which has been suggested to possibly reflect their relatively poor antioxidant protection (Royle *et al.* 1999).

Comparisons of yolk carotenoid levels in free-range and captive domestic geese suggested that birds foraging freely deposit far higher amounts of carotenoids into their eggs than do their counterparts in captivity (mean ± s.e: free-range, 37.30 ± 3.20 $\mu g\ g^{-1}$ yolk; captive 2.10 ± 0.20 $\mu g\ g^{-1}$ yolk) (Speake *et al.* 1999b). It is not clear whether these differences indicate that carotenoid supply is not limiting in nature, or an inadequacy of the captive diet, or a relatively high requirement for carotenoids in wild as compared to captive birds. Recent observational studies of yolk carotenoid levels in wild birds have revealed a high degree of variation both within and between species (Table 1.1). The causes and consequences of such

variation for life history differentiation are poorly understood. Since no data are available on the amounts of carotenoids consumed in natural diets, and no experimental manipulations of carotenoid supply have been conducted in any wild bird species, it remains unclear whether carotenoid supply is limiting for egg production capacity in nature.

As for egg production, there is no direct evidence that carotenoid supply is limiting for parental work rate, or male fertility in wild birds. However, there is some evidence in birds and fish of correlations between male fertility and the expression of ornamental traits, both carotenoid-based traits (e.g. Liljedal *et al.* 1999) and other traits that could potentially be influenced by antioxidant supply (display rate, ornament size; von Schantz *et al.* 1999) (e.g. Mjelstad 1991; Matthews *et al.* 1997; reviewed by Sheldon 1994).

Table 1.1: Concentrations of carotenoids in the egg yolk of wild birds

species	total carotenoids $\mu g\ g^{-1}$ yolk mean (S.D.) n	coefficient of variation (%)	reference
emperor penguin, *Aptenodytes forsteri*	8.60 (*) 6	–	Speake *et al.* (1999a)
lesser black-backed gull, *Larus fuscus*	71.60 (29.06) 20	40.59	Surai *et al.* (2001b)
common moorhen, *Gallinula chloropus*	47.50 (21.80) 10	45.90	Surai *et al.* (2001b)
American coot, *Fulica americana*	131.00 (18.96) 10	14.47	Surai *et al.* (2001b)
northern gannet, *Morus bassanus*	17.70 (3.70) 4	20.90	Surai *et al.* (2001a)
great skua, *Catharacta skua*	12.70 (6.30) 5	49.61	Surai *et al.* (2001a)
American white pelican, *Pelecanus erythrorhynchos*	150.90 (67.80) 8	44.93	Surai *et al.* (2001a)
double-crested cormorant, *Phalacrocorax auritus*	115.70 (79.80) 21	68.97	Surai *et al.* (2001a)
Canada goose, *Branta canadensis*	22.10 (3.14) 5	14.21	Speake *et al.* (1999b)

* value not given in primary reference

Aims of thesis

This thesis aims to investigate experimentally the role of carotenoids as a resource underlying costs of reproduction in birds. If carotenoid supply is limiting, I hypothesise that parents should face a trade-off in the allocation of carotenoids to offspring production (egg production, sperm production, chick-rearing) and to somatic maintenance (immune function, antioxidant protection).

The chapters that follow describe experiments where costs of reproduction were studied in free-living lesser black-backed gulls, *Larus fuscus*, by supplemental feeding with carotenoids and by inoculation with sheep red blood cells (a non-replicating antigen). The effects of such manipulations on maternal and paternal phenotype, egg production and quality, chick-rearing capacity, and sperm quality are examined. It is predicted that 1) increased carotenoid supply (through supplemental feeding) should result in increased reproductive performance; 2) there should be a trade-off between investment in immune function and reproduction (invoked by immune challenge); and 3) this trade-off should be uncoupled by increased carotenoid supply.

Study species and site

The lesser black-backed gull has been used extensively in studies of the costs of reproduction, particularly the costs of egg production, being remarkably tolerant of disturbance and experimental manipulations during breeding, including daily nest visits, supplemental feeding, capture, blood sampling and egg manipulations (e.g. Hiom *et al.* 1991; Bolton 1991; Bolton *et al.* 1992; Monaghan *et al.* 1998; Nager *et al.* 2000a,b; 2001). Moreover, it has been hypothesised that piscivorous birds should have a very high requirement for antioxidants, because of the risk of oxidative stress

Chapter plan

Whether maternal carotenoid supply is limiting for her egg production capacity has not previously been studied in any wild bird species. ***Chapter 2*** describes the effects of dietary carotenoid supplementation during the pre-laying period on maternal phenotype (integument pigmentation, plasma concentrations of carotenoids, antioxidant activity and Ig), egg production, and egg quality measured in terms of yolk concentrations of carotenoids, Ig and antioxidant activity, and within-clutch patterns of these resources.

Little is known about whether yolk carotenoid profiles in wild birds are simply a reflection of diet, or reflect mechanisms of physiological discrimination (differential uptake / transport / deposition, metabolic transformation). Physiological discrimination could represent a cost of egg production. In ***Chapter 3*** I describe an experiment where gulls were provided with a cocktail of four carotenoids as a supplemental food during the pre-laying period, and the differential carotenoid profile of the eggs that they laid was measured. To help elucidate whether the experimental treatment invoked a physiologically normal response, within the ranges of natural variation, I measured whether carotenoid supplementation resulted in reduced yolk susceptibility to lipid peroxidation.

The suggestion that a trade-off between parental chick-rearing capacity and immune function could be shaped by antioxidant capacity has not been studied explicitly. In ***Chapter 4*** I present an experiment where maternal condition was manipulated by carotenoid supplementation and by inoculation, and the capacity to rear a control (foster) brood of young was measured.

Similarly, there has been no previous investigation of the possibility that a trade-off between egg production capacity and immune function is mediated by antioxidant

supply. ***Chapter 5*** describes an experiment where maternal condition was manipulated by carotenoid supplementation and by inoculation, and the quality of the eggs produced by such females was measured in terms of composition or viability. Measures of viability were performed by fostering eggs to control (non-manipulated) breeding pairs, and then measuring the performance (hatchability, growth, immune function and survival) of resultant chicks.

The effects of carotenoid supply on sperm quality have not been studied previously in wild birds. In ***Chapter 6*** I describe the effects of carotenoid supplementation on phenotypic variation in male gulls, measured in terms of carotenoid pigmentation of integument and ejaculate quality, as measured by counting the number of sperm that successfully penetrated the inner perivitelline membrane of eggs.

Chapter 7 consists of a general discussion of the work embodied in this thesis.

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Chapter 2

CAROTENOIDS AND EGG QUALITY IN THE LESSER BLACK-BACKED GULL *LARUS FUSCUS*: A SUPPLEMENTAL FEEDING STUDY OF MATERNAL EFFECTS

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INTRODUCTION

Parental condition at the time of offspring production can have long-lasting effects on phenotypic variation in offspring (reviewed by Bernardo 1996a; Mousseau & Fox 1998). Such 'maternal effects', where offspring phenotype is influenced by parental condition rather than genetic inheritance, have presumably evolved to enable parents to translate their environmental experience into adaptive variation in their offspring (Bernardo 1996a; Mousseau & Fox 1998). Maternal effects are thought to be widespread and potentially of great significance in shaping life history evolution (e.g. as in collared flycatchers, *Ficedula albicollis*; Schluter & Gustafsson 1993), but their underlying proximate mechanisms are poorly understood (Bernardo 1996a).

Egg quality is an important maternal effect; it is a phenotype of, and can profoundly influence fitness in, both mother and offspring (Bernardo 1996b; Mousseau & Fox 1998). In birds (like other oviparous animals), females must invest all the resources required for embryonic development in one self-contained package. The causal mechanisms, and defining features of a good egg are still poorly understood (Bernardo 1996b). Several studies have shown that hatchability, growth and survival of offspring correlate positively with egg size, and egg size correlates positively with maternal condition, but also many studies did not find such relationships (reviewed by Williams 1994). Recently, it has been shown that specific egg components including hormones (e.g. Schwabl 1993) and lipids (Nager *et al.* 2000) can influence fitness-related traits in offspring, independently of egg size.

Carotenoids have been hypothesised to be responsible for egg quality maternal effects in wild birds (Royle *et al.* 1999; Blount *et al.* 2000). Carotenoids are lipid-

soluble hydrocarbons that are synthesised only by photosynthetic plants and bacteria; all animals must obtain them through their diet (Goodwin 1984). Carotenoids are widely used by animals as red and yellow pigments (recently reviewed by Møller *et al.* 2000), but can also act as antioxidants and immunostimulants (for biochemical and immunological reviews see for example Chew 1996; Stahl & Sies 1999). In domestic hens dietary carotenoids are deposited into yolk where they reduce the susceptibility of embryonic tissues to free radical attack (Surai & Speake 1998), and enhance hatchling immune function (McWhinney *et al.* 1989; Haq *et al.* 1996). Free radicals are highly unstable atoms or molecules with unpaired electrons, which arise as metabolic by-products. Free radicals seek to pair with other electrons and in doing so damage other molecules. If not under control, free radical induced changes in membrane properties (fluidity, flexibility) and functions (intercellular signalling, enzymatic activities) can result in impaired immune function (Chew 1996). It has been hypothesised that bird embryos and hatchlings are particularly in need of antioxidants because their rapid metabolism incurs high rates of free radical production, and their tissues are rich in unsaturated lipids that are susceptible to free radical attack (Surai *et al.* 2001). Female birds also deposit immunoglobulins (Ig) into yolk to provide offspring with passive immunity (Kowalczyk *et al.* 1985). Ig is the class of glycoproteins to which antibody belongs (Roitt *et al.* 1998). But the possibility that any influence of carotenoids on maternal immune function, thereby modulating levels of Ig in circulation, has consequences for the passive immunity imparted to offspring has not been studied in any species.

It has been hypothesised that carotenoids are a scarce, limiting resource to wild animals, such that their availability can constrain the expression of antioxidant

activity, immune function and also integument pigmentation (Lozano 1994; von Schantz *et al.* 1999). However, we are not aware of any study that has experimentally tested whether the availability of carotenoids in wild birds might influence the levels of antioxidant activity and Ig in the eggs that they produce. Here, we carry out such a test in a supplemental feeding study of lesser black-backed gulls, *Larus fuscus*. We hypothesised that body carotenoid levels (plasma, integument pigmentation) would be higher in carotenoid supplemented females, and consequently, such females would have enhanced antioxidant protection and immune function, which would be reflected in the composition of their eggs.

MATERIALS AND METHODS

a) *Site and supplemental feeding*

Data were collected at Walney Island, northwest England, where about 24 000 pairs of lesser black-backed gulls breed (Monaghan *et al.* 1998). In mid-April, shortly after arrival of the birds at the colony and ca. 4 weeks before laying started, we randomly allocated breeding pairs in a central part of the colony to a feeding treatment. Thirty-seven nests were given 2 mg total carotenoids in 20 g solid vegetable fat (Van den Bergh Foods Ltd., Crawley, UK), and 37 other nests were given an equal amount of fat (control group), daily. Previous studies have shown that daily supplementation with a considerably larger amount of fat (120 g) does not enhance female condition or egg size in this species (Bolton *et al.* 1992). We used vegetable fat because it facilitates the gut absorption of carotenoids (Klasing 1998; Stahl & Sies 1999). To minimise the risk of theft by non-target birds, food was delivered at night and placed inside a length of PVC pipe (4.5cm diameter, 8 cm deep) that had been buried vertically in the ground, level with the surface and next to the nest, at the start of the experiment.

The carotenoid dose placed daily at each nest equated to twice the amount of carotenoids in an average first-laid egg (= 0.98 mg; PFS unpublished). No data are available on the total carotenoid intake of any wild bird species, although high consumption does not cause toxicity in domestic hens (Klasing 1998). Dietary grade carotenoids were mixed in approximately the same ratio as they occur in gull eggs at the Walney colony (40% yellow xanthophylls, 25% red xanthophylls and 35% β-carotene; PFS unpubl. data). Thus, on a daily basis each nest was given 40 mg Oro Glo Layer™ (containing 1.8% lutein and 0.2% zeaxanthin; Kemin Europa NV,

Herentals, Belgium), 5 mg Carophyll Red™ (10% canthaxanthin; Hoffmann-La Roche, Basel, Switzerland), and 7 mg Rovimix™ (10% β-carotene; Hoffmann-La Roche). Supplements were prepared daily. Fat was heated at 150°C until molten, left to cool until viscous (12-14°C; at which point carotenoids were added), then hardened at –20°C for 2-3 h before delivery to the gulls.

The proportion of supplementally fed nests that yielded eggs did not differ significantly between treatment groups (controls, 34 of 37 nests; carotenoid-fed, 35 of 37 nests; Yates corrected chi-square test, $\chi^2 = 0.01$, $p = 0.936$). In relation to nests that yielded eggs, the date on which supplemental feeding began did not differ significantly between treatment groups (controls, 17.21 ± 0.50 April (mean ± s.e.); carotenoid-fed, 17.97 ± 0.74 April (mean ± 1 s.e.); Mann–Whitney test, $z = 0.261$, $p = 0.794$), nor the period of supplementation prior to laying (controls, 27.76 ± 1.01 d; carotenoid-fed, 28.66 ± 1.42 d; Mann–Whitney test, $z = 0.403$, $p = 0.687$). Hence, control and carotenoid-fed birds did not differ in their timing of laying (Mann-Whitney test, $z = 1.037$, $p = 0.300$). Supplemental feeding continued daily throughout laying.

b) ***Measurement of maternal phenotype***

We measured aspects of maternal phenotype for a sample of supplementally fed nests that had yielded a 3-egg clutch (see below). We attempted to catch all such females at the nest using a walk-in trap within one day of clutch completion; we were able to catch 6 controls and 10 carotenoid-fed females. The yellow colour of the bill and tarsus was measured by visual comparison with a Roche Yolk Colour Fan (RYCF; Hoffman-LaRoche), whereas the orange-red colour of the bill spot, gape

flange and orbital ring was measured using a Dulux Trade Colour Palette (DTCP; Dulux, Slough, UK). These objectively defined colour standards comprise consecutive steps in unique combinations of hue (colour in the colloquial sense, i.e. red, blue, etc.), value (brightness) and chroma (degree of saturation with hue). Observed RYCF scores ranged from 8-15. We numbered consecutive steps in the DTCP that characterised the colours found in our study population (scores ranged from 1-11). Birds were caught by MLT, and all measurements were made by JDB who did not know the treatment group of origin. Measurements were made indoors in indirect natural light and based on the lateral right-hand aspect of each integument trait. Carotenoid pigmentation reflects predominantly in the visible part of the spectrum, and under standardised conditions visual colour measurements have been shown to correlate strongly with scores obtained by spectrophotometry (Hill 1998). Immediately after colour measurements, 0.5 ml of blood was collected from the tarsal vein into a heparin-rinsed syringe (under Home Office license), centrifuged at 14 000 g for 5 min, and plasma stored at –20°C until analysis (see below).

c) *Measurement of egg phenotype*

Three is the modal clutch size in lesser black-backed gulls (e.g. Bolton *et al.* 1992). We collected a random sample of 3-egg clutches for analyses of yolk composition (7 control clutches; 11 from the carotenoid-fed group). We had planned to collect 12 such clutches from each feeding treatment; our sample sizes are smaller and unbalanced because of predation of eggs. Remaining eggs were used in different experiments, the results of which will be published elsewhere. Eggs were collected on the day of laying, replaced with dummies, weighed (± 0.1 g) using an electronic

balance and measured (length and breadth, ± 0.1 mm) using a sliding calliper. Yolk was separated from albumen with a domestic egg separator sieve, then rolled on damp filter paper to remove remaining traces of albumen, weighed (± 0.1 g), homogenised and stored at –20°C until analysis (see below). Clutches sampled for yolk composition analysis were produced during the first half of the laying period (because 3-egg clutches are produced mostly by early layers), and therefore all experimental birds were of relatively high quality. In respect to total clutch volume (equation in Bolton *et al.* 1992), an estimate of maternal investment in the clutch, the subset of clutches sampled for yolk composition analysis did not differ from the remaining 3-egg clutches either in control (*t*–test, $t_{16} = 1.478, p = 0.159$) or carotenoid-fed groups ($t_{25} = 0.783, p = 0.441$). Hereafter we refer to first-laid eggs as a-eggs, second-laid eggs as b-eggs, etc.

d) *Biochemical assays*

Total carotenoid concentrations in female plasma and yolk were determined using HPLC as described in Surai and Speake (1998), using mobile phases of acetonitrile/methanol (85:15) and acetonitrile/dichloromethane/methanol (70:20:10) in gradient elution (see Granado *et al.* 1998). Detection was by absorbence at 445nm. Total carotenoids are reported as μg ml^{-1} plasma or μg g^{-1} yolk.

Antioxidant activity was measured in samples of plasma and a-egg yolk using a decolorization assay (Re *et al.* 1999). Assays were performed on the same yolk lipid-phase used for measurement of carotenoids. The assay measures the rate at which a pre-formed radical monocation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) is quenched by antioxidants in the test sample. Trolox was

used as a standard (Hoffmann-La Roche). Results are expressed as μmol of Trolox equivalent per ml or g of test sample.

e) *Immunological assays*

Total Ig concentrations in blood plasma and yolk were determined using a single radial immunodiffusion assay (Roitt *et al.* 1998). Sheep anti-lesser black-backed gull Ig was produced for us at the Scottish Antibody Production Unit (Carluke, UK) by serially injecting a sheep with gull Ig (obtained by precipitation from yolk). Sheep plasma, containing antibody against gull Ig, was collected and stored at –20°C until use as antiserum. Antiserum was mixed into a preparation of 2 % agar in barbitone buffer at a ratio of 1:14 (v/v) at 56°C, 3 ml of which was poured evenly onto microscope slides and allowed to set. Circular wells (4 mm diameter) were punched into the agar at an equal spacing, and 10 μl of test antigen was added (samples of egg yolk were first diluted 1:1 w/v in PBS). Plates were left for 72 h for the antigen to diffuse out of the wells and bind with the antiserum, precipitating in a ring. Ring diameter was measured using a magnifying lens and sliding calliper (to the nearest 0.02 mm); $(\text{ring diameter})^2$ is proportional to the antigen (i.e. gull Ig) concentration (Roitt *et al.* 1998). Unknowns were determined by interpolation from a standard curve based on six serial dilutions of a pool of non-test samples of gull plasma in PBS.

Samples from control and carotenoid-fed females were allocated among slides at random. Each test sample was included on two slides for calculation of repeatability (Lessells and Boag 1997). Ring diameter was measured two times in different directions. There was significant repeatability between mean ring diameters of the

same test antigen on two different slides (yolk: $r = 0.536$, $F_{53,54} = 3.312$, $p < 0.001$; plasma: $r = 0.707$, $F_{15,16} = 5.810$, $p < 0.001$). Means from the two assays were used in subsequent analyses.

f) *Data analyses*

Principal component analysis (PCA) was used to generate an index of female integument coloration from the five colour measurements made on each bird. Variation in egg mass, yolk carotenoid and Ig concentrations was assessed using repeated measures analysis of covariance (rmANCOVA) with laying sequence (a-, b-, c-egg) as a within-subjects variable, feeding treatment as a between-subjects factor, and female coloration index as a covariate. We used female coloration index as a covariate to represent body carotenoid levels in general, since plasma carotenoid concentrations may reflect relatively ephemeral patterns, as affected by the time since the last meal for example. Within-subjects effects were evaluated according to the multivariate approach; tests based on Pillai's Trace, Wilks' Lambda, Hotelling's Trace and Roy's Largest Root always gave identical F-values. Significant laying sequence effects were followed by post-hoc contrasts (a- versus b-egg; b- versus c-egg; Bonferroni adjusted alpha = 0.025). Where necessary, data were $\log_{10}$-transformed before analysis. Data for Ig indices in yolk remained heteroscedastic after transformation. We only present results of the analysis based on untransformed data, because ANOVA is robust for heteroscedasticity if results are statistically significant (Ito 1980). Information about egg size together with yolk concentrations of resources may directly reflect resource availability to offspring only if yolk size varies proportionately (i.e. isometrically) with egg size. To compare differences in

yolk mass relative to egg mass between treatment groups we tested the allometric relationship between clutch mean yolk mass and egg mass using $\log_{10}$:$\log_{10}$ regression (see for example Williams 1994), including feeding treatment and female coloration index as factors (i.e. ANCOVA). In all models *p*-values correspond to type III sums of squares. Models were developed using backwards elimination starting with the highest order interaction. Other statistical tests are introduced in the text of the Results (two-tailed alpha = 5%). Values are reported as means ± 1 s.e.

RESULTS

a) *Carotenoid effects on maternal phenotype*

Carotenoid supplementation did not affect the frequency of 3-egg clutches (controls, 24 of 34 females; carotenoid-fed, 29 of 35 females; Yates corrected chi-square test, $\chi^2 = 0.06$, $p = 0.799$). The five integument colour measurements made on each bird were significantly intercorrelated (all $p < 0.015$). In a PCA the first factor explained 59.65 % of the variance in female coloration, with a large positive loading on colour scores for gape, orbital ring, bill and leg (eigenvectors of 0.87, 0.84, 0.81 and 0.71, respectively), and a negative loading on bill spot colour (-0.60). Only the first factor accounted for variance greater than 1 (i.e. eigenvalue > 1), and first factor scores were extracted and used as a female coloration index. Carotenoid-fed females had significantly higher coloration indices (Figure 2.1a), plasma carotenoid concentrations (Figure 2.1b) and antioxidant activity (Figure 2.1c), but significantly lower plasma Ig indices compared with control females (Figure 2.1d).

b) *Maternal effects on egg phenotype*

Egg mass declined over the laying sequence in a similar manner in both feeding treatments, and did not differ significantly between control and carotenoid-fed groups (Figure 2.2a and Table 2.1). Yolk mass increased proportionately with egg mass, and this isometric relationship did not differ significantly between control and carotenoid-fed groups (ANCOVA, feeding treatment, $F_{1,15} = 0.002$, $p = 0.966$; female coloration index, $F_{1,12} = 0.02$, $p = 0.884$; $\log_{10}$(clutch mean egg mass), $F_{1,16} = 36.72$, p

< 0.0001; all interactions, N.S.; $\log_{10}$(clutch mean yolk mass) = –0.622 (± 0.311) + 1.003 (± 0.165) $\log_{10}$(clutch mean egg mass); p(slope = 1) > 0.5).

Yolk carotenoid concentrations declined over the laying sequence in a similar manner in both feeding treatments, but were significantly higher in eggs laid by carotenoid-fed females (Figure 2.2b and Table 2.1). This within-clutch pattern varied according to female integument pigmentation (Table 2.1): in more brightly coloured females the decline in carotenoids between the last two eggs was smaller than in dull females (correlation of female coloration index with $\log_{10}$(difference in carotenoid concentration between b- and c-egg): r = –0.522, n = 16, p = 0.038). The concentration of carotenoids in maternal circulation correlated with the mean concentration of carotenoids in the clutch (r = 0.598, n = 16, p = 0.014).

Yolk Ig indices were significantly higher in a- compared to b- and c-eggs, and this within-clutch pattern did not differ significantly among feeding treatments (Figure 2.2c and Table 2.1). But eggs produced by carotenoid-fed females contained significantly lower concentrations of Ig compared with controls (Figure 2.2c and Table 2.1). Antioxidant activity in a-egg yolk did not differ significantly among feeding treatments (controls, 0.59 ± 0.04 μmol g^{-1} yolk; carotenoid-fed, 0.65 ± 0.05 μmol g^{-1} yolk; ANCOVA: feeding treatment, $F_{1,14}$ = 2.72, p = 0.121; female coloration index, $F_{1,13}$ = 2.00, p = 0.181; interaction N.S.), but was positively correlated with the a-egg yolk carotenoid concentration (r = 0.521, n = 18; p = 0.039). As for carotenoids, there was a correlation between the concentration of Ig in maternal circulation and the mean concentration of Ig in the clutch (r = 0.651, n = 16, p = 0.006).

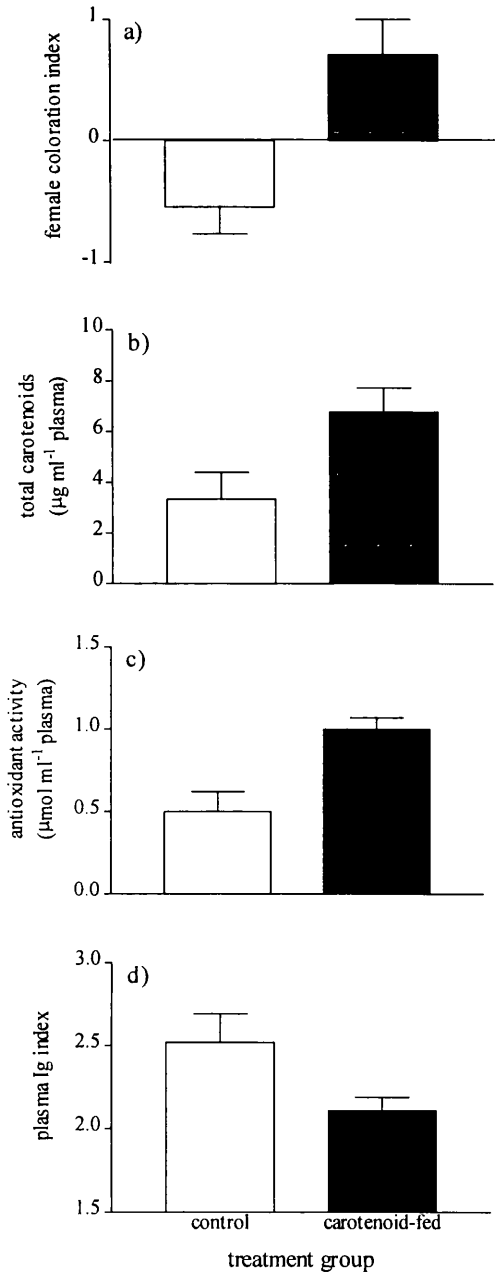

Figure 2.1: Effects of supplemental feeding on maternal phenotype in control (n = 6) and carotenoid-fed lesser black-backed gulls (n = 10, except in c where n = 9 because there was insufficient plasma to assay antioxidant activity for one individual). Means (± 1 s.e.) are shown. a), Female coloration index (first principal component; see text of Results), which differed significantly between feeding treatments (t-test, $t_{14} = 2.88, p = 0.012$). b), Plasma carotenoid concentration, which was significantly higher in carotenoid-fed females (ANCOVA: feeding treatment, $F_{1,14} = 5.52, p = 0.034$; female coloration index, $F_{1,13} = 0.02, p = 0.894$; interaction N.S.). c), Plasma antioxidant activity; which was significantly higher in carotenoid-fed females (ANCOVA: feeding treatment, $F_{1,13} = 14.98, p = 0.002$; female coloration index, $F_{1,12} = 0.00, p = 0.994$; interaction N.S.). d), Plasma Ig index, which was significantly lower in carotenoid-fed females (ANCOVA: feeding treatment, $F_{1,14} = 6.11, p = 0.027$; female coloration index, $F_{1,13} = 0.01, p = 0.936$; interaction N.S.).

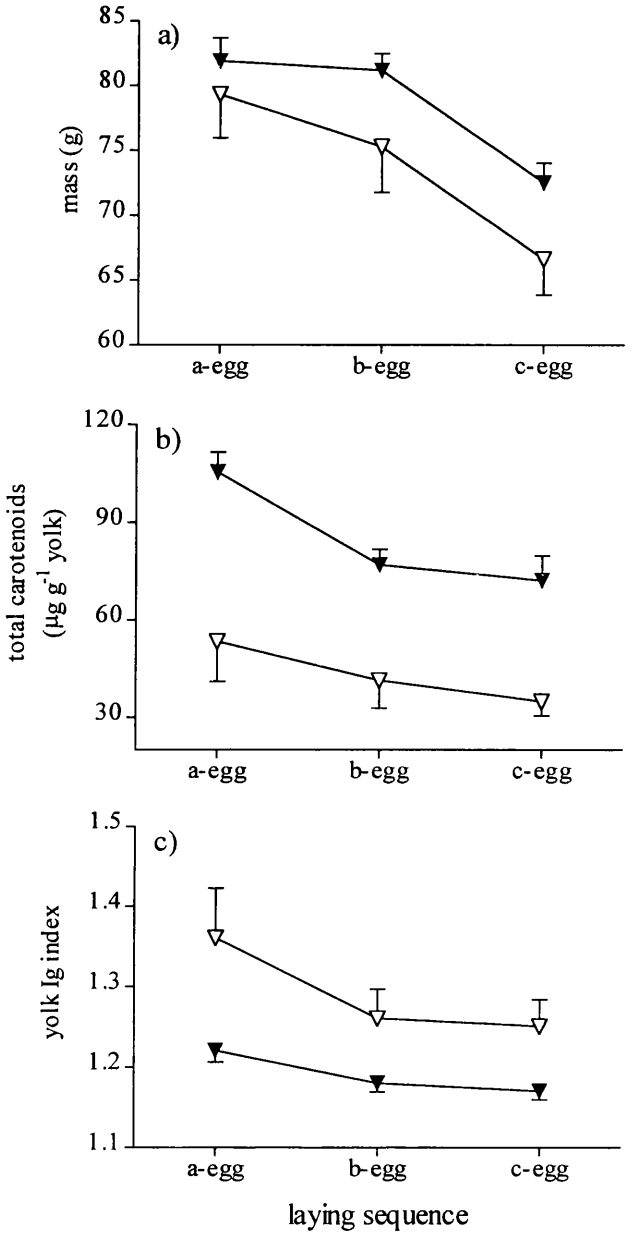

Figure 2.2: Effects of supplemental feeding on egg phenotype in the control (open triangles; $n = 7$ clutches) and carotenoid-fed groups (closed triangles; $n = 11$ clutches). Means (± 1 s.e.) are shown. a), Egg mass. b), Yolk carotenoid concentration. c), Yolk Ig indices. See Table 2.1 for results of statistical analyses.

Table 2.1: Effects of supplemental feeding on egg phenotype in control (n = 7 clutches) and carotenoid-fed (n = 11 clutches) lesser black-backed gulls. Statistical results are from rmANCOVAs with laying sequence as a within-subjects factor, feeding treatment as a between-subjects factor, and female coloration index as a covariate. Models were developed using backwards elimination (see text for details). Only main effects and significant interactions are shown.

source	egg mass			$\log_{10}$(total carotenoids)			total immunoglobulins		
	F	d.f.	p	F	d.f.	p	F	d.f.	p
within-subjects									
laying sequence	43.91	2,16	<0.001[a]	72.98	2,12	<0.001[b]	7.18	2,16	0.006[c]
laying sequence x female coloration index	-	-	-	6.26	2,12	0.014	-	-	-
between-subjects									
feeding treatment	0.58	1,14	0.459	8.32	1,13	0.013	10.01	1,14	0.007
female coloration index	0.03	1,13	0.864	0.27	1,13	0.613	1.38	1,13	0.261

[a], post-hoc contrasts: a- versus b-egg, $F_{1,17} = 9.48$, $p = 0.007$; b- versus c-egg, $F_{1,17} = 67.29$, $p < 0.001$.

[b], post-hoc contrasts: a- versus b-egg, $F_{1,14} = 101.04$, $p < 0.001$; b- versus c-egg, $F_{1,14} = 8.12$, $p = 0.013$.

[c], post-hoc contrasts: a- versus b-egg, $F_{1,17} = 12.79$, $p = 0.002$; b- versus c-egg, $F_{1,17} = 1.65$, $p = 0.216$.

All post-hoc contrasts, Bonferroni adjusted alpha = 0.025.

DISCUSSION

This study has shown that dietary supplementation with carotenoids resulted in almost two-fold increases in carotenoid concentrations and free radical trapping activity in plasma, and carotenoid pigmentation of integument, in breeding female lesser black-backed gulls. The females in the two treatments also differed in their immune function: carotenoid supplemented birds had significantly lower plasma Ig concentrations. The effects of carotenoid supply on maternal phenotype were reflected in the phenotypic variation among the eggs that they laid. Carotenoid-fed birds produced eggs that contained significantly more carotenoids, but less Ig, in comparison with controls. Antioxidant activity in yolk was correlated with its carotenoid concentration. These results suggest that carotenoid supply can profoundly influence maternal phenotype, with consequences for the composition of the eggs that they lay. But do these results indicate that carotenoids are a scarce, limiting resource to breeding females?

It has been suggested that individual wild animals differ in their supplies of carotenoids because of variation in foraging efficiency (access to carotenoids; Endler 1980; Kodric-Brown 1985; Hill 1990), or parasitism and diseases (e.g. Milinski & Bakker 1990; Houde and Torio 1992), which in some instances may directly inhibit carotenoid absorption, or place demands on carotenoid supply for antioxidant activity and immune function (Lozano 1994; von Schantz *et al.* 1999; reviewed by Olson & Owens 1998; Møller *et al.* 2000). The present study indicates that increased carotenoid supply, resulting in higher blood carotenoid levels, can translate into increased antioxidant activity in vitro. Several studies of humans and domesticated

animals have implicated free radicals in the etiology of diseases, and antioxidant activity has been shown to play an important role in disease resistance (reviewed by Chew 1996; Stahl & Sies 1999; Møller *et al.* 2000). However, since we did not measure whether free radical production exceeded the availability of carotenoids to neutralise them in control birds in vivo, it remains unclear whether carotenoid supply is limiting for protection against free radicals in gulls (and see Hill 1999). Our results also show that carotenoid supplementation resulted in lower levels of Ig in circulation. Previous (observational) studies of wild birds that have related body carotenoid levels to indirect measures of immune system activity such as circulating levels of Ig or leukocytes have reported both positive and negative correlations (reviewed by Møller *et al.* 2000). This may seem puzzling, given that studies of humans and domesticated animals have shown that carotenoids can stimulate the production of immune cells, and prevent them being damaged by free radical attack (reviewed by Chew 1996; Stahl & Sies 1999; Møller *et al.* 2000). But levels of Ig in circulation partly reflect the current requirement for antibody (i.e. exposure to antigens). Higher antibody titres should result in faster clearance of antigen, but titres will not remain high unless infection persists (Roitt *et al.* 1998) because Ig turnover is very rapid in birds – being about 1.5 days in adult domestic hens (Patterson *et al.* 1962). Thus, the low plasma Ig indices in carotenoid-fed females possibly reflected their enhanced efficiency at clearing infections over the preceding four weeks, during the period of supplemental feeding. We cannot rule out the alternative possibility that carotenoid supplementation inhibited immune function. There is limited evidence of an association between high carotenoid consumption and disease in humans, possibly because certain carotenoids switch from being

antioxidants to prooxidants above threshold concentrations (Mayne 1996). But this seems an unlikely explanation for our results because carotenoid-fed females had markedly enhanced plasma antioxidant activity. Ultimately, the issue of whether carotenoid supply is limiting for immune function in wild animals will only be resolved by experimentally varying exposure to antigens and then measuring specific responses in individuals with different circulating levels of carotenoids.

It is not possible to conclude whether there were causal relationships between female integument coloration and plasma concentrations of carotenoids or Ig. However, females producing clutches with the largest decline in carotenoids over the laying sequence had the dullest integument pigmentation on clutch completion, consistent with the explanation that carotenoid pigmentation of integument and yolk are associated. Carotenoid investment into eggs has been linked to declining carotenoid pigmentation of integument in domestic hens (Klasing 1998; and see Burley *et al.* 1992; Negro *et al.* 1998). There is increasing interest in the possibility that female ornaments reveal aspects of quality to males (Amundsen 2000). In passerine species of birds, there is some evidence that male mate choice can be influenced by female carotenoid pigmentation (Burley & Coopersmith 1987), but there is presently no evidence that such choices are related to laying date, clutch size or other measures of female reproductive success (Hill 1993). Similarly, in the present study carotenoid supply was not a proximate constraint on laying date, egg or clutch size. But under natural feeding conditions gulls presumably do not obtain carotenoids independently of other nutrients, such as amino acids, that have been shown to be limiting for egg production (Bolton *et al.* 1992). Therefore the

possibility that carotenoid pigmentation of integument could predict female quality in gulls deserves further study.

As in female plasma, high carotenoid concentrations in yolk were associated with high antioxidant activity, but low levels of Ig. Thus it appears that the level of carotenoid and Ig deposition into yolk can simply be a function of the level of carotenoids and Ig circulating in the females themselves. The significance for the offspring of this inverse relationship is not known, because both these resources would seem independently likely to benefit chicks. Embryos and hatchlings are incapable of synthesising Ig, so they rely on passive immunity before their own immune system becomes effective (Kowalczyk *et al.* 1985). It has recently been shown that kittiwakes, *Rissa tridactyla*, breeding in areas that have high prevalence of specific parasites deposit relevant antibody into their eggs, consistent with the suggestion that Ig deposition into yolk is adaptive (Gasparini *et al.* 2001; and see Heeb *et al.* 1999). There is some evidence from captive studies that passive immunity enhances disease resistance (e.g. Fadly & Smith 1991). On the other hand, domestic hen chicks with high tissue concentrations of maternally derived carotenoids have enhanced antioxidant protection (Surai & Speake 1998) and lymphocyte synthesis (Haq *et al.* 1996).

Consistent with earlier studies of this species, egg size declined sequentially through the clutch (e.g. Bolton *et al.* 1992; Royle *et al.* 1999; Nager *et al.* 2000), and since the relationship between egg mass and yolk mass was isometric, our results indicate that the absolute amounts of carotenoids and Ig covaried with egg size. Thus, the mechanisms that bring about a within-clutch hierarchy in carotenoid (sensu Royle *et al.* 1999) and Ig investment into offspring appear to be independent of

maternal supplies of carotenoids. The costs of producing young (rather than eggs per se) could potentially explain the evolution of such mechanisms. It was recently hypothesised that low antioxidant reserves in c-eggs comprised a maternal strategy to facilitate brood reduction in gulls (Royle *et al.* 1999). Our results compliment this suggestion, because c-eggs are also low in passive immunity.

The question arises as to whether deposition of carotenoids and passive immunity into eggs could be costly for females. Costs incurred by females solely during egg production have been linked to increased parasitism (Oppliger *et al.* 1996) and impaired ability to rear chicks (Monaghan *et al.* 1998), but a physiological explanation has not been elucidated. Endocytosis of circulating Ig by developing oocytes means that a domestic hen loses 30-40% of circulating Ig per day in addition to normal turnover (Kowalczyk *et al.* 1985). Could such investment cause increased maternal susceptibility to parasites and diseases, and does maternal deposition of carotenoids into eggs reduce her antioxidant protection? Similarly, it remains to be established whether maternally derived carotenoid supply is limiting to offspring in wild birds. Our results suggest that when carotenoids are in abundant supply to breeding females, this potential benefit can be transferred to their offspring via the egg. But could high antioxidant protection compensate for low levels of passive immunity in determining chick fitness? In conclusion, in lesser black-backed gulls, aspects of maternal and egg phenotypes that seem likely to have potential importance for reproductive success are influenced by carotenoid supply in the maternal diet. Questions that address the fitness consequences of carotenoid induced maternal effects are the focus of our continuing studies.

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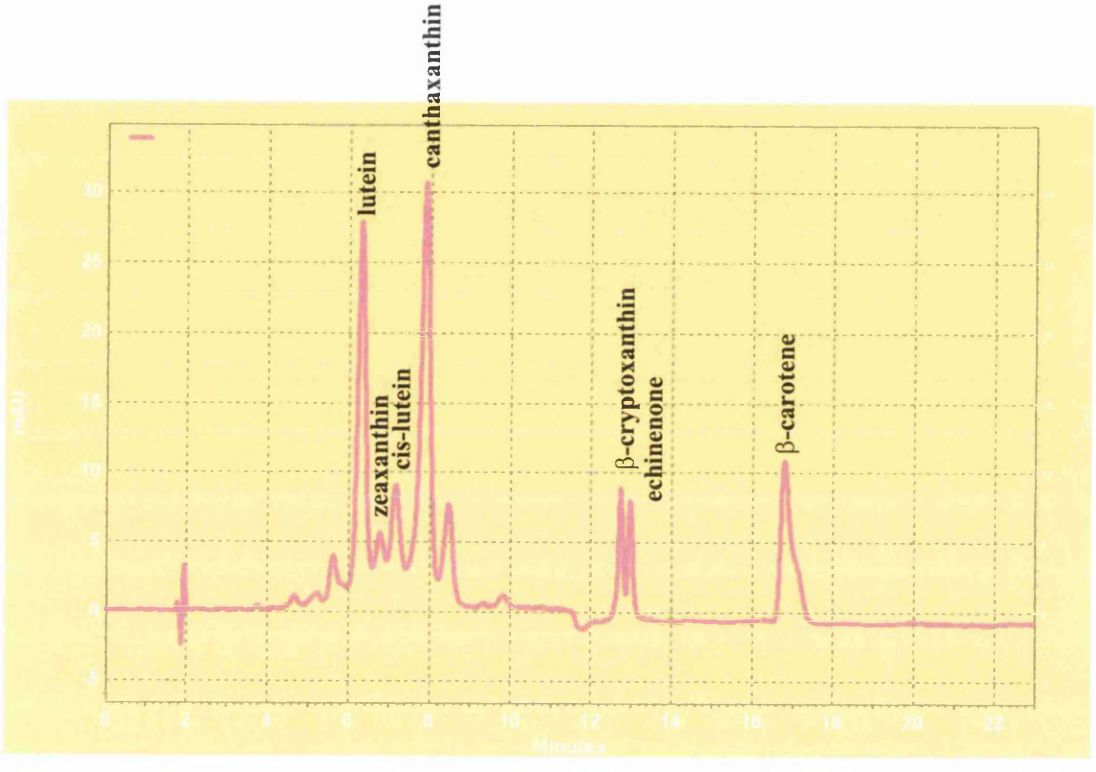
Chapter 3

PATTERNS OF YOLK ENRICHMENT WITH DIETARY CAROTENOIDS IN GULLS: THE ROLES OF PIGMENT ACQUISITION AND UTILIZATION



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INTRODUCTION

Yellow-red coloration is a conspicuous feature of egg yolk in birds. This phenomenon is brought about by carotenoids, lipid-soluble hydrocarbons with both pigmenting and antioxidant properties, that are transferred to developing oocytes from maternal circulation (Goodwin 1984). Only certain bacteria, algae and higher plants can produce carotenoids de novo (Goodwin 1984; Latscha 1990). Animals must obtain carotenoids in their diet, although a certain range of metabolic transformations is possible (reviewed by Brush 1990; Møller *et al.* 2000). It has therefore been hypothesised that carotenoids are a scarce, limiting resource to animals (reviewed by Olson & Owens 1998; Møller *et al.* 2000). However, little is known of the ecological factors that regulate how females provision their eggs with carotenoids in nature (Royle *et al.* 1999; Blount *et al.* 2000).

One possibility is that maternal selective uptake, transport or deposition, or metabolic transformations of ingested carotenoids regulate the pattern of yolk enrichment with carotenoids (i.e. mechanisms of physiological discrimination). Domestic hens characteristically transfer certain carotenoids from the maternal diet into eggs more efficiently than others (e.g. Hencken 1992; Haq & Bailey 1996; Surai & Speake 1998), and can metabolically transform certain carotenoids before deposition into yolk (Hencken 1992). Alternatively, the profile of carotenoids deposited into yolk may directly reflect the relative proportions of carotenoids in the maternal diet, as suggested by Partali *et al.*'s (1987) observational study of wild great tits, *Parus major*. An experimental approach is required to better understand the relationship between diet and yolk carotenoid composition in wild birds.

There are sound theoretical reasons to predict that the particular balance of individual carotenoids in yolk should have been shaped by natural selection. As antioxidants, carotenoids are capable of quenching free radicals generated as by-products of oxidative metabolism, and can thereby protect DNA, proteins and lipids from oxidation (for biochemical reviews, see Edge *et al.* 1997; Stahl & Sies 1999), including in egg yolk and embryo tissues (reviewed by Blount *et al.* 2000; Surai *et al.* 2001a). Carotenoids are a large (more than 600) and diverse group of compounds, with the potential to act as either antioxidants or prooxidants (Burton & Ingold 1984; Edge *et al.* 1997; Stahl & Sies 1999). In vitro studies have revealed that free radical trapping activity is most efficient when certain combinations of antioxidants are involved (e.g. Böhm *et al.* 1997), or within certain ranges of carotenoid concentration and partial oxygen pressure (Burton & Ingold 1984). It has been shown that individual yolk carotenoids are differentially incorporated into specific embryo tissues, consistent with the suggestion that they serve particular roles (Surai *et al.* 2001b). But it remains unclear whether mechanisms operate in females to produce eggs with a specific yolk carotenoid profile.

Understanding whether physiological discrimination among carotenoids occurs during egg production in wild birds is of interest from the perspective of costs of reproduction. Carotenoids are widely distributed and probably abundant in the food of most animals (Goodwin 1984; Latscha 1990; Hudon 1994), but an imbalance between dietary supply and physiological 'preference' for specific compounds could mean that carotenoids are nonetheless limiting (Hill 1996; Olson & Owens 1998). Moreover, metabolic transformations of carotenoids are limited in scope, far less than

completely efficient (Fox & Hopkins 1966; Fox *et al.* 1967), and expend energy (Brush 1990).

This paper reports on a study in which we provided a cocktail of four carotenoids as a supplemental food to free-living, pre-laying lesser black-backed gulls, *Larus fuscus*, a species known to have a complex yolk carotenoid profile involving at least eight different types of carotenoid (P. F. Surai unpublished data). We tested whether carotenoid-supplementation invoked increased yolk carotenoid concentrations of (1) the supplemental carotenoids only, or (2) additional carotenoids not included in the supplement, and (3) whether the percentage profiles of yolk carotenoids differed between control and carotenoid-supplemented groups. To help elucidate whether the experimental treatment invoked a physiologically normal response, within the ranges of natural variation, we compared yolk susceptibility to lipid peroxidation in control and carotenoid-enriched eggs in vitro.

MATERIALS AND METHODS

a) *Supplemental feeding and collection of eggs*

As part of a larger experiment in 1999 lesser black-backed gulls breeding on Walney Island, Cumbria, U.K., were supplementally fed with either 20 g solid vegetable fat (Van den Bergh Foods Ltd., Crawley, UK), or an equal amount of fat mixed with 2 mg total carotenoids (carotenoid-supplemented) daily before and during laying. Details of the full sample size of nests that were supplementally fed are given by Blount *et al.* (2002; *Chapter 2*); sample sizes of nests used in the present study are given below. Pairs of birds were allocated to a feeding treatment at random as soon as their nest site was identified, and food was placed next to the nest at night. The supplemental food had always gone by the following morning.

Vegetable fat was chosen as a control supplement because it is carotenoid-free, but facilitates the uptake of dietary carotenoids (Stahl & Sies 1999). Moreover, relatively large amounts of supplemental fat (120 g day^{-1}) do not affect female condition or egg quality in this species (Bolton *et al.* 1993). There are no data on the quantity of ingested carotenoids needed to produce a particular concentration or profile of yolk pigmentation in any wild bird species. The daily amount of supplemental total carotenoids equated to twice that contained in an average first-laid egg produced at this colony (P. F. Surai, unpublished data from 1998). Seven types of carotenoid have previously been identified in such eggs, but only four of these are available commercially as dietary grade additives. These are lutein and zeaxanthin (yellow xanthophylls) that comprise ca. 30% and 4%, respectively, canthaxanthin (an orange-red xanthophyll) ca. 6%, and β-carotene (yellow-orange carotene) ca. 33%. The remainder comprises ca. 4% cis-lutein (yellow xanthophyll), 4% β-cryptoxanthin

and 13% echinenone (orange-red xanthophylls), and 6% unidentified carotenoids (P. F. Surai, unpublished data from 1998). The categorisation of carotenoids as xanthophylls and carotenes refers to their molecular structure (reviewed by Edge *et al.* 1997; Stahl & Sies 1999), and our nominal categorisation of yellow and orange-red xanthophylls, and carotenes, respectively, does not imply any particular functional separation of these compounds. For example, β-carotene is a potential precursor of various orange-red xanthophylls, and orange-red xanthophylls can be converted into yellow xanthophylls by metabolic transformation (e.g. Brush & Power 1976; Brush 1990; Møller *et al.* 2000). We designed the supplement using lutein and zeaxanthin to represent all yellow xanthophylls, canthaxanthin to represent all orange-red xanthophylls, and β-carotene, respectively, mixed such that the ratio of yellow xanthophylls / orange-red xanthophylls / β-carotene equated to that in the total identified carotenoids in gull eggs (40:25:35%). On a daily basis each carotenoid-supplemented nest received 40 mg Oro Glo Layer™ (active ingredients 1.8% lutein and 0.2% zeaxanthin by mass; Kemin Europa NV, Herentals, Belgium), 5 mg Carophyll Red™ (10% canthaxanthin by mass; La Roche, Basel, Switzerland), and 7 mg Rovimix™ (10% β-carotene by mass; La Roche). The non-active ingredient in all these products is a starch-gelatine matrix that helps to stabilise the carotenoids in storage. Carotenoids were stored in the dark at 4 °C before the preparation of supplemental food to prevent their oxidation by heat or light.

Fat was heated in an oven to 150 °C then allowed to cool to 12-14 °C before carotenoids were added and mixed thoroughly. Fat destined for control nests was similarly heated and cooled. Both carotenoid and control supplements were hardened by storage at –20 °C for 2-3 h before presentation to the gulls.

A random sample of first-laid eggs (hereafter, 'a-eggs') was collected from the control (n = 13) and carotenoid (n = 14) feeding treatments on the day of laying and used for the present study. Egg mass and wet yolk mass were measured (± 0.1 g) on the day of laying using an electronic balance. Yolk was collected, homogenised and stored at –20 °C until biochemical analyses. Neither the date on which supplemental feeding began, laying date, egg or yolk mass differed significantly among feeding treatments in this sample of a-eggs (date feeding began: control, 17.00 ± 0.69 April (mean ± 1 s.e.); carotenoid, 17.07 ± 1.15 April; Mann–Whitney test, $z = 0.518$, $p = 0.550$; laying date: control, 17.31 ± 2.06 May (mean ± 1 s.e.); carotenoid, 17.43 ± 1.54 May; t-test, $t = 0.047$, d.f. = 25, $p = 0.963$; egg mass: control, 81.28 ± 2.08 g; carotenoid, 81.31 ± 1.95 g; $t = 0.008$, d.f. = 25, $p = 0.994$; yolk mass: control, 19.52 ± 0.35 g; carotenoid, 19.43 ± 0.51 g; $t = 0.138$, d.f. = 25, $p = 0.891$). Thus, the duration of supplemental feeding prior to laying was about 4 weeks in both treatments. In lesser black-backed gulls clutch size is modally three, and correlates positively with female condition (e.g. Bolton *et al.* 1993). We sampled a-eggs from clutches that ranged in size from one to three. However, this seems unlikely to have introduced a bias because the ratio of different clutch sizes in the samples did not differ significantly among feeding treatments (chi-square test, $\chi^2 = 0.630$, d.f. = 1, $p > 0.5$), and the a-egg yolk concentration of total carotenoids (see below) did not covary with clutch size (Kruskal Wallis ANOVA with clutch size as a factor: control, $\chi^2 = 5.563$, d.f.= 2, $p = 0.062$; carotenoid, $\chi^2 = 1.194$, d.f. = 2, $p = 0.384$).

b) *Biochemical analyses*

Concentrations of yolk carotenoids were measured using high performance liquid chromatography (HPLC). An aliquot of yolk was vortexed in 0.7 ml 5% NaCl for 10 s, then 0.5 ml ethanol was added and homogenised (20 s), then 0.5 ml hexane was added and homogenised (20 s). Samples were then centrifuged and the hexane phase, containing the carotenoids, was collected. Extraction with hexane was performed twice, and the combined phase was dried under N_2 gas, then re-eluted in dichloromethane/methanol (1:1 v/v) ready for HPLC. Carotenoids were quantified using a Spherisorb type S30DS2, 3μ C_{18} reverse-phase column (25 cm x 4.6 mm) (Phase Separations, Clwyd, UK) with a mobile phase of acetonitrile-methanol (85:15) and acetonitrile-dichloromethane-methanol (70:20:10) in gradient elution (see Granado *et al.* 1998), using detection by absorbence at 445 nm. Peaks were identified by comparison with carotenoid standards (Sigma, U.K.; La Roche, Switzerland). Carotenoid concentrations are reported as μg g^{-1} yolk.

Yolk susceptibility to lipid peroxidation was estimated in a random sample of eight eggs from each treatment. These comprised a representative sub-sample of eggs, since the yolk concentration of total carotenoids did not differ significantly from the treatment average (one-sample *t*-tests: controls, $t = 0.711$, d.f. = 7, $p = 0.5$; carotenoid-supplemented, $t = 0.433$, df = 7, $p = 0.678$). Assays involved measurement of the colorimetric reaction of thiobarbituric acid (TBA) with molondialdehyde (MDA; a secondary product of lipid peroxidation) (e.g. Ohkawa *et al.* 1979). An aliquot of yolk was homogenised in phosphate-buffered saline (10% w/v) containing 1.15% (w/v) KCl. Samples were incubated at 37 °C for 60 min in the presence of $FeSO_4$ under an air atmosphere with gentle shaking. At the end of this period butylated hydroxytoluene

(0.01% v/v) was added. Then, 0.2 ml of sodium dodecyl sulphate (8%) was added, samples were vortexed, and 1.5 ml of 20% CH_3COOH at pH 3.5 (adjusted by KOH) and 1.5 ml of TBA (0.8% w/v in water) were added. Samples were vortexed then incubated at 95 °C for 60 min. After cooling down, MDA-TBA adduct was extracted using butanol and spectrophotometric measurements were made at 532 nm. 1,1,3,3-Tetramethoxypropane (Sigma, U.K.) was used as a standard, and results are expressed as μg MDA g^{-1} yolk h^{-1}.

c) *Data analyses*

Variation in concentrations and percentages of individual carotenoids (proportion of total), respectively, was assessed using MANOVA with feeding treatment as a factor (tests based on Pillai's Trace, Wilks' Lambda, Hotelling's Trace and Roy's Largest Root gave identical *F*-values). Concentration data were first $\log_{10}$-transformed to achieve homoscedasticity, whereas percentage data were log-ratio-transformed (Reyment 1989). MANOVA was followed by univariate ANOVAs to identify effects of individual carotenoids. Significance criteria in these tests were adjusted sequentially (Sokal & Rohlf 1995) because the Bonferroni correction is excessively conservative where k is large (e.g. Chandler 1995). Thus, the comparison-wise error rates were 0.006 (where k = 8), then 0.007 (k = 7), etc. Other statistical tests are introduced in the text of the Results, and two-tailed $\alpha = 0.05$. Values are reported as means ± 1 s.e.

RESULTS

Seven carotenoids were identified in the gull yolk, plus a further group of carotenoids that could not be identified specifically. Yolk concentrations of carotenoids differed significantly between feeding treatments (MANOVA based on $\log_{10}$ values: $F_{8,18}$= 6.407, p = 0.001; Figure 3.1a). Post-hoc univariate ANOVAs showed that in comparison with controls, eggs produced by carotenoid-supplemented females were significantly enriched with lutein, zeaxanthin, canthaxanthin and β-carotene, all of which featured in the supplemental food, but also cis-lutein, β-cryptoxanthin, echinenone and unidentified carotenoids, which did not (Table 3.1).

The proportions of individual carotenoids in yolk differed significantly between feeding treatments (MANOVA based on log-ratio values: $F_{8,18}$ = 4.800, p = 0.003), specifically because eggs produced by carotenoid-supplemented females contained relatively lower proportions of lutein and zeaxanthin, and higher proportions of canthaxanthin (Figure 3.1b and Table 3.1). However, proportions of the other five classes of carotenoids in yolk did not differ significantly in comparison with controls (Figure 3.1b and Table 3.1). Consequently, the overall balance of individual carotenoids was similar in eggs produced by control and carotenoid-supplemented females (Spearman's correlation based on percentages, r_s = 0.762, n = 8, p = 0.028). Yolk susceptibility to lipid peroxidation was significantly lower in eggs produced by carotenoid-supplemented females in comparison with controls (t-test, t_{14}= 2.411, p = 0.030, Figure 3.2).

To help elucidate whether yolk carotenoid profiles simply directly reflect diet, or involve physiological discrimination, we produced correlation matrices between yolk concentrations of individual carotenoids, and their relative proportions, respectively,

based on control eggs. Positive correlations would be consistent with the explanation that yolk carotenoid profiles reflect diet (but would not rule out the action of physiological discrimination), whereas negative correlations would suggest physiological discrimination. Of 28 possible correlations among the concentrations of individual carotenoids, nine relationships were significantly positive and none were significantly negative (Table 3.2). In contrast, of 28 possible correlations among the proportions of individual carotenoids, five were significantly negative and none were significantly positive (Table 3.2).

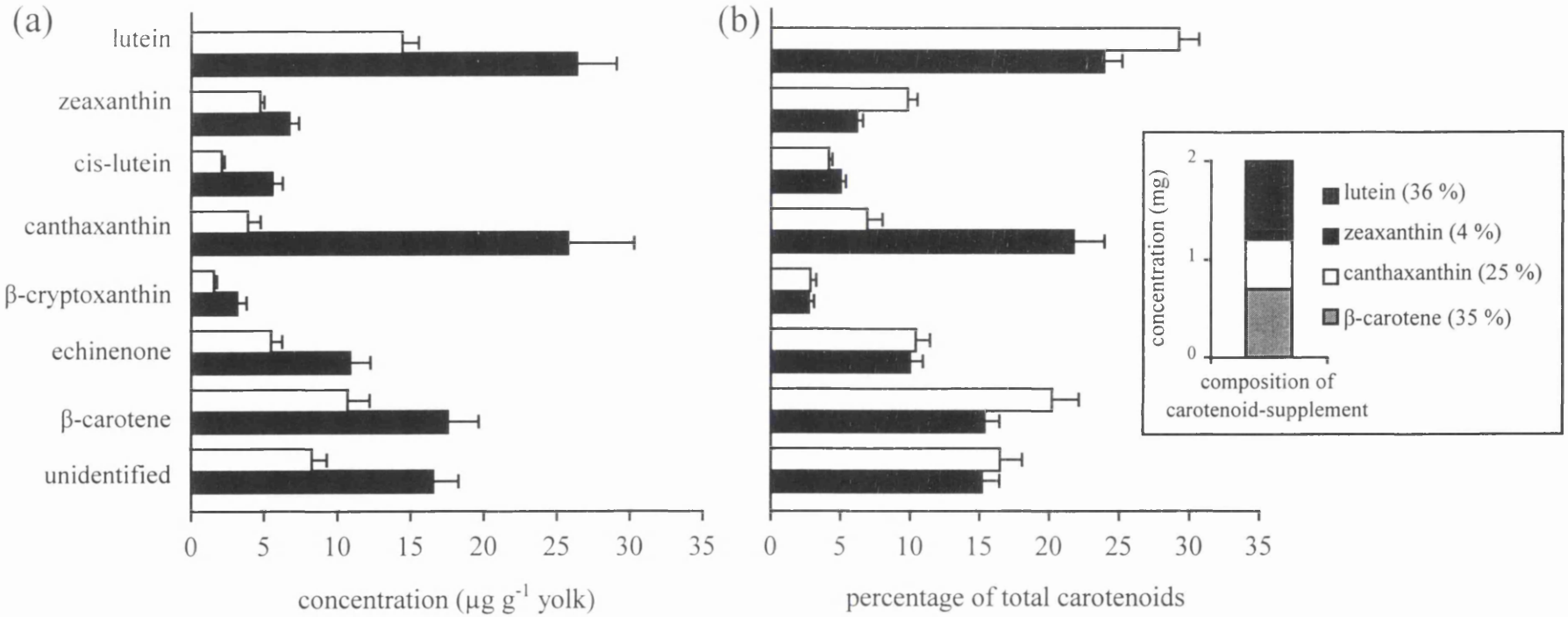

Figure 3.1: Mean ± s.e. (*a*) concentrations and (*b*) relative proportions of carotenoids in the yolk of eggs produced by wild lesser black-backed gulls given a carotenoid-free (n = 13; open bars) or carotenoid supplement (n = 14; closed bars) in the diet for about one month prior to laying. See Table 3.1 for results of statistical analysis. Concentrations and relative proportions of carotenoids in the supplement are shown, inset.

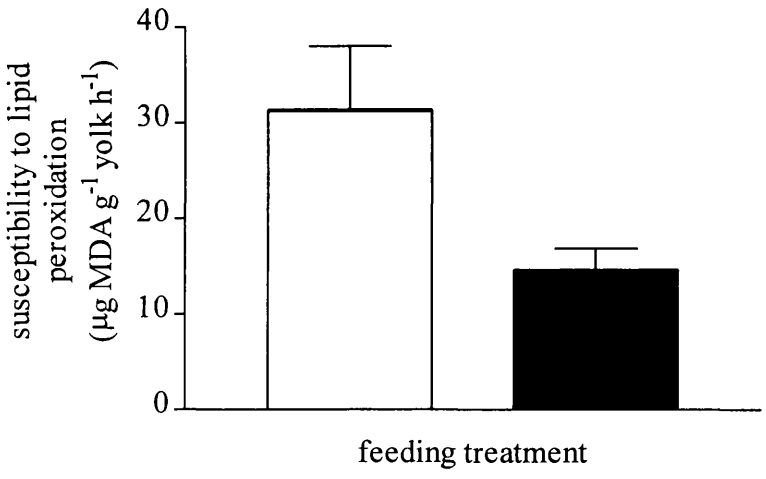

Figure 3.2: Mean ± s.e. susceptibility to lipid peroxidation in the yolk of eggs produced by wild lesser black-backed gulls given a carotenoid-free (n = 8; open bars) or carotenoid supplement (n = 8; closed bars) in the diet for about one month prior to laying. See text for results of statistical analysis.

Table 3.1: Variation in yolk carotenoid profiles in eggs produced by control and carotenoid-supplemented lesser black-backed gulls, resulting from univariate ANOVAs with feeding treatment as a factor. *P*-values marked with an asterisk remained statistically significant after sequential Bonferroni correction (see Materials and methods).

	$\log_{10}$ concentration ($\mu g\ g^{-1}$ yolk)			log-ratio percentage of total carotenoids ($\mu g\ g^{-1}$ yolk)		
source	$F_{1,25}$	p		$F_{1,25}$	p	
lutein	19.478	< 0.0001	*	7.778	0.010	*
zeaxanthin	6.488	0.017	*	20.652	< 0.0001	*
cis-lutein	39.729	< 0.0001	*	3.960	0.058	
canthaxanthin	46.338	< 0.0001	*	34.947	< 0.0001	*
β-cryptoxanthin	8.703	0.007	*	0.603	0.445	
echinenone	12.833	0.001	*	0.079	0.782	
β-carotene	5.748	0.024	*	3.910	0.059	
unidentified carotenoids	17.335	< 0.0001	*	0.291	0.595	

Table 3.2: Spearman rank correlation coefficients between yolk concentrations (uppermost values) and percentages (proportion of total carotenoids; lowermost values) of individual carotenoids in eggs produced by control-supplemented lesser black-backed gulls ($n = 13$). *P*-values marked with asterisk(s) are statistically significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

	zeaxanthin	cis-lutein	canthaxanthin	β-cryptoxanthin	echinenone	β-carotene	unidentified
lutein	0.454	0.918 ***	0.714 **	0.280	0.462	0.456	0.747 **
	0.401	0.495	-0.297	0.055	-0.758 **	-0.665 *	0.390
zeaxanthin		0.534	0.501	0.091	0.669 *	0.366	0.462
		0.099	-0.374	0.016	-0.093	-0.423	0.077
cis-lutein			0.780 **	0.273	0.473	0.352	0.670 *
			0.115	0.115	-0.280	-0.742 **	0.423
canthaxanthin				-0.140	0.593 *	0.555 *	0.456
				-0.236	0.192	0.104	-0.181
β-cryptoxanthin					0.245	0.091	-0.168
					0.099	0.093	-0.324
echinenone						0.852 ***	0.269
						0.505	-0.665 *
β-carotene							0.253
							-0.747 **

DISCUSSION

This study has shown that dietary supplementation with a mixture of four carotenoid types resulted in the production of eggs that contained increased yolk concentrations of seven individual carotenoids, and also unidentified carotenoids, in comparison with controls. However, despite these changes, the overall percentage profile of yolk carotenoids was broadly similar (i.e. positively correlated) between control and carotenoid-supplemented groups. In an observational study of egg composition in lesser black-backed gulls at a different study colony, Royle *et al.* (1999) noted that the relative proportions of yolk carotenoids were remarkably similar among eggs despite large variation in yolk total carotenoid concentrations. The carotenoid profile of yolk reported here is qualitatively similar to that study – but what mechanism could result in an apparently characteristic profile of yolk carotenoids in this species? Could yolk carotenoid profiles simply reflect relative dietary supplies of these pigments, or could there be an additional influence of physiological discrimination?

Several lines of evidence point to an influence of physiological discrimination among ingested carotenoids in shaping yolk carotenoid profiles in gulls. The relative concentrations of the four carotenoids in the supplement (lutein > β-carotene > canthaxanthin > zeaxanthin) were similar to their representation in yolk (lutein > canthaxanthin > β-carotene > zeaxanthin), but there were differences between treatments in the yolk percentages of these carotenoids. Canthaxanthin was only the third most abundant carotenoid in the supplement, but represented the highest proportional increase in eggs produced by carotenoid-supplemented females in comparison with controls. This could be explained by canthaxanthin out-competing β-carotene during gut absorption (van den Berg 1999). In contrast, despite the

inclusion of lutein and zeaxanthin in the carotenoid supplement, these compounds comprised lower proportions of total carotenoids in eggs produced by carotenoid-supplemented than control females. These patterns mirror those observed in domestic hens, which transfer canthaxanthin from diet to yolk far more efficiently than zeaxanthin (Hencken 1992).

Interestingly, unlike domestic hens (Hencken 1992), our results suggest that gulls readily transfer β-carotene from diet to yolk. It has previously been reported that β-carotene comprises a relatively high percentage of yolk total carotenoids in lesser black-backed gulls at a different study colony (Royle *et al.* 1999), and also in American coots, *Fulica Americana*, and common moorhens, *Gallinula chloropus* (Surai *et al.* 2001b). β-Carotene is possibly the most abundant carotenoid produced in nature (Latscha 1990). However, our results suggest that its relatively high representation in yolk is not simply a reflection of superabundance in the diet; carotenoid-supplementation resulted in a significant increase in yolk concentrations of β-carotene, but its percentage profile did not differ between control and carotenoid-supplemented groups. β-Carotene is among the most powerful antioxidants, but can become a prooxidant above threshold concentrations and in conditions of high partial oxygen pressure (Burton & Ingold 1984; Edge *et al.* 1997), and possibly it plays an important role in the antioxidant protection of embryos in wild birds (Surai *et al.* 2000a,b).

Specificity of carotenoid uptake and assimilation in wild birds has previously been reported in studies of integument pigmentation (e.g. Fox & McBeth 1970; Fox, McBeth & Mackinney 1970; Brush & Power 1976; Hill 1992; Negro & Garrido-Fernández 2000; Negro *et al.* 2000; McGraw *et al.* 2001). The mechanisms involved

have not been fully elucidated, although it is known that selective absorption can occur in the gut (Fox & McBeth 1970), and individual carotenoids have affinities for specific lipoproteins in circulation responsible for their transportation and deposition (Brush 1990; Surai & Speake 1998; Surai *et al.* 2000). In this study, increases in the yolk concentrations of carotenoids that were not included in the carotenoid supplement (cis-lutein, β-cryptoxanthin, echinenone, unidentified), could have arisen by metabolic transformations (e.g. β-carotene conversion into echinenone). Such a mechanism could also in part explain the particularly high representation of canthaxanthin in the eggs of carotenoid-supplemented females (echinenone conversion into canthaxanthin; see Fox *et al.* 1970; Brush 1990; Hencken 1992; Møller *et al.* 2000; McGraw *et al.* 2001).

To conclude incontrovertibly that yolk synthesis in gulls involves physiological discrimination among ingested carotenoids would require a comparison of the relative profiles of carotenoids in the natural diet, maternal body stores and in egg yolk – a difficult task under field conditions. As recently pointed out by McGraw *et al.* (2001), our understanding of the interactions between carotenoid acquisition and physiological utilization in determining patterns of pigmentation in birds could benefit from studies tracing the fate of labelled dietary pigments. The natural diet of lesser black-backed gulls includes fish, crustaceans, molluscs and also domestic waste (Cramp 1983; own observations), which certainly seems likely to include all the types of carotenoids that were identified in control gull yolk in the present study (see Goodwin 1984; Latscha 1990). However, our exploratory analysis of carotenoid profiles in control eggs revealed further evidence to suggest the action of physiological discrimination. Among control eggs, a) relatively few potential

correlations between carotenoid concentrations were significantly positive (9 of 28 relationships); and b) increasing proportions of certain carotenoids were associated with decreasing proportions of other carotenoids (5 of 28 potential correlations were significantly negative), suggesting interactions among carotenoids during uptake / assimilation, and calling into doubt the possibility that the natural diet and yolk have identical carotenoid profiles in gulls.

Some additional mechanisms could contribute to explain out results. We recently reported that carotenoid-fed female gulls have increased plasma antioxidant activity and reduced immunoglobulin levels in comparison with controls, suggesting improved health (Blount *et al.* 2002, *Chapter 2*). Thus, in theory carotenoid-supplemented females may have been healthier, and thus had improved foraging efficiency or carotenoid assimilation (since gut parasitism is known to impair carotenoid absorption; e.g. Ruff *et al.* 1974). However, we think such positive-feedback explanations are unlikely to entirely explain our results, because they predict that positive correlations should exist between concentrations and proportions of individual yolk carotenoids, respectively. Our correlation analysis based on control eggs revealed limited evidence of positive correlations between carotenoid levels, but also negative correlations, consistent with a physiological discrimination explanation (see above).

Some supplies of carotenoids deposited into yolk could have been derived from body stores (body fat, liver, integument) rather than metabolic transformation. However, the possible generality of this mechanism is unclear, because body stores of carotenoids are insufficient to meet the demands of egg production in domestic hens (Klasing 1998), and a decline in female integument pigmentation at the time of

egg production has been reported in various wild bird species (Burley *et al.* 1992; Negro *et al.* 1998; Blount *et al.* 2002, *Chapter 2*).

Carotenoid supplementation gave rise to yolk carotenoid concentrations that in some instances exceeded the range of variation in controls. Thus, we cannot exclude the possibility that the physiological mechanisms hypothesised to explain patterns of yolk carotenoid deposition in carotenoid-supplemented gulls differ from those operating under natural feeding conditions. However, we think that the similarity of the overall percentage profile of individual yolk carotenoids between treatments, and the reduced yolk susceptibility to lipid peroxidation following carotenoid-supplementation, supports the conclusion that the mechanisms involved were physiologically normal. Had levels of carotenoid supplementation been excessive, we may have found a prooxidant rather than antioxidant effect (see Introduction). Interestingly, the average total carotenoid concentration in control eggs in this study (circa 51 $\mu g\ g^{-1}$ yolk) is qualitatively lower than reported by Royle *et al.* (1999) (circa 56 $\mu g\ g^{-1}$ yolk), and our data from the Walney colony from the previous year (65.18 $\mu g\ g^{-1}$ yolk, range 43.54 – 92.94 μg / g yolk, $n = 5$ eggs; P. F. Surai, unpublished data). Perhaps therefore natural carotenoid supplies were particularly low in the present study. To our knowledge this paper is the first to have demonstrated experimentally that carotenoid assimilation (at any physiological level) is sensitive to dietary carotenoid supplies in any free-living animal species, and future studies may help to elucidate a normal range of carotenoid intake.

We cannot rule out the possibility that yolk carotenoid profiles were a by-product of differential carotenoid use for alternative somatic demands (e.g. antioxidant protection, immune function), rather than arising through mechanisms to achieve a

recipe for a good egg. Studies of domestic hens have shown that antioxidant activity can vary markedly according to the type of carotenoid supplied in the diet (Woodall *et al.* 1996) and its interaction with antioxidant vitamins (Tengerdy *et al.* 1990), but no information is available about whether differential use of carotenoids for such somatic processes influences yolk carotenoid profiles. Since eggs produced by carotenoid-supplemented birds exhibited lower yolk susceptibility to peroxidation than controls in vitro, possibly the balance of yolk carotenoids in this species serves to maximise antioxidant protection of offspring. This hypothesis could be investigated in vitro by experimentally altering the carotenoid profile of yolk, and measuring the effects on susceptibility to lipid peroxidation.

Possibly, mechanisms of physiological discrimination among ingested carotenoids have been shaped by natural selection in response to stochasticity and unpredictability in the supply of carotenoids in the diet. Variation in food supply (temporal and geographic) has been invoked to explain intraspecific variation in plasma or integument levels of carotenoids in birds (e.g. Slagsvold & Lifjeld 1985; Partali *et al.* 1987; Hill 1993; Linville & Breitwisch 1997; Bortolotti *et al.* 2000; Negro *et al.* 2000; Negro & Garrido-Fernández 2001). The carotenoid intake of lesser black-backed gulls seems likely to be highly variable, since they feed opportunistically (Cramp 1983). In apparent contrast to our findings, Partali *et al.* (1987) reported a positive correlation between the relative proportions of carotenoids in the diet and deposited into yolk in wild great tits. Perhaps physiological discrimination among ingested carotenoids for the purposes of egg production is characteristic of opportunistic feeders, whereas specialised feeders have evolved foraging strategies that maximise their intake of certain carotenoids required for egg

production. This may be an interesting direction for further study, particularly in relation to the relative costs of alternative strategies.

The pattern of carotenoid deposition into yolk must ultimately reflect some interplay between a female's diet and her capacity to absorb, transport, store and (or) modify carotenoids, whilst reconciling any competing somatic demands for their use (e.g. antioxidant protection, immune function). Our results suggest a role of physiological discrimination among ingested carotenoids during egg production in gulls. Several lines of evidence support the suggestion that such mechanisms could be costly. Birds preferentially use those dietary carotenoids that do not require transformation for integument pigmentation (Fox *et al.* 1970; Goodwin 1984; Brush 1990), consistent with the idea that carotenoid metabolism is energetically costly (Hill 1996). There is growing evidence that increased investment in egg production causes reduced maternal condition in bird species (e.g. Heaney & Monaghan 1995; Oppliger *et al.* 1996), including gulls (Monaghan *et al.* 1998), but the physiological basis of these costs has not been elucidated. We have so far not found any evidence to suggest that supplementation with a carotenoid cocktail causes reduced egg production capacity (Blount *et al.* 2002; and see Materials and methods, this study), as may be expected if supplementation invoked energetically costly metabolism transformations of carotenoids. However, it seems plausible that such costs could be offset by reduced effort in foraging for carotenoids. The maternal fitness consequences of yolk enrichment with carotenoids await study.

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Chapter 4

Carotenoid Supply Mediates a Trade-Off Between Immune Defence and Chick-Rearing Capacity in Gulls *Larus fuscus*

INTRODUCTION

Life history theory predicts that reproduction is costly, and consequently individuals should face a trade-off between investment in current and future reproduction (Williams 1966; Stearns 1992). In birds, several studies have reported a positive correlation between reproductive effort and susceptibility to parasitism (e.g. Ots & Hõrak 1996; Allander 1997), with resultant fitness costs (Gustaffson *et al.* 1994; Richner *et al.* 1995; reviewed by Møller 1997). One possible explanation for such findings is a trade-of between the allocation of resource(s) to reproduction and immune defence. However, the identity of the limiting resource(s) and the mechanism in question remains a contentious issue in evolutionary ecology (Gustafsson *et al.* 1994; Sheldon & Verhulst 1996; Råberg *et al.* 1998; Westneat & Birkhead 1998; Norris & Evans 2000).

It has traditionally been viewed that life history trade-offs, including costs of reproduction, are mediated by energy limitation (Williams 1966; Stearns 1992), although the empirical evidence has been equivocal (reviewed by Råberg *et al.* 1998; Norris & Evans 2000). Recently, there has been growing interest in the possibility that other mechanisms may be involved. One particular hypothesis posits that trade-offs between life-history activities that incur increased metabolic energy turnover (e.g. reproduction and immune defence) could arise because of free radical-induced cell damage (Svensson *et al.* 1998). Free radicals are highly unstable atoms or molecules that are by-products of oxidative metabolism. If not under control (= oxidative stress), free radicals can damage DNA, proteins and lipids, and thereby impair physiological processes including immune function (for mechanistic reviews see Chew 1996; Stahl & Sies 1999). It is known from studies of humans that

strenuous physical exercise can trigger oxidative stress (reviews by Ji 1995; Sen 2001). Similar studies in birds are lacking. However, brood size manipulations have shown that parents working harder in chick-rearing consequently have depressed immune function (Deerenberg *et al.* 1997; Nordling *et al.* 1998; Moreno *et al.* 1999), increased levels of parasitism (Richner *et al.* 1995), and reduced future fecundity (Gustafsson & Sutherland 1988), and survival (Daan *et al.* 1996). Similarly, increased energy expenditure (experimentally invoked by exposure to cold stress) has been shown to result in reduced antibody responses in blue tits *Parus caeruleus*, which was hypothesised to reflect impaired immune function caused by oxidative stress (Svensson *et al.* 1998). Like work rate, immune system activation causes free radical production due to increased energy turnover, and because macrophages and neutrophils generate free radicals as part of their mechanism for destroying antigens (Chew 1996). Studies of birds have shown that parents mounting an immune response (following experimental inoculation) have a reduced chick-rearing capacity (Ilmonen *et al.* 2000; Råberg *et al.* 2000; but see Williams 1999), consistent with an oxidative stress explanation.

The risk of oxidative stress is modulated by carotenoids and other antioxidants, which can neutralize free radicals. Increased carotenoid intake has been shown to improve antioxidant protection and immune responses in humans, laboratory animals (e.g. Jyonouchi *et al.* 1994; Nakano *et al.* 1999) and domestic hens (McWhinney *et al.* 1989; Tengerdy *et al.* 1990; Woodall *et al.* 1996; reviewed by Chew 1996; Stahl & Sies 1999; Møller *et al.* 2000). Immune system activation has been shown to result in oxidation and depletion of body levels of carotenoids in birds (Allen 1997; Saino *et al.* 2000). Like all animals, birds must ultimately obtain carotenoids from their diet (Goodwin 1984), which raises the possibility that carotenoid supply could

be physiologically limiting (Lozano 1994; von Schantz *et al.* 1999). We recently reported that wild female lesser black-backed gulls, *Larus fuscus*, provisioned with carotenoids had twofold higher plasma levels of carotenoids and antioxidant activity, and reduced immunoglobulin levels in comparison with controls, possibly indicating improved health (Blount *et al.* 2002a, *Chapter 2*). However, it remains to be tested whether a trade-off between reproductive effort and immune function is modulated by carotenoid supply.

Here, we carry out such a test in a study of wild lesser black-backed gulls. We manipulated maternal carotenoid supplies upward by supplemental feeding, and invoked females to mount an immune response by inoculating them with a non-replicating antigen. We measured the effects of these manipulations on the capacity to rear a control (foster) clutch of young in comparison with controls. We hypothesised that (1) increased carotenoid supply would result in improved offspring-rearing capacity; (2) there would be a trade-off between investment in immune function and offspring-rearing capacity; and (3) the trade-off would be disengaged by increased carotenoid supply.

MATERIALS AND METHODS

a) *Manipulation of carotenoid supplies upward*

This study was carried out at the southern peninsula of Walney Island, Cumbria, UK, where about 24 000 pairs of lesser black-backed gulls breed (Monaghan *et al.* 1998). As part of a larger experiment (see *Chapter 5*), on 9 April 2000, shortly after arrival of gulls at the colony and about 4 weeks before laying started, we randomly allocated breeding pairs in a central part of the colony to a feeding treatment. We supplementally fed females daily at the nest either with a control supplement of 20 g solid vegetable fat (Van den Bergh Foods Ltd., Crawley, UK), or an equal amount of fat mixed with 2 mg total carotenoids (carotenoid-diet group). Daily supplementation with a considerably larger amount of fat (120 g) has previously been shown to have no effect on reproductive performance in this species (Bolton *et al.* 1992). To minimise the risk of theft by non-target birds, food was placed at night inside a length of PVC pipe (4.5cm diameter, 8 cm deep) buried vertically in the ground next to the nest. Further details of the preparation of supplemental food, and the ratio of different carotenoids in the supplement, are given elsewhere (Blount *et al.* 2002a,b, *Chapters 2 & 3*). Supplemental feeding continued daily throughout laying (see below).

Eggs were collected on the day of laying, weighed using an electronic balance (± 0.1 g) and replaced with dummies. Gulls can be caught most readily at the nest after they have initiated incubation. Thus females were captured at the nest using a walk-in trap within one day of clutch completion for measurement of maternal phenotype and allocation to an inoculation treatment (see below; uncaught females were dropped from the experiment). We were able to determine the sex of birds in the hand based on body mass and size because lesser black-backed gulls are sexually

dimorphic (Cramp 1983). The ratio of nests at which females were caught did not differ significantly between diet treatments (overall, 55.6 % of 187 nests; chi-square test, $\chi^2 = 0.20$, d.f. = 1, $p = 0.656$). Carotenoid pigmentation of integument was measured by visual comparison with objectively defined colour standards under standardised conditions (bill and leg: Roche Yolk Colour Fan; Hoffman-LaRoche, Basel, Switzerland; bill spot, gape flange and orbital ring: Dulux Trade Colour Palette; Dulux, Slough, UK). Full details are given by Blount *et al.* (2002a, *Chapter 2*). Immediately after colour measurements, 0.5 ml of blood was collected from the tarsal vein. Blood was stored in heparin-coated tubes at 4-6°C in the field, then within 4 h of collection plasma was collected by centrifugation and stored at –20°C until analysis (see below).

b) *Inoculation of females*

Immediately after blood samples were collected, females were assigned randomly to either an immune-challenge treatment (and received an intraperitoneal injection with 1.5 ml washed sheep red blood cells (SRBC; Scottish Antibody Production Unit, Carluke, UK) 2 % vol/vol in PBS), or to a control-challenge treatment (and received an intraperitoneal injection with 1.5 ml PBS). The proportion of females that received the immune-challenge did not differ significantly between diet treatments (overall, 51.9 % of 104 nests; chi-square test, $\chi^2 = 0.13$, d.f. = 1, $p = 0.714$). The inoculum was stored at 5°C and used within 4 days. Inoculum dose was based on that used by Ros *et al.* (1997) in black-headed gulls, *L. ridibundus*, increased in proportion to the larger body mass of female *L. fuscus* (mean of 762 g; Cramp 1983). After injection females were released at the nest. The sample sizes of females in the four treatments were:

1) immune-challenge carotenoid-diet (n = 22 females);

2) control-challenge carotenoid-diet (n = 25 females);

3) immune-challenge control-diet (n = 32 females);

4) control-challenge control-diet (n = 25 females).

Dummy eggs were removed on the day after females were captured to induce the production of replacement clutches. Supplemental feeding continued until 4 days after laying of replacement clutches began, which typically is the day of completion of a modal-sized clutch of three (Bolton *et al.* 1992). Birds that failed to re-lay were supplementally fed until 20 days after removal of the first clutch (no birds laid after that interval). For analysis of changes in maternal phenotype between producing first and replacement clutches, we attempted to recapture all females 4 days after laying of replacement clutches began. We were able to recapture 15 immune-challenge carotenoid-diet females, 15 control-challenge carotenoid-diet females, 12 immune-challenge control-diet females, and 11 control-challenge control-diet females. We recorded integument coloration and body mass, and collected a 0.5 ml blood sample (methods as above).

c) *Chick-rearing capacity*

Since carotenoid supply influences egg quality in this species (Blount *et al.* 2002a,b, *Chapters 2 & 3*), we isolated the effects of parental quality on offspring-rearing capacity from effects operating via egg quality by replacing gulls' own clutches with a control (foster) clutch. Replacement clutch eggs were removed on the day of laying for use in a different experiment (*Chapter 5*), and replaced with dummies. Five days after the laying of replacement clutches began we provided a sample of nests with a clutch of eggs the same size as their own, produced by a non-

manipulated neighbouring pair that had completed laying on the same day. Nests that received a foster clutch were randomly assigned; 33.3 % of all 87 nests that re-laid were not given a foster clutch because a suitable clutch was not available on the required day within the study area. In particular, the proportion of experimental nests excluded from the analysis of chick-rearing capacity increased with advancing laying date (logistic regression: $\chi^2 = 25.56$, d.f. = 1, $p < 0.001$), but did not differ significantly among treatments (maternal challenge, $\chi^2 = 3.32$, d.f. = 1, $p = 0.069$; maternal diet, $\chi^2 = 0.05$, d.f. = 1, $p = 0.827$; all interactions NS). Since birds laying later are of relatively lower quality with lower egg production capacity (Hipfner *et al.* 1999), our study of chick-rearing capacity excluded relatively low quality females and also low quality foster clutches. Three is the modal clutch size in lesser black-backed gulls (Cramp 1983). Among nests used in the study of chick-rearing capacity, 87.9 % of replacement clutches comprised three eggs, 8.7 % comprised two eggs and 3.4 % comprised one egg. Replacement clutch mass did not differ significantly between treatments (ANOVA: maternal challenge, $F_{1,56} = 1.04$, $p = 0.313$; maternal diet, $F_{1,55} = 0.40$, $p = 0.529$; interaction NS). Similarly, among experimental nests receiving a foster clutch there was no difference in the proportion of females that had been caught twice (65.5 % of 58 nests; logistic regression: maternal challenge, $\chi^2 = 0.01$, d.f. = 1, $p = 0.909$; maternal diet, $\chi^2 = 0.53$, d.f. = 1, $p = 0.467$; interaction NS). Thus, all experimental nests in the study of chick-rearing capacity had made a similar level of primary reproductive investment and had experienced a similar level of disturbance. The mass of foster clutches did not differ significantly between treatments (ANCOVA: maternal challenge, $F_{1,54} = 0.00$, $p = 0.956$; maternal diet, $F_{1,55} = 0.38$, $p = 0.542$; clutch size, $F_{1,56} = 160.37$, $p < 0.0001$; all interactions NS).

Nests were followed through incubation and rearing until chicks were 35 days of age and close to fledging (Bolton *et al.* 1992). To facilitate the location of chicks a fence of chicken wire (ca. 0.5 m high x 4 m diameter) was placed around nests 3-4 days before the anticipated day of hatching. On the day of hatching, before individuals had emerged fully from their egg, we colour marked the bill uniquely with a small dab of nail polish. We subsequently colour marked hatchlings with a small strip of PVC tape secured loosely around the tarsus. We recorded any disappearance of eggs or chicks from the territory (i.e. assumed to be losses due to predation), and the hatching and fledging rates among remaining eggs and chicks, respectively. Chicks were recorded as dead only if their corpse was found. Chick body mass is reported for hatchlings (< 24 h of age) and at 28 days of age. We report mass at d 28 as fledgling mass because that was the last age at which data could be obtained for all chicks that subsequently fledged.

d) *Biochemical and immunological assays*

Total carotenoid concentrations ($\mu g\ ml^{-1}$ plasma) were determined using HPLC with detection by absorbence at 445nm as described previously (Surai & Speake 1998, as modified by Blount *et al.* 2002a, *Chapter 2*). We measured anti-SRBC antibody titre in pre- and post-inoculation maternal plasma using a haemagglutination assay (e.g. Roitt *et al.* 1998). Plasma was heated at 56°C for 30 min to inactivate complement, then serially diluted (twofold) in PBS in U–bottomed microtitre plates. An equal volume of a fourfold dilution of the inoculum was added to each well, and plates were covered and left to develop at 4°C for 24 h. Titres were scored as the highest dilution of plasma showing agglutination: post- minus pre-inoculation titre is reported as the antibody response (Ros *et al.* 1997).

e) *Data analyses*

Principal component analysis (PCA) was used to generate indices of integument coloration from the five colour measurements made on each bird. PCA indices were based on measurements made on completion of the first clutch, and the change in coloration between first and replacement clutches (i.e. for each integument trait, $value_2 - value_1$ was entered into the PCA). Variation in the proportions of eggs that disappeared or that hatched, and chicks that disappeared or fledged, respectively, was assessed using binomial regression with a logit link function in GLIM (NAG 1986); thus clutches or broods rather than individual eggs or chicks were the units in the analyses. In most models the ratio deviance/df exceeded 1, so we assessed the statistical significance of independent variables using *F*-ratios (Crawley 1992).

Data for anti-SRBC antibody titres were skewed to the right because of several titres of zero, and a normal distribution could not be achieved by data transformation. Thus, changes in rank(antibody responses) were assessed using parametric ANCOVA, with significance tests based on the Kruskal-Wallis *H* statistic (Zar 1984, page 249).

In all models we included maternal challenge and maternal diet as factors. Since variation in egg production effort has been linked to variation in parasitism (Oppliger *et al.* 1996) and chick-rearing capacity (Heaney & Monaghan 1995; Monaghan *et al.* 1998), we also included the total mass of the eggs produced in the period preceding measurement of the dependent variable as a covariate ('total egg mass'). Total egg mass also correlated negatively with laying date ($r = -0.246$, $n = 104$, $p = 0.012$), and thus accounts for any seasonal effects.

In the analyses of changes in maternal phenotype (antibody response; integument coloration; plasma carotenoid concentration; and body condition, calculated as residuals from body mass regressed on tarsus length), we also included the initial value of the trait (i.e. on first clutch completion) as a covariate (e.g. Ots *et al.* 2001). In the analysis of change in rank(antibody response), values for the covariates were substituted with ranks (Zar 1984).

All models were developed using backwards elimination starting with the highest order interaction; only significant interaction terms are reported. Other statistical tests are introduced in the text of the Results. Two-tailed alpha = 5%, unless stated otherwise. Values are reported as means ± 1 s.e.

RESULTS

a) Effects of carotenoid supply on maternal and egg phenotypes pre-inoculation

As expected, in agreement with our previous findings (Blount *et al.* 2002a, *Chapter 2*), carotenoid supplementation did not influence the timing of laying of first clutches ($t_{102} = 0.28$, $p = 0.779$), clutch mass ($t_{102} = 0.57$, $p = 0.570$), or female body condition indices ($t_{102} = 0.08$, $p = 0.936$), but carotenoid-diet females had significantly higher integument coloration indices ($t_{102} = 5.72$, $p < 0.0001$) and plasma carotenoid concentrations than controls on completion of the clutch ($t_{102} = 5.27$, $p < 0.0001$). Thus, just prior to experimental inoculation, females in the two feeding treatments differed with respect to their carotenoid supplies but not their body condition or gross measures of primary reproductive investment.

b) Effects of inoculation and carotenoid supply on maternal and egg phenotypes

Immune-challenge females exhibited changes in antibody titre and integument coloration consistent with having mounted an immune response. A larger proportion of immune-challenge females (overall, 44.4 % of 27) exhibited a positive antibody response than control-challenge females (overall, 11.5 % of 26) (logistic regression: maternal challenge, $\chi^2 = 6.41$, d.f. = 1, $p = 0.011$), independent of maternal diet or the overall investment in egg production (maternal diet, $\chi^2 = 3.67$, d.f. = 1, $p = 0.055$; total egg mass, $\chi^2 = 0.46$, d.f. = 1, $p = 0.498$). Antibody responses were significantly higher in immune-challenge compared to control-challenge females, and also in control-diet compared to carotenoid-diet females, independent of the overall investment in egg production (Figure 4.1a).

In females of both feeding treatments carotenoid-based integument coloration diminished following immune-challenge, and increased following control-challenge (Figure 4.1b). There was no effect of maternal challenge or diet on changes in plasma carotenoid concentrations (on average, $+0.58 \pm 1.26$ μg ml^{-1}; maternal challenge, $F_{1,48} = 0.02$, $p = 0.877$; maternal diet, $F_{1,50} = 2.72$, $p = 0.105$; total egg mass, $F_{1,49} = 0.40$, $p = 0.531$; initial value, $F_{1,51} = 34.17$, $p < 0.0001$). Similarly, we found no evidence to suggest that maternal challenge or diet influenced the change in body condition that occurred during re-laying. Condition indices diminished to a similar extent in all treatments, independent of the overall investment in egg production (on average, -7.80 ± 8.58; maternal challenge, $F_{1,50} = 0.55$, $p = 0.462$; maternal diet, $F_{1,49} = 0.33$, $p = 0.568$; total egg mass, $F_{1,48} = 0.06$, $p = 0.809$; initial value, $F_{1,51} = 23.85$, $p < 0.0001$).

The proportion of fostered eggs that gave rise to fledged young did not differ significantly among treatments (Figure 4.2a). Similarly, there were no significant effects of maternal challenge or diet on chick-rearing capacity when each stage of the rearing attempt was considered independently (overall: 20.0 % of 165 eggs disappeared; 92.4 % of 132 remaining eggs hatched; 13.9 % of 122 chicks disappeared; and 62.9 % of 105 remaining chicks fledged; Table 4.1). However, the hatching rate was higher among eggs incubated by females that had invested most in the production of their own eggs (Table 4.1, footnote).

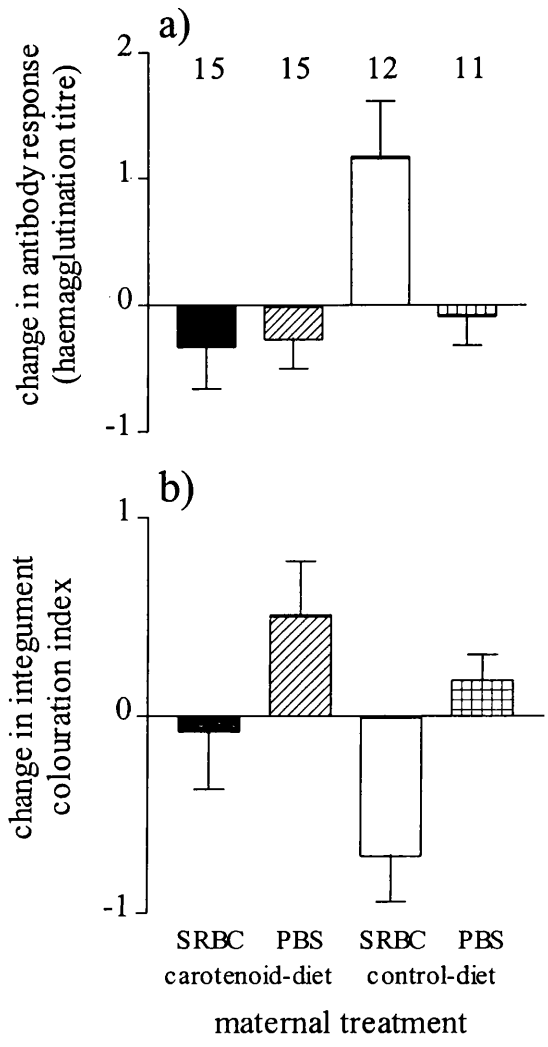

Figure 4.1: Changes in maternal phenotype in relation to challenge (immune challenge, SRBC; or control, PBS) and diet treatments (carotenoid; or control). Means (± 1 s.e.) are shown. Numbers in parentheses are the sample sizes of females. a), Antibody response, which was significantly higher in immune- than control-challenge females (ANCOVA based on ranks: maternal challenge, $H_{1,49} = 29.73, p < 0.0001$) and in control- compared to carotenoid-diet females (maternal diet, $H_{1,49} = 18.85, p < 0.0001$), but did not covary with the overall investment in egg production (total egg mass, $H_{1,48} = 0.26, p = 0.610$; initial value, $H_{1,49} = 405.05, p < 0.0001$). b), Change in integument coloration index (first factor from a principal component analysis, see Methods), which declined in immune-challenge females compared to control-challenge females (maternal challenge, $F_{1,51} = 7.93, p = 0.007$; maternal diet, $F_{1,50} = 3.60, p = 0.064$; total egg mass, $F_{1,49} = 1.15, p = 0.290$; initial value, $F_{1,48} = 0.99, p = 0.325$).

As expected given that foster clutches were of 'standard' mass (see Methods), the mass of a-, b- and c-chicks at hatching did not differ significantly among treatments (ANOVAs, all $F < 3.79$ and $p > 0.05$). Total brood mass at fledging did not differ significantly among treatments when controlling for the number of fledglings (maternal challenge, $F_{1,32} = 1.55, p = 0.223$; maternal diet, $F_{1,31} = 0.13, p = 0.716$; total egg mass, $F_{1,30} = 0.20, p = 0.888$; number of fledglings, $F_{1,33} = 320.43, p < 0.0001$). Thus, variation in brood mass at fledging was largely a function of offspring number. However, the total biomass of offspring reared (total brood mass without controlling for brood size, an overall measure of parental investment) was significantly lower in broods reared by immune-challenge than control-challenge females that had received the control-diet (Figure 4.2b). In contrast, this relationship was not found for carotenoid-diet females (Figure 4.2b).

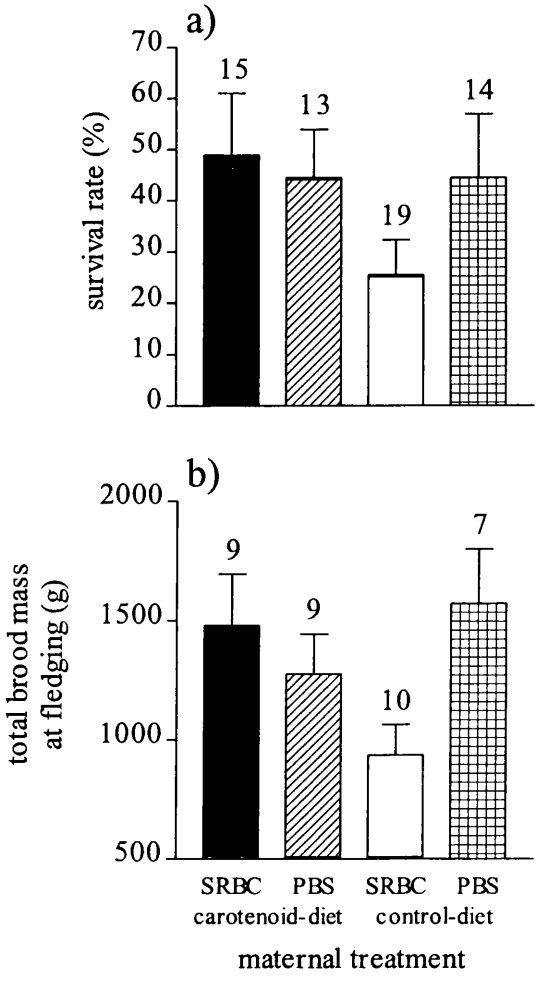

Figure 4.2: Chick-rearing capacity in relation to challenge (immune challenge, SRBC; or control, PBS) and diet treatments of female parent (carotenoid or control). a), Survival rate of offspring (proportion of fostered eggs that gave rise to fledged young; numbers above bars are the sample sizes of foster clutches in each treatment), which did not differ significantly among treatments (binomial regression with number of eggs fostered as denominator: maternal challenge, $F_{1,54} = 0.58, p = 0.448$; maternal diet, $F_{1,56} = 1.01, p = 0.318$; total egg mass, $F_{1,55} = 0.74, p = 0.393$; deviance of null model, 131.48 and 57 d.f; deviance of final model, 129.15 and 56 d.f.). b), Total mass of foster broods at fledging. Means (± 1 s.e.) are shown, and numbers above bars are the sample sizes of broods in each treatment. The effect of maternal immune-challenge on brood mass at fledging differed between the two maternal diet treatments (maternal challenge, $F_{1,31} = 1.38, p = 0.250$; maternal diet, $F_{1,31} = 0.46, p = 0.501$; maternal challenge by maternal diet interaction, $F_{1,31} = 5.21, p = 0.029$), and was not influenced by the overall maternal investment in egg production (total egg mass, $F_{1,30} = 0.38, p = 0.542$). The source of the significant interaction effect was largely attributable to control-diet females: total brood mass at fledging was influenced significantly by maternal immune-challenge in broods reared by control-diet females ($t_{15} = 2.61, p = 0.020$), but not carotenoid-diet females ($t_{16} = 0.75, p = 0.465$).

Table 4.1: Variation in disappearance and hatching rates of foster eggs, and disappearance and fledging rates of chicks hatching from those eggs, in relation to the challenge (immune challenge or control) and diet treatment of the female parent (carotenoid or control). Results are from binomial regression analyses using GLIM; models were developed using backwards elimination, and only main effects are shown because all interaction terms were non significant (see Methods for details).

Source	egg disappearance rate				hatching rate				chick disappearance rate				fledging rate			
	deviance	d.f.	*F*	*p*	deviance	d.f.	*F*	*p*	deviance	d.f.	*F*	*p*	deviance	d.f.	*F*	*p*
null model	116.53	57			41.32	51			75.61	49			98.99	46		
final model	113.29	56			37.93	50			72.90	48			94.49	45		
maternal challenge		1,55	0.75	0.389		1,48	1.59	0.213		1,46	0.03	0.863		1,43	0.38	0.543
maternal diet		1,54	0.00	1.000		1,49	2.23	0.142		1,47	1.21	0.277		1,45	2.14	0.150
total egg mass		1,56	1.60	0.211		1,50	4.46	0.039[†]		1,48	1.78	0.189		1,44	0.93	0.339

[†] slope ± s.e. = 0.016 ± 0.009

DISCUSSION

This study has shown that there was a trade-off between immune function and chick-rearing capacity in female lesser black-backed gulls, which was mediated by carotenoid supply. There was no significant effect of maternal immune-challenge on the total number of offspring reared to fledging, or brood total mass at fledging whilst statistically controlling for brood size, but the total biomass of fledglings (i.e. the product of both offspring number and their mass; a measurement analogous to clutch mass in the study of egg production capacity) was lower in broods reared by immune-challenge than control-challenge females that had received the control-diet. The number of surviving young produced per breeding event is obviously a central component of fitness, and relatively light mass at fledging is likely to translate into poor post-fledging survival (e.g. Magrath 1991; Both *et al.* 1999; Naef-Daenzer *et al.* 2001). Therefore, total fledgling mass is a reasonable estimate of parental investment. As hypothesised, experimentally increased carotenoid supply disengaged the trade-off: there was no difference in the total mass of broods reared by immune-challenge and control-challenge females that had received the carotenoid-diet. We found no evidence to suggest that carotenoid supply was limiting for chick-rearing capacity *per se* – that may simply reflect the fact that birds used in the analysis of chick-rearing capacity were relatively early layers and therefore of high quality (see Materials and Methods). However, carotenoid supply was limiting for chick-rearing capacity in females that had also faced the demands of mounting an acquired immune response.

Activation of the immune system can require increased energy turnover (reviewed by Lochmiller & Deerenberg 2000). However, evidence of energetic modulation of trade-offs between immune function and reproduction in birds has so far been

equivocal (reviewed by Råberg *et al.* 1998; Norris & Evans 2000). In a study of captive blue tits, *Parus caeruleus*, Svensson *et al.* (1998) reported that increased energy turnover (invoked by exposure to cold stress) resulted in lowered antibody responses following inoculation with diphtheria-tetanus. But since inoculation did not induce any change in body mass, or basal metabolic rate (when statistically controlling for variation in body mass), they concluded that energy limitation was unlikely to explain the reduced immune function in cold-exposed birds. In contrast, it has recently been reported that inoculation with SRBCs resulted in increased basal metabolic rate and reduced body mass in wild great tits, *Parus major*, consistent with a high energetic cost of immune function (Ots *et al.* 2001). These discordant results may simply reflect differences in the scale of immune system activity invoked by different antigens, or differences in energy supply under captive and wild conditions (Ots *et al.* 2001). Alternatively, it has been suggested that energy supply *per se* may not be the limiting factor for immune function and other energy-demanding activities such as reproduction, but rather it is energy turnover that has a marked effect on free radical production (Svensson *et al.* 1998). By this scenario, antioxidants are the limiting resource rather than energy supply *per se*.

Females that invested most in producing their own eggs, as measured by total egg mass, hatched a larger proportion of their foster clutch. This is consistent with the explanation that higher quality females, which produced heavier clutches, also had better incubation performance. Alternatively, perhaps earlier-laid foster clutches were of inherently higher quality and therefore more likely to hatch (total egg mass and laying date were negatively correlated; see Methods). However, we found no evidence that maternal challenge or carotenoid supply influenced the hatching rate of foster eggs.

Mounting an immune response incurred a decline in carotenoid-based integument coloration in both carotenoid- and control-diet females, consistent with the explanation that carotenoids were limiting for antioxidant activity and immune function (and for similar results in barn swallow, *Hirundo rustica*, chicks see Saino *et al.* 2000). It has been demonstrated in domestic hens that immune system activation incurs diminished plasma carotenoids due to their oxidation by free radicals produced during the immune response (Allen 1997). In contrast, plasma carotenoids did not decline in immune-challenge females. However, plasma carotenoids seem likely to reflect relatively short-term patterns of change whereas a decline in integument pigmentation is likely to reflect a progressive depletion of body stores of carotenoids (Klasing 1998).

All females received a supplement of equal calorific value that differed only with respect to its carotenoid content, suggesting that carotenoid limitation rather than energy supply was the mechanism underlying the observed effects on offspring-rearing capacity. Possibly, carotenoid-diet females were able to expend more energy in provisioning their young without incurring oxidative stress. It has been shown that strenuous physical exercise can result in oxidative stress in humans, which can be mitigated by increased dietary intake of antioxidants (review by Ji 1995; Sen 2001). We have previously shown that carotenoid supplementation resulted in increased plasma antioxidant activity in lesser black-backed gulls (Blount *et al.* 2002a, *Chapter 2*). Carotenoid supplementation may also have resulted in more efficient immune function, thereby reducing the total energy requirement of dealing with SRBC challenge, resulting in higher energy availability for other activities (i.e. foraging). Carotenoid-diet females exhibited lower antibody responses than control-diet females following the immune-challenge, which may indicate more efficient

clearance of SRBCs in females with high carotenoid supplies. Other studies of gulls have shown that peak antibody titres are attained 6 days post-inoculation (Grasman *et al.* 1996; Ros *et al.* 1997), but we collected post-inoculation plasma samples after an interval of 12 – 20 days. Thus, the positive antibody responses that we reported are likely to reflect the regression phase of the immune response, and apparent non-responders may in fact be birds in which the immune response had already subsided by the day of sample collection. Clearance rate of antigen has been shown to correlate positively with plasma carotenoid supplies in humans (Metzger *et al.* 2001), and with the brightness of carotenoid-based tail-patches among male greenfinch, *Carduelis chloris* (Lindström & Lundström 2000). Alternatively, SRBCs may have been cleared by the operation of innate immune defences. Carotenoids can stimulate the phagocytic ability of neutrophils and macrophages by inactivating 'surplus' free radicals produced by these immune cells whilst attacking antigens (reviewed by Chew 1993, 1996). It has been reported that phagocytic clearance of antigen during the initial, inflammatory stage of an immune response may mean that insufficient antigen remains to invoke an acquired immune response (Weiner & Bandieri 1974; Cheng & Lamont 1988). It is also possible that individuals with low antibody responses were facultatively tolerating SRBC antigen whilst maximising their reproductive investment (see Deerenberg *et al.* 1997). Although the mechanism remains unclear, our results clearly show that high carotenoid supply enabled immune-challenge females to cope with inoculation and invest more in parental care, as reflected in the relatively high mass of the broods that they reared.

Our results suggest that variation in carotenoid supply could explain the observed deleterious effects of mounting an acquired immune response on chick-rearing capacity reported in earlier studies (Ilmonen *et al.* 2000; Råberg *et al.* 2000). It

would also be interesting to test the complimentary hypothesis that increased carotenoid supply uncouples the observed trade-off between high work rate and condition in parent birds (Gustafsson & Sutherland 1988; Richner *et al.* 1995; Daan *et al.* 1996; Deerenberg *et al.* 1997; Nordling *et al.* 1998; Moreno *et al.* 1999). In conclusion, our results provide support for the suggestion that reproductive effort and immune defence are limited by competition over shared resource(s) (Gustasson *et al.* 1994; Sheldon & Verhulst 1996), and that carotenoid supply is one resource that underlies a trade-off between these activities.

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Chapter 5

The Influence of Carotenoid Supply and Immune Challenge on Egg Production Capacity: an Experimental Study in Gulls *Larus fuscus*

INTRODUCTION

Costs of reproduction, arising through trade-offs between physiological activities competing for the same resource, have an important influence on the evolution of life histories (Williams 1966; Stearns 1992). Birds are good model organisms for the study of reproductive costs because the stages of each reproductive attempt (egg production, incubation and chick rearing) can be manipulated independently to elucidate the relative costs (e.g. Monaghan & Nager 1997). Most such studies have focussed on trade-offs between chick-rearing effort and subsequent parental survival and fecundity, the traditional assumption being that investment in earlier stages of breeding imposes relatively trivial demands (reviewed by Stearns 1992). However, it is now recognised that egg production can also be costly, and should be taken into account in order to fully understand the constraints on reproductive investment (Partridge & Harvey 1985; Stearns 1992; Monaghan & Nager 1997).

In birds, females must provision the egg with all the resources required for embryonic development in one investment. It has recently become clear that females can incur considerable costs solely through egg production (increased parasitism: Oppliger *et al.* 1996; reduced chick-rearing capacity: Heaney & Monaghan 1995; Monaghan *et al.* 1995, 1998; reduced future breeding rate: Nager *et al.* 2001a). There is also some evidence that egg quality declines with increasing egg number (reviewed by Williams 1994; Bernardo 1996). For example, increased egg-production (through egg removal during laying) resulted in additionally-laid eggs that were less likely to give rise to a fledged chick than eggs from a normal-sized clutch (Nager *et al.* 2000). Therefore, studies of birds have suggested that increasing egg production effort can trade against the phenotypic quality of females and also

their eggs. However, the mechanisms underlying such trade-offs are still poorly understood (Williams 1994; Bernardo 1996; Perrins 1996; Monaghan & Nager 1997; Meijer & Drent 1999).

One little explored possibility is that maternal supplies of carotenoids are limiting both for her capacity to produce eggs and to maintain her health. Carotenoids are antioxidant pigments present in many tissues in animals, including avian egg yolk (Goodwin 1984). As antioxidants, carotenoids can neutralise free radicals that arise through oxidative metabolism; if not under control (= oxidative stress), free radicals can damage DNA, proteins and lipids, and thereby impair physiological processes (reviewed by Chew 1996; Edge *et al.* 1997; Stahl & Sies 1999). There is also some evidence that carotenoids can stimulate the expression of cytokines, interleukins, genes and intercellular signalling responsible for the development of immune function (reviews by Stahl & Sies 1999; Møller *et al.* 2000). Animals cannot synthesise carotenoids de novo, and ultimately must obtain these pigments in their diet (Goodwin 1984). It has therefore been suggested that carotenoids could be limiting for immune function (Lozano 1994), antioxidant activity (von Schantz *et al.* 1999), and the production and quality of eggs in birds (Royle *et al.* 1999; Blount *et al.* 2000). These suggestions stem from evidence that antioxidant activity and immune function correlate positively with carotenoid intake in humans, laboratory mammals and domesticated bird species (e.g. Allard *et al.* 1994; Jyonouchi *et al.* 1994; McWhinney *et al.* 1989; Tengerdy *et al.* 1990; Woodall *et al.* 1996; Cheng *et al.* 2001; reviewed by Møller *et al.* 2000). Similarly, experiments have shown that female domestic hens fed additional carotenoids transfer them to their eggs, resulting in enhanced antioxidant activity (Mayne & Parker 1989; Lawlor & O'Brien 1995; Surai & Speake 1998), and immune function in offspring (Haq, Bailey & Chinnah

1996). Whether carotenoid supply underlies a trade-off between egg-production and immune function in female wild birds remains to be seen. Evidence of carotenoid-limitation of these activities comes mostly from correlative studies that have considered only one or other component of the putative trade-off (e.g. Royle *et al.* 1999; Saino *et al.* 1999; reviewed by Møller *et al.* 2000). We recently found that wild female gulls provisioned with carotenoids during the pre-laying period had higher levels of carotenoids and antioxidant activity, and lower levels of immunoglobulins (Ig) in plasma compared to controls, which was mirrored in the composition of the eggs that they laid (Blount *et al.* 2002a, *Chapter 2*). Possibly, these results indicate that carotenoids were limiting for egg quality and maternal immune function. However, to elucidate whether a trade-off exists between carotenoid allocation to these activities requires manipulation both of carotenoid supply and immune system activation.

Here, we carry out such a test in a study of wild lesser black-backed gulls, *Larus fuscus*. In a factorial experiment we manipulated maternal carotenoid supplies during the pre-laying period by supplemental feeding with either carotenoids or a control (carotenoid-free) diet, and manipulated maternal investment in immune function by injecting females with either a benign antigen or saline as a control. We subsequently measured egg-production capacity (laying rate, egg and clutch size), and egg quality in terms of composition (yolk concentrations of carotenoids and Ig) and resultant chick performance (immune function, growth and survival) when reared singly by foster parents. We hypothesised that 1) under a natural diet there would be a trade-off between immune function and egg production capacity; and 2) supplemental feeding with carotenoids would improve egg production / quality, and there would no longer be a trade-off between the two.

MATERIALS AND METHODS

a) *Manipulation of maternal carotenoid supplies and immune system activation*

The experiment was carried out between April – August 2000 at a colony of ca. 24 000 pairs of lesser black-backed gulls, Walney Island, Cumbria, UK. As part of a larger experiment (see *Chapter 4*), in early April about one month before laying started we randomly allocated nests in a central part of the colony to a feeding treatment. We supplementally fed females daily at the nest either with 2 mg carotenoids in a 20 g bolus of vegetable fat (Van den Bergh Foods Ltd., Crawley, UK) (carotenoid-diet; $n = 91$ nests) or a carotenoid-free ration of 20 g fat only (control-diet; $n = 96$ nests). Further details of supplemental feeding are given by Blount *et al.* 2002a,b (*Chapters 2 & 3*).

We attempted to capture all females at the nest on completion of their first clutch (uncaught females were dropped from the experiment). The ratio of females that were caught did not differ significantly between feeding treatments (overall, 55.6 % of 187 nests; chi-square test, $\chi^2 = 0.02$, d.f. = 1, $p = 0.656$). The carotenoid-based integument pigmentation of females was measured by visual comparison with colour standards, and a small blood sample (0.5 ml) was collected from the tarsal vein, for use in another experiment (*Chapter 4*). Females were then assigned randomly to either an immune-challenge treatment (and received an intraperitoneal injection of 1.5 ml 2 % (vol/vol) washed sheep red blood cells (SRBC) in phosphate-buffered saline (PBS)), or to a control-challenge treatment (and received a similar injection of 1.5 ml PBS), then released at the nest. Thus, ultimately the sample sizes of females in the four treatments were:

1) immune-challenge carotenoid-diet ($n = 22$ females);

2) control-challenge carotenoid-diet (n = 25 females);

3) immune-challenge control-diet (n = 32 females);

4) control-challenge control-diet (n = 25 females).

There was no significant difference between diet treatments in the proportion of females that responded to inoculation with a positive antibody response; the effects of the treatments on maternal phenotype are presented fully elsewhere (see *Chapter 4*). The day after females were captured, first clutches were removed to induce the production of replacement clutches, which are the main focus of the present paper. Supplemental feeding continued until after the laying of replacement clutches was complete (for further details see *Chapter 4*).

b) *Measurement of egg and chick phenotype*

First and replacement clutch eggs were collected on the day of laying, weighed using an electronic balance (± 0.1 g) and replaced with dummies. Hereafter we refer to eggs as b_1-eggs, a_2-eggs etc, where the letter denotes the position in the laying sequence and the subscript denotes the clutch number. In b_1-eggs and b_2-eggs, yolk was separated from albumen, weighed (± 0.1 g) then homogenised and stored at –20°C until compositional analysis (carotenoid and immunoglobulin concentrations; see below). All a_1-eggs and c_1-eggs were collected for use in other experiments, the results of which will be presented elsewhere.

All a_2-eggs and c_2-eggs were fostered to control parents, to isolate the effects of egg phenotype on offspring performance from effects of maternal condition operating during the rearing period (Monaghan *et al.* 1998). Foster parents were all high quality pairs, having completed a 3-egg first clutch of their own on the same day as experimental pairs completed their replacement clutch. Foster nests received

a single experimental egg in place of one of their own eggs; to eliminate any effects of sibling competition on chick performance we replaced foster parents' remaining two eggs with dummies.

All nests were visited once daily through incubation and the nestling period until fledging. To facilitate the location of chicks we encircled nests with a fence of chicken wire (ca. 0.5 m high x 4 m diameter) 3 or 4 days before eggs were due to hatch. We recorded any disappearance of eggs and the hatching rate among remaining eggs, and similarly, we recorded any disappearance of chicks and the survival rate among remaining chicks. Ultimately, we concluded that chicks had survived if they were alive at 35 days of age, which is close to fledging (Bolton *et al.* 1992). Chicks were deemed to have died only if their corpse was found on the territory. Some replacement clutch eggs (22.9 % of 157) and chicks (21.5 % of 79) disappeared from their foster parents' territory, presumably due to predation by other gulls, and some additional eggs (1.3 % of 157) were abandoned by their foster parents. There was no evidence that these causes of loss were related to egg quality, as influenced by the treatment group of the original parents or the position (a or c) in the laying sequence (logistic regressions: egg loss, maternal challenge, $\chi^2 = 2.34$, d.f. $= 1, p = 0.126$; maternal diet, $\chi^2 = 0.20$, d.f. $= 1, p = 0.657$; egg position, $\chi^2 = 1.42$, d.f. $= 1, p = 0.233$; interactions NS; chick loss, maternal challenge, $\chi^2 = 0.00$, d.f. $= 1, p = 0.998$; maternal diet, $\chi^2 = 2.45$, d.f. $= 1, p = 0.117$; egg position, $\chi^2 = 2.35$, d.f. $= 1, p = 0.126$; interactions NS). Thus, we excluded these cases from our analyses of offspring performance.

We measured chick body mass and tarsus length within 24 h of hatching, then on three occasions during the linear phase of nestling growth (d 4, 13 and 22), and just prior to fledging (d 28). We measured body mass using an electronic balance (chicks

< 200 g, ± 0.1 g) or a spring balance (chicks > 200 g, ± 2.5 g), and tarsus length using a sliding calliper (± 0.1 mm). On each of days 4, 13 and 22, we collected 0.5 ml of blood from the tarsal vein. Blood was stored in heparin-coated tubes at 4-6°C in the field, then within 4 h of collection plasma was collected by centrifugation and stored at –20°C until analysis (see below).

On day 4, following blood collection, we tested two independent branches of immune function in chicks. To assay cell-mediated immune function we injected the right foot web intradermally with 0.5 mg phytohemagglutinin (PHA; Sigma, UK) in 0.1 ml PBS using a 29 g needle. The left foot web was injected with the same volume of PBS as a control challenge. PHA is a lectin that induces induration, associated with perivascular accumulation of T-lymphocytes and other leukocytes when injected intradermally (McCorkle *et al.* 1980). Immediately before and 24 ± 0.5 h post-injection, we measured the thickness of foot webs (± 0.02 mm) using a Köfer K50 gauge (Coventry Gauge Ltd., Poole, UK). We modified the gauge by reversing its spring action, such that the feelers closed when released and exerted a constant, light pressure on the foot web. PHA response is reported as the post- minus pre-injection thickness of the right (experimental) foot web, minus any change in thickness of the left (control) foot web. Immune sensitisation to PHA has been studied previously in gulls (Grasman *et al.* 1996; Alonso-Alvarez & Tella 2001). Following injection of foot webs, chicks were injected intraperitoneally with 0.2 ml of washed *Brucella abortus* cells (BA strain 99; Veterinary Laboratories Agency, Weybridge, UK) 5 % vol/vol in PBS. BA is a T lymphocyte-independent antigen (e.g. McCorkle & Thaxton 1988; Montgomery *et al.* 1991). The inoculum was stored at 5°C and used within 4 days. Inoculum dose was the same as that used by Montgomery *et al.* (1991) in 7-day-old domestic hen chicks. Anti-BA antibody titres

were measured in blood collected on days 4 (pre-) and 13 (post-inoculation) (see below).

d) *Biochemical and immunological assays*

Total carotenoid concentrations in yolk and plasma were determined using HPLC with detection by absorbence at 445nm as described previously (Surai & Speake 1998, as modified by Blount *et al.* 2002a, *Chapter 2*). Total carotenoid concentrations are reported as $\mu g\ g^{-1}$ yolk or $\mu g\ ml^{-1}$ plasma.

Total immunoglobulin (Ig) concentrations in yolk and plasma were measured using a single radial immunodiffusion assay as described previously (Roitt *et al.* 1998; Blount *et al.* 2002a, *Chapter 2*), with slight modifications. Sheep plasma containing anti-lesser black-backed gull Ig was obtained from the Scottish Antibody Production Unit (Carluke, UK), and stored at –20°C until use as antiserum. Antiserum was mixed into a preparation of 2 % agar in barbitone buffer at a ratio of 1:14 (v/v) at 56°C, 10 ml of which was poured evenly onto radial immunodiffusion plates (The Binding Site Ltd., Birmingham, UK) and allowed to set. Circular wells (4 mm diameter) were punched into the agar at an equal spacing, and 10 µl of test antigen was added (samples of egg yolk were first diluted 1:1.5 w/v in PBS). Plates were left for 72 h for the antigen to diffuse out of the wells and bind with the antiserum, precipitating in a ring. Plates were viewed at 6.5 x magnification using a video camera (TK-1381; JVC) attached to a binocular microscope (M3Z; Wild, Heerbrugg, Switerland). Images were captured using Falcon PCI Image Lab software (MCM Design, Birkerød, Denmark), and ring diameter was measured using Scion Image Beta 3b software (Scion Corporation, Maryland, USA). Ring diameter was measured two times in different directions and the mean used for subsequent

analysis; (ring diameter)^2 is proportional to the antigen (i.e. gull Ig) concentration (Roitt *et al.* 1998). Unknowns were determined by interpolation from a standard curve based on six serial dilutions of a pool of non-test samples of gull plasma in PBS. Samples were allocated among plates at random.

We measured BA antibody responses using an agglutination assay (e.g. Birkhead *et al.* 1998; Roitt *et al.* 1998). Plasma was serially diluted (twofold) in PBS in U–bottomed microtitre plates. An equal volume of a fourfold dilution of the inoculum was added to each well, and plates were covered and left to develop at 4°C for 24 h. Titres were scored as the highest dilution of sample showing agglutination, and the BA antibody response is reported as the post- minus pre-inoculation titre.

f) *Data analyses*

In analyses of maternal egg production capacity and egg phenotype we included maternal challenge (immune-challenge or control-challenge) and maternal diet (carotenoid or control) as factors. We also included laying date as a covariate because earlier layers are higher quality females with a higher egg production capacity (e.g. Hipfner *et al.* 1999; Nager *et al.* 2000). Changes in b-egg phenotype (clutch mass; carotenoid and Ig concentrations) between first and replacement clutches were assessed in terms of value$_2$ minus value$_1$ (= dependent variable), with the initial value of the trait (i.e. at first clutch completion) as a covariate, thus controlling for effects of inter-individual differences in phenotype at first capture on subsequent changes in phenotype (Merilä & Wiggins 1997; Ots *et al.* 2001). Allometric variation in b-egg yolk mass relative to egg mass was assessed using $\log_{10}$:$\log_{10}$ regression in ANCOVA models (i.e. including explanatory variables) (Ricklefs 1984).

Variation in chick phenotype over the nestling period (plasma carotenoids and Ig) was analysed using repeated-measures ANCOVA (rmANCOVA) with age (d 4, 13 and 22) as a within-subjects variable, maternal diet, maternal challenge and position in the laying sequence (a- or c-egg) as between-subjects factors, and instantaneous growth rate over the period preceding measurement of the dependent variable of interest as a covariate. We included position in the laying sequence as a factor because a-eggs are larger than c-eggs in gulls (e.g. Blount *et al.* 2002a, *Chapter 2*) and have been shown to have an inherently higher survival probability (Parsons 1975). The difference in mass between a_2-eggs and c_2-eggs did not differ significantly between treatments (ANCOVA: maternal challenge, $F_{1,79} = 3.31$, $p = 0.073$; maternal diet, $F_{1,78} = 0.37$, $p = 0.543$; initial value, $F_{1,80} = 17.33$, $p < 0.0001$). Instantaneous growth rates between hatching and various intervals during the nestling period were calculated for each chick using the equation (for d 0 – 4, and d 0 – 13, respectively):

$$R = (\log_{10} W_2 - \log_{10} W_1)/(t_2 - t_1)$$

where W is mass and t is time. For chicks that survived until at least d 22, we calculated R as $(R_{d\,0-4} + R_{d\,4-13} + R_{d\,13-22})/3$, and similarly, for chicks that survived until at least d 28 we calculated R as $(R_{d\,0-4} + R_{d\,4-13} + R_{d\,13-22} + R_{d\,22-28})/4$. We used growth rate as a covariate because resource allocation to growth may trade-off with other life history activities (Metcalfe & Monaghan 2001).

In all rmANCOVA models we evaluated within-subjects effects by the multivariate approach; tests based on Pillai's Trace, Wilks' Lambda, Hotelling's Trace and Roy's Largest Root always gave identical F-values. Significant effects

were followed by post-hoc contrasts (Bonferroni adjusted alpha = 0.025). Data for chick plasma carotenoids remained heteroscedastic after logarithmic transformation; we only present results of the analysis based on untransformed data, because ANOVA is robust for heteroscedasticity if results are statistically significant (Ito 1980).

The survival rate of offspring was analysed using logistic regression with maternal diet, maternal challenge, and position in the laying sequence as factors. That analysis used the number of chicks as the sampling units, and thus involved more degrees of freedom than if the number of nests had been used as the sampling units. Thus, we tested whether the conclusions of the analysis of offspring survival differed in a logistic regression with binomial error distribution and a logit link function, with *n*(surviving chicks per brood) as the response variable, and *n*(chicks hatched per brood, excluding losses due to predation) as the denominator. The ratio between the explained deviance and the d.f. was less than 1, so the significance test was based on the chi-square distribution (Crawley 1992).

All models were developed using backwards elimination starting with the highest order interaction; we only report significant interaction terms. Other statistical tests are introduced in the text of the Results. Two-tailed alpha = 5%. Values are reported as means ± 1 s.e.

RESULTS

a) *Effects of carotenoid supply on egg production pre-inoculation*

As expected, in agreement with our previous findings (Blount *et al.* 2002a, *Chapter 2*), carotenoid supplementation did not influence the maternal capacity to produce a first clutch (proportion of females that laid: carotenoid-diet, 72.5 % of 91 nests; control-diet, 77.1 % of 96 nests; chi-square test, $\chi^2 = 0.03$, d.f. = 1, $p = 0.873$; timing of laying: carotenoid-diet, 10.40 ± 0.94 May; control-diet, 10.05 ± 0.83 May; $t_{102} = 0.28$, $p = 0.779$; clutch mass: carotenoid-diet, 219.90 ± 5.94 g; control-diet, 224.15 ± 4.66 g; maternal diet, $F_{1,101} = 0.27$, $p = 0.604$; laying date, $F_{1,102} = 4.60$, $p = 0.034$; heavier clutches were produced by earlier layers). However, also as expected (see Blount *et al.* 2002a, *Chapter 2*), carotenoid-supplementation gave rise to first clutch eggs that contained higher concentrations of carotenoids and lower concentrations of Ig compared to controls (b_1-egg yolk carotenoids: carotenoid-diet, 61.22 ± 3.82 μg g^{-1} yolk; control-diet, 38.38 ± 2.50 μg g^{-1} yolk; ANOVA, maternal diet, $F_{1,101} = 26.76$, $p < 0.0001$; laying date, $F_{1,100} = 0.59$, $p = 0.445$; yolk Ig indices: carotenoid-diet, 1.13 ± 0.02; control-diet, 1.19 ± 0.02; ANOVA, maternal diet, $F_{1,101} = 4.58$, $p = 0.035$; laying date, $F_{1,100} = 0.74$, $p = 0.391$). Thus, prior to capture and inoculation, females in the two feeding treatments did not differ with respect to gross measures of primary reproductive investment, but carotenoid-diet females had produced eggs containing higher concentrations of carotenoids and lower concentrations of Ig than control-diet females. As previously reported in gulls (reviewed by Williams 1994), lighter first clutch eggs contained disproportionately lighter yolks in both feeding treatments (ANCOVA with $\log_{10}(b_1$-yolk mass) as a dependent variable: maternal diet, $F_{1,100} = 0.62$; $p = 0.433$; laying date, $F_{1,99} = 0.01$; p

= 0.931; $\log_{10}(b_1\text{-egg mass})$, $F_{1,101} = 70.85$; $p < 0.0001$; slope, 0.717 ± 0.085; $F_{1,101}(\text{slope} = 1) = 11.08$, $p = 0.001$).

b) ***Effects of maternal immune challenge and carotenoid supply on egg production capacity***

There was no significant effect of maternal immune challenge on the proportion of females that re-laid following the removal of first clutches, but a significantly larger proportion of carotenoid-diet females re-laid then control-diet females (Figure 5.1a). Maternal treatment did not influence the latency to re-lay, being on average 11.46 ± 0.18 d after first clutch removal (all effects $F < 0.01$, $p > 0.915$). Similarly, maternal immune challenge and carotenoid supply did not influence changes in the mass of replacement compared to first clutches (an average decrease of 3.2 %; maternal challenge, $F_{1,82} = 0.06$; $p = 0.810$; maternal diet, $F_{1,83} = 1.91$; $p = 0.170$; initial value, $F_{1,84} = 30.38$; $p < 0.0001$; laying date, $F_{1,84} = 4.03$; $p = 0.048$; later layers produced relatively light replacement clutches compared to first clutches: partial correlation coefficient (controlling for initial value), $r = -0.214$). However, whereas maternal challenge did not influence changes in the size of replacement compared to first clutches, but there was a smaller decline in clutch size in the carotenoid-diet than in the control-diet group (Figure 5.1b).

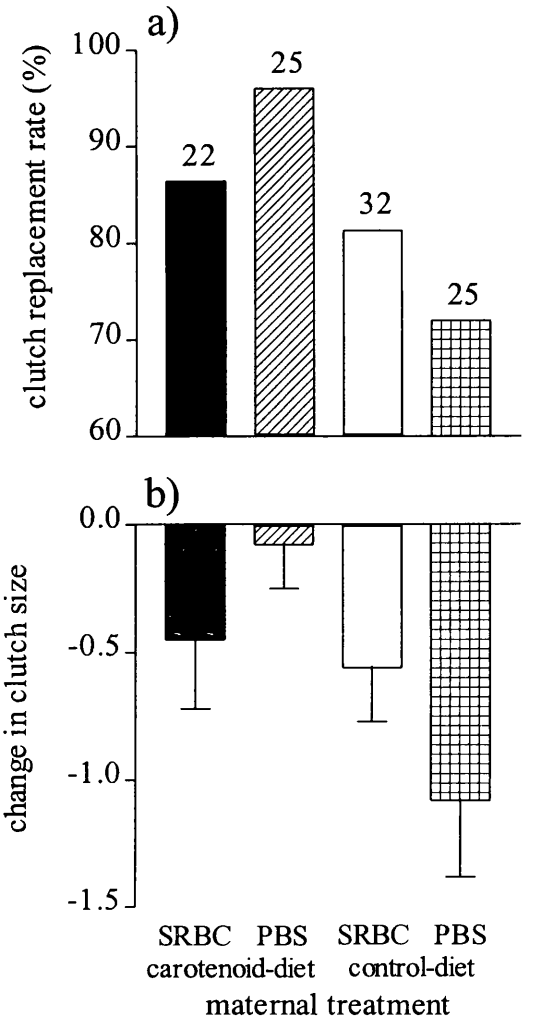

Figure 5.1: Egg production capacity in relation to the challenge (immune challenge, SRBC; or control challenge, PBS) and diet treatments of female gulls (carotenoid; or control). Numbers above bars are sample sizes of females in each treatment. a), Clutch replacement rate. Bars represent the proportion of females that re-laid. There was no effect of immune-challenge or laying date on clutch replacement rate, but a significantly larger proportion of carotenoid-diet females re-laid in comparison with control-diet females (logistic regression: maternal challenge, $\chi^2 = 0.00$, d.f. = 1, $p = 0.974$; maternal diet, $\chi^2 = 4.23$, d.f. = 1, $p = 0.040$; laying date, $\chi^2 = 0.98$, d.f. = 1, $p = 0.322$). b), Change in clutch size, which was not influenced by immune-challenge or laying date, but declined more in the control-diet than in the carotenoid-diet group (ANOVA: maternal challenge, $F_{1,99} = 0.19$; $p = 0.667$; maternal diet, $F_{1,101} = 5.19$; $p = 0.025$; initial value, $F_{1,101} = 17.12$; $p < 0.0001$; laying date, $F_{1,100} = 3.16$; $p = 0.079$).

c) *Effects of maternal immune challenge and carotenoid supply on egg quality: egg composition*

The mass of b_2-egg yolk correlated with egg mass differently between the two maternal feeding treatments, but was not influenced by maternal immune-challenge (ANCOVA with $\log_{10}(b_2\text{-yolk mass})$ as a dependent variable: maternal challenge, $F_{1,78} = 2.16$; $p = 0.146$; maternal diet, $F_{1,79} = 8.22$; $p = 0.005$; laying date, $F_{1,77} = 1.72$; $p = 0.194$; $\log_{10}(b_2\text{-egg mass})$, $F_{1,79} = 35.51$; $p < 0.0001$; diet-by-$\log_{10}(b_2\text{-egg mass})$ interaction, $F_{1,79} = 8.17$; $p = 0.005$). Yolk mass varied proportionately with egg mass in b_2-eggs of carotenoid-diet females (slope, 0.980 ± 0.186; $F_{1,40}(\text{slope} = 1) = 0.01$, $p = 0.921$), but smaller b_2-eggs contained disproportionately less yolk in the control-diet group (slope, 0.333 ± 0.116; $F_{1,39}(\text{slope} = 1) = 33.22$, $p < 0.0001$). Thus larger eggs produced by carotenoid-diet females contained relatively more lipid reserves than larger eggs produced by control-diet females.

There was no effect of maternal challenge or diet on changes in yolk carotenoid concentrations between b_1- and b_2-eggs (an average increase of 0.59 ± 2.18 ug g^{-1} yolk, or 2.6 %; maternal challenge, $F_{1,78} = 0.64$; $p = 0.425$; maternal diet, $F_{1,77} = 0.00$; $p = 0.964$; laying date, $F_{1,79} = 0.82$; $p = 0.994$; initial value, $F_{1,80} = 18.10$; $p < 0.0001$). Thus, as in first clutches (see above), b_2-eggs produced by carotenoid-diet females were substantially enriched with carotenoids in comparison with control-diet females (Figure 5.2a). There was no effect of maternal challenge or diet on changes in yolk Ig indices between b_1- and b_2-eggs (an average increase of 0.16 ± 0.02, or 13.4 %; maternal challenge, $F_{1,78} = 0.71$; $p = 0.401$; maternal diet, $F_{1,77} = 0.48$; $p = 0.489$; laying date, $F_{1,79} = 2.60$; $p = 0.111$; initial value, $F_{1,80} = 46.30$; $p < 0.0001$), and b_2-egg Ig indices did not differ significantly between treatments (Figure 5.2b).

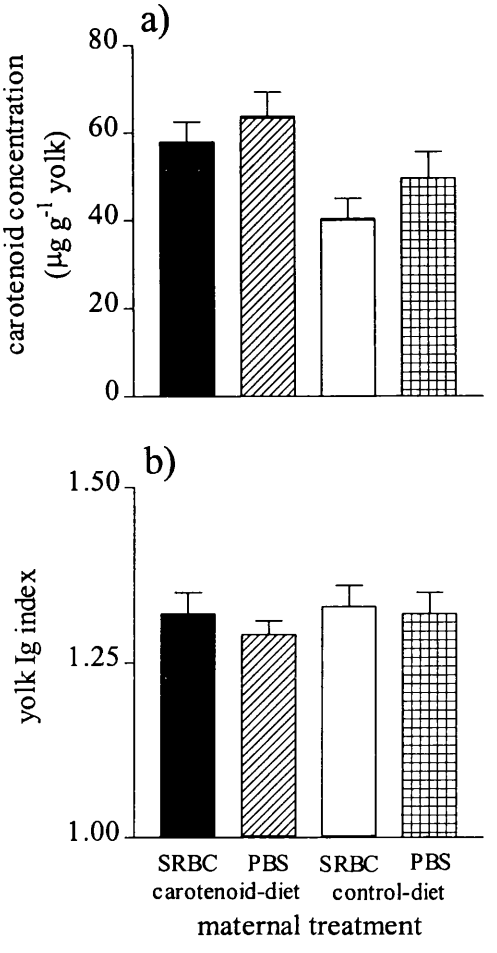

Figure 5.2: Variation in carotenoid and immunoglobulin concentrations in b_2-eggs, in relation to maternal challenge (SRBC or control) and diet treatments (carotenoid or control). Numbers in parentheses are the sample sizes of eggs. a), yolk carotenoid concentration, which did not differ significantly according to maternal immune-challenge or laying date, but was significantly higher in eggs produced by carotenoid-diet than control-diet females (maternal challenge, $F_{1,80} = 1.97, p = 0.165$; maternal diet, $F_{1,81} = 10.91, p = 0.001$; laying date, $F_{1,79} = 0.07, p = 0.797$). b), yolk Ig index, which did not differ significantly according to treatment or laying date (maternal challenge, $F_{1,79} = 0.11, p = 0.742$; maternal diet, $F_{1,80} = 0.26, p = 0.612$; laying date, $F_{1,81} = 2.04$, $p = 0.157$).

d) *Effects of maternal immune challenge and carotenoid supply on egg quality: chick performance*

Having excluded eggs that were lost due to predation or abandonment from the analysis (see Materials and Methods), hatching rate did not differ significantly according to maternal treatment, or egg position in the laying sequence (overall mean, 66.4 % of 119 eggs; logistic regression: maternal diet, $\chi^2 = 0.13$, d.f. = 1, $p =$

0.724; maternal challenge, $\chi^2 = 1.90$, d.f. = 1, $p = 0.169$; egg position, $\chi^2 = 0.02$, d.f. = 1, $p = 0.902$). Hatchling mass did not differ significantly according to maternal diet or immune challenge treatments, but a-chicks were heavier than c-chicks by on average 3.54 g or 7.2 % (ANOVA: maternal diet, $F_{1,75} = 0.02$; $p = 0.896$; maternal challenge, $F_{1,76} = 0.14$; $p = 0.709$; egg position, $F_{1,77} = 7.62$; $p = 0.007$). The effect of maternal treatment on hatchling tarsus length depended on the position in the laying sequence. In a-chicks, tarsus length did not differ according to maternal treatment. However, c-chicks produced by carotenoid-diet females had significantly shorter tarsi than c-chicks produced by control-diet females, being a difference of on average 4.62 % or 1.49 mm (ANOVA: maternal challenge, $F_{1,16} = 0.29$; $p = 0.600$; maternal diet, $F_{1,17} = 3.77$; $p = 0.069$; position, $F_{1,17} = 0.71$; $p = 0.410$; maternal diet by egg position interaction, $F_{1,17} = 5.20$; $p = 0.036$). (Data for hatchling tarsus length were missing for some individuals because of equipment failure in the field. However, the results of the analysis of tarsus length were qualitatively similar and the conclusions unchanged when the analysis was based on tarsus length at day 4, when data were available for all individuals (results not shown)).

Carotenoid concentrations in chick plasma diminished during the first half of the linear growth period (i.e. days 4-13) in a-chicks, whereas in c-chicks levels were at a consistently low level throughout the linear growth period (Figure 5.3a and Table 5.1). Plasma carotenoid concentrations were significantly higher in chicks produced by mothers that received a control challenge, and in a- compared to c-chicks, but did not differ significantly according to maternal diet or chick growth rate (Figure 5.3a and Table 5.1).

Concentrations of Ig in chick plasma diminished markedly and significantly during the first half of the linear growth period, then increased slightly but

significantly during the second half of the linear growth period (Figure 5.3b and Table 5.1). That pattern was similar across all a- and c-chicks (Table 5.1). Plasma Ig concentrations did not differ significantly according to maternal treatment, or egg position in the laying sequence, but chicks with the lowest plasma Ig concentrations were those that had grown fastest since hatching (Table 5.1; correlation of mean(d 4, 13 and 22) plasma Ig concentration and growth rate d 0 – 22, $r = -0.267$).

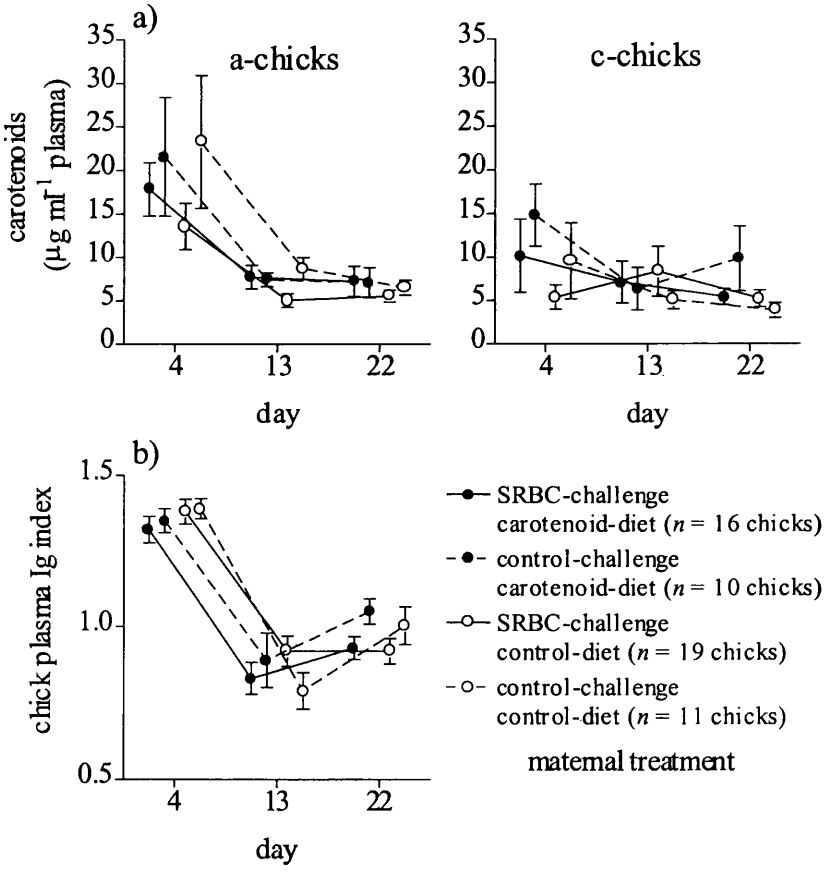

Figure 5.3: Variation in carotenoid and immunoglobulin concentrations in chick plasma over the nestling period (d 4, 13 and 22), in relation to maternal challenge (immune-challenge or control-challenge) and diet treatments (carotenoid or control). Numbers in parentheses are the sample sizes of chicks. a), plasma carotenoid concentration. b), plasma Ig index. See Table 5.1 for results of statistical analyses.

Table 5.1: Variation in carotenoid and Ig concentrations in chick plasma resulting from rmANCOVAs with age (d 4, 13 and 22) as a within-subjects factor, maternal challenge (control or SRBC), maternal diet (control or carotenoid), and position in the laying sequence (a- or c-chick) as between-subjects factors. Chick instantaneous growth rate, for the period relevant to the dependent variable of interest, was included as a covariate (see Methods for details). The models were developed using backwards elimination (see Materials and Methods for details). Only main effects are shown; all interaction terms were non-significant.

source	plasma carotenoid concentration			plasma Ig concentration		
	F	d.f.	*p*	*F*	d.f.	*p*
within-subjects						
day	10.52	2,53	<0.0001[a]	142.42	2,54	<0.0001[c]
day by egg position	4.02	2,53	0.024[b]			
between-subjects						
maternal challenge	4.24	1,53	0.044	1.32	1,53	0.256
maternal diet	2.47	1,52	0.122	0.21	1,52	0.650
egg position	7.58	1,53	0.008	0.11	1,51	0.742
growth rate (d 0-22)	0.74	1,51	0.395	4.13	1,54	0.047

[a], post-hoc contrasts: d 4 versus d 13, $F_{1,54} = 17.67$, $p < 0.0001$; d 13 versus d 22, $F_{1,54} = 0.89$, $p = 0.349$.

[b], post-hoc contrasts: d 4 versus d 13, $F_{1,54} = 8.07$, $p = 0.006$; d 13 versus d 22, $F_{1,54} = 0.10$, $p = 0.759$.

[c], post-hoc contrasts: d 4 versus d 13, $F_{1,55} = 203.40$, $p < 0.0001$; d 13 versus d 22, $F_{1,55} = 7.30$, $p = 0.009$.

All post-hoc contrasts, Bonferroni adjusted alpha = 0.025.

All chicks produced a positive PHA response. However, chicks of carotenoid-diet females produced significantly larger PHA responses than chicks of control-diet females, independently of whether their mother received an immune challenge or the egg position in the laying sequence (Figure 5.4a). Chicks that produced the smallest PHA responses were those that had grown fastest since hatching (correlation of PHA response and growth rate d 0 – 4, $r = -0.313$). PHA responses did not covary with body mass on day 4 in any treatment group (all $p > 0.05$).

The proportion of chicks that exhibited a positive BA antibody response did not differ significantly according to maternal challenge or egg position in the laying

sequence (logistic regression: maternal challenge, $\chi^2 = 0.42$, d.f. = 1, $p = 0.515$; egg position, $\chi^2 = 0.80$, d.f. = 1, $p = 0.371$), but was significantly higher in chicks produced by carotenoid-diet mothers (65.4 % of 26) compared to control-diet mothers (38.7 % of 31) (maternal diet, $\chi^2 = 4.08$, d.f. = 1, $p = 0.043$). The scale of BA antibody responses did not differ significantly according to maternal treatment or egg position in the laying sequence (Figure 5.3b).

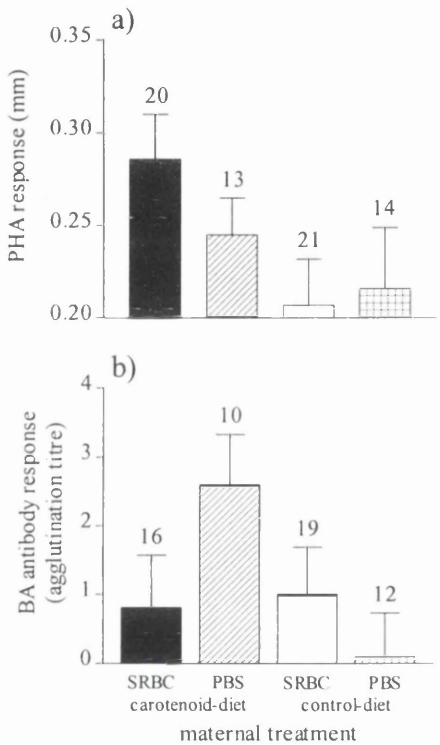

Figure 5.4: Immune responses of offspring hatching from eggs of differing quality, as influenced by the diet (carotenoid or control) and challenge treatment (SRBC or control) of the original female parent. Means (± 1 s.e.) are shown. Numbers above bars are the sample sizes of chicks. a), PHA response, which was higher in chicks hatching from eggs produced by carotenoid-diet females in comparison with controls, independently of whether female parents had received an immune challenge, or egg position in the laying sequence (ANCOVA: maternal challenge, $F_{1,63} = 0.10$; $p = 0.752$; maternal diet, $F_{1,65} = 4.12$; $p = 0.047$; egg position, $F_{1,64} = 2.56$; $p = 0.114$). Chicks that grew fastest as hatchlings produced smaller PHA responses (growth d 0–4, $F_{1,65} = 6.12$; $p = 0.016$; correlation of PHA response and growth rate d 0-4, $r = -0.313$). b), BA antibody response, which did not differ significantly according to maternal treatment, egg position in the laying sequence or chick growth rate (maternal challenge, $F_{1,53} = 0.38$, $p = 0.543$; maternal diet, $F_{1,52} = 0.31$, $p = 0.580$; egg position, $F_{1,51} = 0.01$, $p = 0.910$; growth rate d 0-13, $F_{1,54} = 1.06$, $p = 0.308$; initial value, $F_{1,55} = 78.46$, $p < 0.0001$).

Fledgling body mass did not differ significantly according to the treatment group of the original mother (ANOVA, maternal challenge, $F_{1,51} = 0.81$; $p = 0.372$; maternal diet, $F_{1,50} = 0.26$; $p = 0.614$), but a-chicks were heavier than c-chicks by on average 77.55 g or 11.5 % which was statistically significantly (ANOVA: egg position, $F_{1,52} = 6.48$; $p = 0.014$). However, fledging tarsus length did not differ significantly according to maternal treatment (ANOVA, maternal challenge, $F_{1,52} = 1.82$; $p = 0.183$; maternal diet, $F_{1,51} = 0.43$; $p = 0.516$), or egg position in the laying sequence (ANOVA: egg position, $F_{1,53} = 3.12$; $p = 0.083$). Consequently, c-chicks were in poorer body condition at fledging than a-chicks (ANOVA based on residual body mass: maternal challenge, $F_{1,49} = 0.02$; $p = 0.885$; maternal diet, $F_{1,50} = 0.80$; $p = 0.376$; egg position, $F_{1,51} = 4.63$; $p = 0.036$).

Chick mortality occurred mostly during the first week after hatching (average age of death, 8.11 ± 3.41 d, $n = 9$; half of all mortalities occurred ≤ d 2). The survival rate of chicks did not differ significantly according to maternal challenge, or egg position in the laying sequence. However, chicks arising from eggs produced by carotenoid-diet mothers had a significantly higher rate of survival in comparison with controls (Figure 5.5).

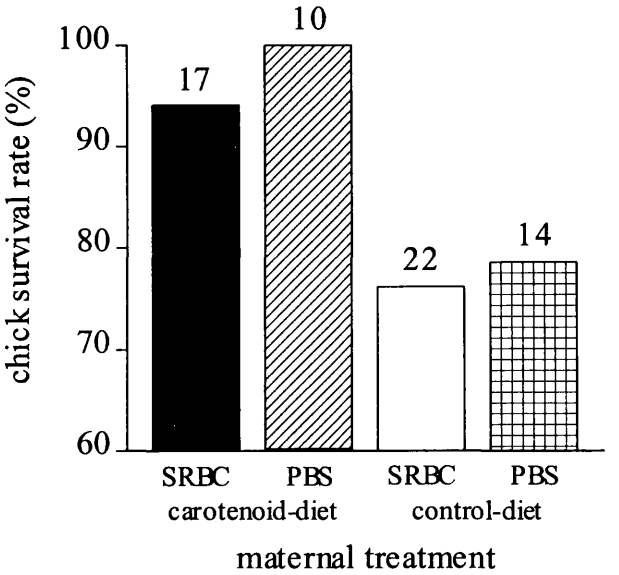

Figure 5.5: Survival rate of offspring hatching from eggs of differing quality, as influenced by maternal diet (carotenoid or control) and challenge treatment (SRBC or control). Numbers above bars are sample sizes of females in each treatment. A significantly larger proportion of chicks that hatched from eggs produced by carotenoid-diet females survived in comparison with controls, independently of whether their mother had received an immune challenge, or egg position in the laying sequence (logistic regression: maternal diet, $\chi^2 = 4.56$, d.f. = 1, $p = 0.033$; maternal challenge, $\chi^2 = 0.02$, d.f. = 1, $p = 0.887$; egg position, $\chi^2 = 0.19$, d.f. = 1, $p = 0.662$). Females that produced high quality a_2-eggs (i.e. surviving chicks) tended to also produce high quality c_2-eggs (McNemar's chi-square test based on replacement clutches of 3 eggs ($n = 13$): both chicks survived in 76.9 % of broods whereas both died in 0 % of broods; $\chi^2 = 8.10$, d.f. = 1, $p = 0.004$). However, the conclusion that carotenoid supplementation in the maternal diet resulted in enhanced egg quality (chick survival) was not confounded by a conflated number of degrees of freedom arising through sibling non-independence, as shown by a binomial regression using n(nests) rather than n(chicks) as the sampling units (see Methods for details; maternal diet, $\chi^2 = 4.10$, d.f. = 1, $p = 0.043$; maternal challenge, $\chi^2 = 0.02$, d.f. = 1, $p = 0.888$), and was not influenced by seasonality (laying date, $\chi^2 = 0.70$, d.f. = 1, $p = 0.403$; null model, deviance = 39.37, d.f. = 48; final model, deviance = 35.27, d.f. = 47).

DISCUSSION

a) *Was carotenoid supply limiting for egg production capacity?*

This study has shown that carotenoid supply was limiting for egg production capacity. As hypothesised, a larger proportion of carotenoid-diet females re-laid following first clutch removal than control-diet females (15 % more), and the decline in the size of replacement compared to first clutches was smaller in the carotenoid- than the control-diet group. Replacement clutch eggs produced by carotenoid-diet females were not larger, but contained relatively large yolks and higher concentrations of carotenoids, and gave rise to chicks that mounted larger immune responses and had a 20 % higher rate of survival than controls. These effects were mediated entirely through egg quality, because parental care (incubation and provisioning) was standardised, being carried out by foster parents. Moreover, since foster parents were assigned the relatively easy task of rearing a single chick, our test is likely to be a conservative one with respect to the importance of carotenoid-mediated maternal effects on egg quality.

There are at least three possible mechanisms by which higher maternal carotenoid supply could have translated into increased egg production capacity. Yolk synthesis depends on hepatic production of vitellogenin and very low-density lipoprotein (VLDL) that are the main sources of yolk protein and lipid (Speake *et al.* 1998). Possibly, increased carotenoid supply enhanced the protection of vitellogenin and VLDL from oxidation. Carotenoids have been shown to confer increased protection of proteins and lipids against free radical attack in various model systems in vivo and in vitro (e.g. Burton & Ingold 1984; Mayne & Parker 1989; Palozza & Krinsky 1992; Lawlor & O'Brien 1995; Woodall *et al.* 1996; Surai & Speake 1998; reviewed by Stahl & Sies 1999; Møller *et al.* 2000). Alternatively, increased carotenoid supply

could have altered temporal patterns in the endocrine regulation of follicle development. Hepatic expression of the genes that code for vitellogenin and VLDL synthesis is induced by oestrogen (Speake *et al.* 1998), whereas increased release of follicle-stimulating hormone from the pituitary, resulting in ovulation, is triggered by progesterone (reviewed by Carey 1996). Palm oil (containing α- and β-carotene) and retinoic acid (a vitamin A metabolite, ultimately derived from carotenes) have recently been shown to stimulate the expression of estrogenic enzymes, resulting in increased oestrogen production in various cell types in vitro (e.g. Ng *et al.* 2000; Hughes *et al.* 2001), and increased dietary β-carotene resulted in decreased progesterone secretion in Japanese quail, *Coturnix japonica* (Pusztai *et al.* 2000). Thus perhaps increased carotenoid supply promoted greater rates of synthesis of vitellogenin and VLDL, and more deposition into the follicle before its growth was terminated. Finally, in theory increased carotenoid supply could have resulted in improved maternal health and thus foraging efficiency (i.e. acquisition of all nutrients and energy required for egg formation). However, this seems an unlikely explanation for our results because we have not found any evidence to suggest that carotenoid-fed gulls develop improved body condition (Blount *et al.* 2002a, *Chapter 2*; *Chapter 4*). To our knowledge, this study provides the first direct evidence that egg production capacity is enhanced by maternal carotenoid supply in any wild bird species. Recent studies of captive fish have yielded similar findings (Verakunpiriya *et al.* 1997; Vassalo-Agius *et al.* 2001), but the causal mechanisms have not been elucidated. Our results suggest that carotenoid supply was limiting for the mass and size of replacement but not first clutches. Natural carotenoid availability can vary widely between geographic locations and years (e.g. Slagsvold & Lifjeld 1985; Hill 1993; Linville & Breitwisch 1997; Bortolotti *et al.* 2000), and thus further studies in

a range of environmental scenarios may help to elucidate the extent to which carotenoid supply underlies variation in egg production capacity in birds.

By what mechanism could maternal carotenoid supplementation have led to larger PHA responses and survival rates in chicks? Since Ig levels in b_2-eggs did not differ significantly between treatments, this suggests that differences in chick immune function and survival were independent of passive immunity. One possibility is that relatively large yolk size conferred increased energy and protein supplies. It has been estimated that yolk fatty acids are responsible for providing more than 90 per cent of the total energy required by the embryo (Noble & Cochi 1990). Since hatchling body mass did not differ significantly according to maternal diet treatment, chicks hatching from carotenoid-enriched eggs seem likely to have had higher potential energy and protein supplies as neonates – the period when chick mortality was most likely to occur. Such chicks did not exhibit higher growth rates, but possibly they utilised their larger yolk reserves to fuel immune function. There is some evidence that avian immune function is limited by energy (Glick *et al.* 1981; Ots *et al.* 2001; but see Glick *et al.* 1983; Svensson *et al.* 1998) and protein supplies (Glick *et al.* 1983; Tsiagbe *et al.* 1987; Lochmiller *et al.* 1993; Gonzalez *et al.* 1999; reviewed by Lochmiller & Deerenberg 2000; Norris & Evans 2000). Alternatively, increased antioxidant activity could have enabled chicks to benefit from high energy-turnover (Svensson *et al.* 1998), or high immune system activity without incurring oxidative stress (Chew 1996; von Schantz *et al.* 1999). We have shown previously that susceptibility to free radical attack correlates negatively with the carotenoid content of gull yolk (Blount *et al.* 2002a,b, *Chapters 2 & 3*), suggesting that high carotenoid deposition into eggs promotes antioxidant protection in offspring. It also seems possible that increased carotenoid supplies directly enhanced chick antioxidant

protection, immune function and resistance to pathogens, as suggested by studies of domesticated hens and ducks in vivo (McWhinney *et al.* 1989; Tengerdy *et al.* 1990; Woodall *et al.* 1996; Cheng *et al.* 2001) and in vitro (Mayne & Parker 1989; Lawlor & O'Brien 1995; Haq *et al.* 1996; Surai & Speake 1998; see Introduction). By whatever mechanism, increased maternal carotenoid supply resulted in enhanced offspring quality, with the effects being mediated via the egg.

Anti-BA antibody titres did not differ between treatments, possibly because we inoculated chicks at a relatively early stage during B-lymphocyte maturation; this process is not complete until 6 weeks of age in turkeys (McCorkle & Thaxton 1988). The fact that plasma Ig levels increased between 13-22 days old could reflect increasing levels of mature B-lymphocytes; similar ontogenetic patterns of Ig synthesis have been reported in other bird species (Apanius 1998). However, a higher proportion of chicks hatching from carotenoid-enriched eggs produced a positive BA antibody response in comparison with controls, possibly indicating an effect of carotenoid supply on B-lymphocyte maturation rate.

It is interesting that inter-treatment differences in PHA responses were evident even though plasma carotenoid concentrations were similar across treatments on the day of injection (day 4). Since it was clear that maternal carotenoid supplementation gave rise to a marked increase in yolk carotenoid concentrations (b_2-eggs), this suggests that considerable depletion of yolk carotenoids must occur during embryogenesis / hatching, possibly due to carotenoid utilization during the ontogeny of immune function. In domestic hens, T lymphocyte production can be detected in embryos by about two-thirds of the way through incubation (Toivanen *et al.* 1981). Indeed, chick plasma carotenoids declined markedly during the first half of the linear growth period in a-chicks, and remained at a consistently low level from hatching in

c-chicks. Similarly, it has been shown that maternally derived plasma carotenoids decrease with increasing age in domestic hen chicks (Haq *et al.* 1996). Thus dilution and (or) depletion of yolk-derived carotenoids in chicks must exceed dietary carotenoid intake. Avian embryos and neonates may be particularly at risk of oxidative stress: their lipid-rich tissues are an ideal substrate for free radical attack, at a time in their life that is characterized by high rates of metabolic activity and thus free radical production, particularly during hatching (Surai *et al.* 2001a). Plasma Ig levels also diminished markedly during the first half of the linear growth period across all treatments, probably reflecting a decline in levels of yolk-derived passive immunity (see Apanius 1998). Taken together, these lines of evidence suggest that the level of maternal carotenoid deposition into yolk may be critical factor that determines her offspring's capacity to develop efficient immune function during embryogenesis and the early post-hatching period.

PHA responses and plasma Ig concentrations correlated negatively with chick growth rate. Such patterns could indicate trade-offs between resource allocation to immune function and growth (Metcalfe & Monaghan 2001; Lochmiller & Deerenberg 2000), but we found no evidence to suggest that carotenoids were the limiting resource in question (the slopes of the relationships between the measures of immune function and growth were similar across treatments). However our experiment design, whereby all chicks received the same immune challenges, could have masked such an effect. That is, chicks hatching from carotenoid-enriched eggs could have allocated relatively more carotenoids to mounting a PHA response than to growth, whereas chicks hatching from control eggs could have adopted the opposite strategy. On the basis of our present data we cannot rule out the possibility

circulating immune cells simply became more diluted with increasing growth (i.e. blood volume).

Since eggs produced by carotenoid-diet females contained relatively large yolks compared to controls, it may seem puzzling that such eggs did not give rise to heavier hatchlings, and gave rise to c-chicks with shorter tarsi. As previously hypothesised (Blount *et al.* 2002a, *Chapter 2*), in natural feeding conditions it seems unlikely that gulls obtain carotenoids independently of sources of proteins, calcium or other nutrients that contribute to their egg production capacity. Thus in the present study carotenoid supplementation could have induced some females to re-lay, when under natural feeding conditions they would not have, resulting in the production of eggs lacking in nutrients required for embryo skeletal growth. Alternatively, carotenoid supplementation could have altered calcium metabolism. High carotenoid intake has been shown to result in reduced plasma calcium levels in rainbow trout, *Oncorhynchus mykiss*, although the mechanism has not been elucidated (Rehulka 2000). Ultimately, c-chick tarsus length did not differ between treatments at fledging, suggesting that c-chicks hatching from carotenoid-enriched eggs exhibited compensatory growth. However, we found no evidence to suggest that such chicks incurred higher costs (i.e. reduced immune function or survival).

It has previously been shown in gulls that c-eggs are of inherently lower quality than a-eggs, because c-chicks perform less well than a-chicks even when clutches are manipulated such that c-chicks hatch first (Parsons 1975). We found that plasma carotenoid levels were higher in a-chicks than c-chicks across all treatments, which is not surprising because gull eggs exhibit a within-clutch decline in yolk carotenoids independently of maternal supplies of carotenoids (Blount *et al.* 2002a, *Chapter 2*). However, the only effects of egg position in the laying sequence on chick phenotype

were related to chick size, and condition at fledging. The lack of a significant interaction between egg position and maternal diet in the analyses of chick immune function or survival probably reflects insufficient statistical power.

Several cross-fostering studies have shown that egg quality (usually measured simply in terms of egg size) has important consequences for offspring survival, with the effects usually being manifest during the first few days post-hatching (e.g. Amundsen & Stokland 1990; Reid & Boersma 1990; Bolton 1991; Amundsen *et al.* 1996; Styrsky *et al.* 1999). However, the compositional features that underlie variation in egg quality have largely been speculative, and most attention has been focused on the importance of macronutrients (reviewed by Williams 1994; Bernardo 1996). This study has shown for the first time in a wild bird species that maternal carotenoid supply has profound consequences for her capacity to produce eggs, and for the quality of those eggs. It remains unclear whether the benefits of high maternal carotenoid supply for egg quality are mediated via effects on lipid supply, or directly via effects of carotenoids on embryo and neonate development. Nager *et al.* (2000) recently showed that chick survival and egg lipid content both declined with increasing egg number in gulls, but they did not measure the carotenoid content of eggs. Thus it would be interesting to investigate whether egg lipid and carotenoid content generally covary in nature.

b) *Was there a trade-off between immune function and egg production capacity?*

Contrary to our prediction, SRBC-challenge did not significantly influence maternal egg-production capacity (laying rate, latency to lay, clutch mass and size) or egg quality (yolk carotenoid and Ig concentrations, chick mass and size, immune function and survival). It has previously been shown that SRBC-challenge invoked a

decline in carotenoid-based integument pigmentation in barn swallow chicks, *Hirundo rustica* (Saino *et al.* 2000), and in gulls (see *Chapter 4*). Similarly, experimental infection with coccidial parasites was shown to result in reduced plasma carotenoids in domestic hens, which covaried with increased free radical production by macrophages and neutrophils during immune system activation (Allen 1997). If carotenoid supply was limiting for egg-production capacity and egg quality, as suggested by our results, why then did not SRBC-challenge amplify that effect? It seems unlikely that there was simply a temporal mismatch in the demands of immune function and egg production: antibody titres peak at 6 days post-inoculation (Grasman *et al.* 1996; Ros *et al.* 1997) and eggs are formed over a period of 9 – 10 days in gulls (Houston *et al.* 1983; this study). We can therefore suggest two possible explanations for the lack of an effect of immune challenge on egg production. First, perhaps inoculation with SRBCs failed to deplete maternal supplies of carotenoids sufficiently to limit egg production. It has been suggested that non-replicating antigens, such as SRBCs, may be less demanding on the immune system than replicating antigens such as bacteria (Westneat & Birkhead 1998). SRBC-challenge has also been shown to have no effect on egg-production capacity in European starlings, *Sturnus vulgaris* (Williams *et al.* 1999). However, this seems an unlikely explanation for our results, because we have previously found that SRBC-challenge causes reduced body carotenoid levels, and a reduced ability to rear a foster brood of young in female gulls (*Chapter 4*). Alternatively, SRBC-challenge females may have aimed at increasing their fitness benefits by maintaining their level of investment into egg production, albeit at the expense of their own condition. Other studies have inferred that the level of passive immunity deposited into eggs correlates positively with the level of pre-laying exposure to parasites, which has

been suggested to be an adaptive response to enhance offspring condition (Heeb *et al.* 1998; Gasparini *et al.* 2001), although the cost to the female is not known.

There was one indication of an effect of maternal immune challenge on the quality of the eggs that she laid. Plasma carotenoid concentrations were significantly lower in chicks hatching from eggs produced by immune-challenge mothers compared to controls. This suggests that a trade-off between carotenoid allocation to immune function and egg quality could operate, but the effect was apparently not strong enough to result in diminished chick immune function or survival.

In conclusion, consistent with the results of an earlier study (Williams *et al.* 1999), we found little evidence to suggest that mounting a humoral immune response during egg formation affected egg production. Since the same method of experimental inoculation used in this study is known to result in reduced condition in females with relatively poor carotenoid supplies (*Chapter 4*), we hypothesise that females facing an immune challenge might maximise their breeding success by striving to produce high quality eggs rather than by striving to maintain somatic condition. This could be achieved by maintaining high levels of lipid and carotenoid investment into eggs, thereby increasing chick survival prospects. This explanation is not inconsistent with the results of previous experimental studies of birds, which have shown that high egg production effort can result in increased parasitism (Oppliger *et al.* 1996), reduced chick-rearing capacity (Heaney & Monaghan 1995; Monaghan *et al.* 1998), and reduced future breeding rate (Nager *et al.* 2001). The possibility that these effects are mediated by carotenoid-limitation of immune function owing to carotenoid and lipid investment into egg production deserves further study.

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Chapter 6

ANTIOXIDANTS, SHOWY MALES AND SPERM QUALITY: A PRELIMINARY INVESTIGATION IN LESSER BLACK-BACKED GULLS *LARUS FUSCUS*

The hypothesis presented in this *Chapter* forms the basis of a paper published as:

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INTRODUCTION

Females often prefer to mate with the most elaborately ornamented males (Andersson 1994). One hypothesis to explain this association posits that the fertilising capacity of a male is revealed by the quality of his ornamental display, and thus choosy females seek the direct benefit of fertility insurance (the phenotype-linked fertility hypothesis; Sheldon 1994, and see references therein). But how could a male's ornamental display reveal his fertility? It has previously been suggested that testosterone could mediate a connection between male ornamentation and fertility (Folstad & Skarstein 1997; Hillgarth *et al.* 1997). However, this probably cannot apply to plumage expression, which is not testosterone-dependent in most species (Owens & Short 1995). Thus, a mechanistic basis to link a wide array of ornamental traits and sperm quality has been lacking.

Recently, it has been hypothesised that free radicals could impair ornament expression in animals (von Schantz *et al.* 1999). Free radicals are atoms or molecules with unpaired electrons that arise as metabolic by-products, that seek to pair with other electrons and in so doing damage the associated atom or molecule (Block 1999). Studies of humans and domesticated animals in vivo and in vitro have shown that free radicals cause strand breakage of hyaluronic acid (Hawkins & Davis 1996), deplete carotenoid pigments (Allen 1997), and inhibit keratin synthesis (O'Toole *et al.* 1996). Hyaluronic acid, carotenoids and keratin are all substrates of a wide variety of ornamental displays, including the cockerel's comb, integument pigmentation, and feathers and spurs, respectively (von Schantz *et al.* 1999). Brain tissue is particularly vulnerable to free radical attack (Halliwell & Gutteridge 1985),

which raises the further possibility that display traits directly influenced by neural function, such as song repertoire and spatial memory, could potentially be affected by free radicals (von Schantz *et al.* 1999). It has not been shown directly that free radicals impair ornament expression, but studies have shown that high levels of parasitism, which can induce increased production of free radicals by neutrophils and macrophages during the inflammatory stage of an immune response (Allen 1997), can cause reduced comb size in domestic hens (Zuk *et al.* 1990). It has also been shown that inoculation of birds with non-replicating antigens resulted in reduced carotenoid-based integument pigmentation (Saino *et al.* 2000; *Chapter 4*), presumably because of oxidation of carotenoids by free radicals.

Studies across a variety of taxa show that free radicals can also impair sperm quality. Sperm display high rates of metabolic activity (and consequent free radical production) and are rich in polyunsaturated fatty acids, traits that render sperm particularly susceptible to oxidation by free radicals (humans: e.g. Aitken *et al.* 1989; various domestic bird species: Wishart 1984; Surai *et al.* 1998; fish: e.g. Liu *et al.* 1997). Some degree of vulnerability to free radical attack of sperm may be a fact of life for reproductive males. But excessive free radical attack can inhibit the synthesis of DNA and RNA (Compori 1989), modify the spermatozoan cytoskeleton and axoneme (Hindshaw *et al.* 1986), causing reduced sperm motility (de Lamirande & Gagnon 1992) and inhibition of sperm-oocyte fusion (Aitken *et al.* 1989). Lipid peroxidation in semen is, therefore, a likely major cause of reduced fertility (e.g. Wishart 1984; Aitken *et al.* 1989; Cecil & Bakst 1993; Sikka *et al.* 1995). Free radicals can also attack the DNA within the sperm nucleus; recent studies suggest

that such damage to the genome may translate into infertility or neoplastic disease in offspring. Free radical induced DNA fragmentation in the non-recombining long arm of the Y chromosome has been linked to disruptions of spermatogenesis and infertility in humans (Roberts 1998). These deletions are not observed in fertile men, or in a majority of the fathers of affected offspring. They must therefore arise de novo in the germ line of affected offspring's fathers (Roberts 1998). Oxidative stress (arising from heavy smoking) in fathers is associated with a marked increase in the incidence of neoplastic disease in their offspring (e.g. Ji *et al.* 1997). This could be because homologous recombination repair to double-strand breaks on the autosomes post-fertilisation automatically results in a loss of heterozygosity – a key feature in children with neoplastic disease (Roberts 1998; Aitken 1999). Thus, studies of humans and domesticated animals suggest that males that have incurred high levels of free radical induced damage to their sperm could be infertile, or father infertile or unhealthy offspring. If the same processes operate in natural populations of animals, we hypothesise that selection should have favoured females that can discriminate such males.

In theory, a link between free radical induced deterioration of sperm quality and ornamental display could exist if there were insufficient supplies of antioxidants in circulation (i.e. in conditions of oxidative stress). Animals use dietary antioxidants including carotenoids, vitamins C and E, and antioxidant enzymes including superoxide dismutase and glutathione peroxidase, to inactivate free radicals (reviewed by Surai 1999; von Schantz *et al.* 1999). There is some direct evidence that carotenoid supply can constrain the development of integument pigmentation in

captive birds and fish (e.g. Kodric-Brown 1989; Hill 1992), and in wild offspring (Saino *et al.* 2000) and adult female birds (Blount *et al.* 2002, *Chapters 2 & 4*). The idea that antioxidant supply is physiologically limiting to male animals, specifically (Lozano 1994; von Schantz *et al.* 1999), has not been tested experimentally in free-living animals.

Here, in a supplemental feeding study of wild lesser black-backed gulls *Larus fuscus*, we measured covariation between the carotenoid pigmentation of males and an index of their sperm quality. We hypothesised that males with high body supplies of carotenoids would consequently develop brighter carotenoid pigmentation and higher quality sperm.

MATERIALS AND METHODS

a) *Study species and site*

Data were collected during April – May 2000 at a colony of about 24 000 pairs of lesser black-backed gulls breeding on Walney Island, Cumbria, U.K.

b) *Supplemental feeding*

Daily from 9 April 2000, 100 nests were given 2 mg total carotenoids mixed with 20 g fat (Spry solid vegetable fat; Van den Bergh Foods Ltd., Crawley, UK), whilst 100 other nests were given an equal amount of fat (control group), placed next to the nest during the night. Supplemental feeding continued daily throughout laying. Full details of the composition, preparation and delivery of the supplements is given elsewhere (Blount *et al.* 2002a,b; *Chapters 2 & 3*).

c) *Collection of eggs and estimation of sperm quality*

We collected a random sample of 20 first-laid eggs from each treatment group on the day that they were laid. The intact yolk sac was separated from albumin and weighed to the nearest 0.1 g. The perivitelline membrane was removed by cutting the ovum into two with a scalpel, flushed carefully with sterile water to remove yolk, and stored in sterile water at 4 °C for 4-10 days before analysis. We counted the number of spermatozoa penetrating the inner perivitelline layer, as described by Birkhead *et al.* (1994). At fertilization in the infundibulum one or more sperm undergo the acrosome reaction leaving a relatively large hole in the inner

perivitelline layer in the region of the germinal disc. We randomly chose five round areas of 4.1 mm diameter and counted the number of holes under a microscope using x 100 magnification and dark field optics. This count does not include additional sperm that are trapped in the outer perivitelline layer without making a hole. The number of holes, however, is very closely related to the total number of spermatozoa (Birkhead *et al.* 1993, 1994). Data were expressed as the total sperm count per sampling area (number of holes per 207.44 mm^2). Due to time constraints in the field, we only obtained estimates of sperm abundance from stored perivitelline membrane samples if we also had corresponding data for the male's pigmentation (see below). Hereafter, all data reported refer to this sub sample, which comprised 14 males in the control group and 11 males in the carotenoid-fed group.

Laying date did not differ significantly among feeding treatments (control group, 24.29 ± 1.60 May (mean ± 1 s.e.); carotenoid-fed group, 21.82 ± 1.28 May (mean ± 1 s.e.); Mann-Whitney test, $z = 1.044$, $p = 0.296$). Thus, the period of supplemental feeding prior to laying was similar in both treatment groups.

d) *Capture of males and measurement of integument coloration*

We caught males at the nest using a walk-in trap within one day of clutch completion. The yellow colour of the bill and tarsus was measured by visual comparison with a Roche Yolk Colour Fan (RYCF; Hoffman-LaRoche), whereas the orange-red colour of the bill spot, gape flange and orbital ring was measured using a Dulux Trade Colour Palette (DTCP; Dulux, Slough, UK). These colour standards are objectively defined, comprising consecutive steps along a colour scale

in unique combinations of hue (colour in the colloquial sense, i.e. red, blue, etc.), value (brightness) and chroma (degree of saturation with hue). The RYCF ranges from scores of 1-15, and is intended for the measurement of coloration in domestic hen egg yolk. We numbered certain consecutive steps in the DTCP that characterised the range of colours of the gape flange and orbital ring found in our study population (scores ranged from 1-11). All colour measurements were made indoors in indirect natural light and based on the lateral right-hand aspect of each integument trait. Measurement of animal coloration by visual comparison with colour standards is subjective, but under standardized conditions results can correlate with scores obtained objectively by spectrophotometry (Zuk & Decruyenaere 1994). Data for bill, bill spot, gape flange, orbital ring and leg coloration were summarized using principal component analysis (PCA). Scores were extracted for the first factor and used as an index of male coloration.

RESULTS

In a PCA the first factor explained 49.4 % of the variance in male coloration, with a large positive loading on colour scores for gape flange, orbital ring, leg and bill (eigenvectors of 0.77, 0.71, 0.67 and 0.62, respectively), and a negative loading on bill spot colour (-0.74). First factor scores were extracted and used as an index of male coloration. Carotenoid-fed males had significantly higher coloration indices than controls (Figure 6.1).

There was a negative correlation between sperm count and yolk size, as measured by the wet mass of yolk (linear regression: $F_{1,23} = 8.326$, $r^2 = 0.266$, $p = 0.008$; sperm count = 16.662 – 0.642 yolk mass). Thus, we statistically removed the effect of yolk mass by using the residuals from the regression model for subsequent analysis. Residual sperm counts (hereafter, 'sperm count index') did not differ significantly between feeding treatments (control males, -0.32 ± 0.52; carotenoid-fed males, 0.41 ± 0.55; Table 6.1), but were positively correlated with male coloration indices (Figure 6.2 and Table 6.1). That result was not confounded by any consistent difference in yolk mass among treatment groups (control group, 19.65 $\pm$ 0.42; carotenoid-fed group, 20.44 $\pm$ 0.60; t-test, $t_{23} = 1.104$, $p = 0.281$).

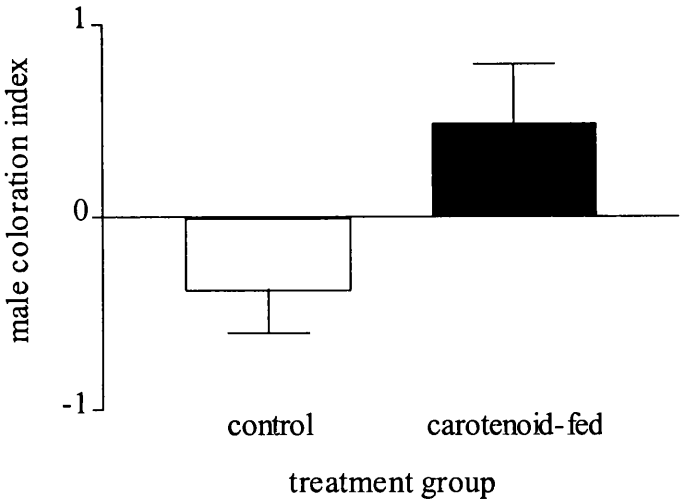

Figure 6.1: Effects of supplemental feeding with carotenoids on integument coloration in control (n = 14) and carotenoid-fed males (n = 11). Bars denote the mean (± 1 s.e.) index of integument coloration resulting from a principal components analysis (see text of Results), which was significantly higher in carotenoid-fed males (t-test, $t_{23} = 2.30$, $p = 0.031$).

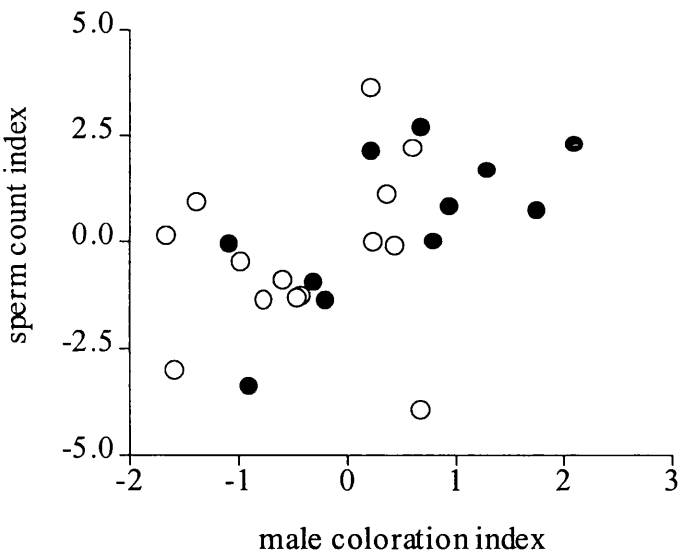

Figure 6.2: Relationship between indices of sperm quality and integument coloration (see text of Results for details) in control (open circles) and carotenoid-fed males (closed circles). See Table 6.1 for results of statistical analysis.

Table 6.1: Variation in sperm quality indices (see text of Results for details), resulting from an ANCOVA with treatment group as a factor and male coloration index as a covariate. The model was developed using backwards elimination starting with the interaction term.

source	*F*	d.f.	*p*
diet	0.00	1, 22	0.974
male coloration index	6.46	1, 23	0.018
diet x male coloration index	0.69	1, 21	0.415

DISCUSSION

This study has shown that carotenoid supplementation in the diet of pre-laying lesser black-backed gulls resulted in increased carotenoid-based integument coloration in males, and in both feeding treatments, males' coloration indices correlated positively with the number of their sperm that successfully reached the ovum and penetrated the inner perivitelline membrane. The difference in sperm count indices between control and carotenoid-fed males per se was non-significant, possibly because of the small sample size. But the spread of the correlation between male coloration and sperm count index increased along the same axis when carotenoid-fed males were considered together with controls (Figure 6.2). We have shown previously that increased integument coloration in female lesser black-backed gulls, resulting from dietary supplementation with carotenoids, was associated with increased plasma levels of carotenoids (Blount *et al.* 2002a, *Chapters 2 & 4*). Thus, it is probable that differences in male phenotype invoked in the present study arose because of elevated circulating levels of carotenoids in the carotenoid-fed group.

Studies of humans and captive animals have provided some evidence that increased antioxidant supply can improve sperm quality. For example, dietary supplementation with vitamins C or E in humans, fish, domesticated mammals and hens has variously been shown to increase the concentrations antioxidants in semen, increase the polyunsaturation of sperm phospholipids and protection against lipid peroxidation, increase sperm concentration and motility and reduce sperm abnormalities, and improve oocyte penetration rate and functional fertility (Friedrichsen *et al.* 1980; Erdinc *et al.* 1986; Brzezinska-Slebodzinska *et al.* 1995; Geva *et al.* 1996; Liu *et al.* 1997; Surai *et al.* 1997, 1998; Hsu *et al.* 1998). There is

no evidence that semen contains carotenoids in any species (Surai 1999), but that does not undermine the hypothesis that sperm quality may be influenced by body carotenoid levels. The action of one antioxidant can save another from oxidation, and antioxidants can repair each other from the oxidised (post radical trapping) state (e.g. Böhm *et al.* 1997, 1998; Mortensen & Skibstead 1997; Edge *et al.* 1998). These actions have been invoked to explain increased tissue vitamin E concentrations in domestic hens following carotenoid supplementation in the diet (Mayne & Parker 1989). This could potentially explain why an absence of lutein from the diet resulted in reduced male fertility in domestic hens (Ferrand & Bohren 1948). Thus, the correlation between indices of integument coloration and sperm counts that we observed could be explained by an interaction with other types of antioxidant.

Since the hypothesis that a male's ornamentation could reveal his fertility was evoked (Sheldon 1994), studies designed to test the idea have mostly been correlative (but see Birkhead *et al.* 1998), yielding mixed results (e.g. positive correlation: Matthews *et al.*, 1997; negative correlation: Liljedal *et al.*, 1999; no correlation: Birkhead & Fletcher, 1995; Birkhead *et al.* 1997). One possible reason for these discordant results is antioxidant supply was not controlled for. Also, by the mechanism that we have hypothesised, females could be choosing showy males to obtain sperm with undamaged genomes rather than for high fertility per se. There is increasing evidence that female choice for the showiest males translates into genetic benefits in the offspring (Hasselquist *et al.* 1996; Sheldon *et al.* 1997). Conventional logic suggests that this relationship is underpinned by the inheritance of some male quality trait, but the further influence of free radical induced damage to the genome is an untested possibility.

The mechanism that we have suggested could in theory apply to any male ornament that is vulnerable to free radical attack. Ornate male plumage is often produced weeks before the period of female choice, but could this investment have lasting effects on sperm quality? It was recently suggested that males should have evolved behavioural and physiological mechanisms to offload older sperm, because free radical attack causes sperm to deteriorate with age (Siva-Jothy 2000). Similarly, if antioxidants are limiting, perhaps males investing high concentrations of antioxidants into plumage development early in the season subsequently need to offload damaged sperm that were produced during that period, and increase the rate at which new sperm are metabolised ready for the period of female choice (thus increasing the likelihood of oxidative stress).

We cannot altogether exclude the alternative explanation that carotenoid-fed males became more attractive to their mates, simply because of their appearance, which resulted in increased copulation frequency. Similarly, we cannot exclude the possibility that carotenoid supplementation affected the general condition of males, via effects on immune function or antioxidant defences, which influenced their copulation frequency. These possibilities could be checked in a future study using behavioural observations. We have shown before that body carotenoid levels can be manipulated in female gulls using a similar supplemental feeding design (Blount *et al.* 2002a,b, *Chapters 2, 3 and 4*). Therefore, a further untested possibility is that carotenoid supplementation at the nest influenced the rate at which ova moved along the infinibundum and were exposed to sperm. However, this seems unlikely because we have found no evidence to suggest that carotenoid supplementation increases the rate at which eggs are produced (Blount *et al.* 2002a; *Chapters 2 & 5*). Future studies could attempt to supplementally feed males but not females.

In conclusion, the mechanism that we have suggested may explain how a male's ornamentation could reveal not only his fertility, but also the structural integrity of the DNA within his sperm. By this mechanism choosy females could obtain both direct (i.e. fertility) and indirect (genetic) benefits. Consistent with our hypothesis, the results of this study show that experimentally increased carotenoid supply resulted in elevated body carotenoid levels in gulls, and there was a correlation between male coloration and an index of sperm quality. To confirm the causal mechanism underlying these results and the ecological importance of our hypothesis, further studies could measure whether increased carotenoid supply resulted in reduced susceptibility of semen to free radical attack in wild birds, and whether there was a complimentary increase in male attractiveness to females.

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Chapter 7

General Discussion

Carotenoids and the costs of reproduction

The concept that reproduction is costly, and consequently that physiological trade-offs will arise between reproduction and other demanding activities, like immune function, is a cornerstone of life history theory (Stearns 1992). The question of which physiological resource(s) could be limiting both for reproduction and immune function has attracted much interest and debate (Gustafsson *et al.* 1994; Sheldon & Verhulst 1996; Råberg *et al.* 1998; Westneat & Birkhead 1998; Norris & Evans 2000). It has traditionally been assumed that energy is limiting (Stearns 1992), but empirical evidence gathered over the last decade has provided only equivocal support for this idea (reviewed by Råberg *et al.* 1998; Norris & Evans 2000). In this thesis I have investigated whether carotenoid supply could play a role in mediating such costs of reproduction, using free-living lesser black-backed gulls as a study species. I hypothesised that increased carotenoid supply (through supplemental feeding) would result in increased reproductive performance, there would be a trade-off between investment in immune function and reproduction (invoked by an immune challenge), and this trade-off would be uncoupled by increased carotenoid supply.

Each chapter of this thesis has been written as a separate piece of research intended for publication in the scientific literature, and as such includes a discussion related to its specific content. Here, I attempt to draw together elements of those individual chapters, and set the work in a broader context.

Was carotenoid supply limiting for reproduction per se?

It has recently been suggested that carotenoids cannot be considered an essential nutrient in birds (Klasing 1998a). Similarly, Hill (1999) has proposed that carotenoid

supplies are unlikely to be physiologically limiting in wild birds (Hill 1999). This latter suggestion is defended on the basis that plasma carotenoid concentrations are often far higher in birds than mammals, and in male compared to female birds, yet there is no reason to think that birds and mammals, or male and female birds, should differ consistently in their needs for carotenoids (Hill 1999). However, all the supporting evidence cited by these studies comes from studies of humans and captive animals, such as domestic hens (see Klasing 1998a; Hill 1999). There has been no evidence available to help judge whether carotenoid supplies are limiting in nature, where acquisition of carotenoids presumably involves a relatively higher investment of time and energy than in captivity. If a high work rate increases the risk of oxidative stress (Svensson *et al.* 1998; Sen 2001), then it seems plausible that foraging itself, investment in sexual display, or the provision of parental care, could increase an individual's needs for carotenoids. It also seems likely that wild birds will be exposed to parasites and diseases, or at least novel antigens, to a greater extent than domestic hens (e.g. due to prophylactic medication and inoculation). This thesis supports a different conclusion to that proposed by Klasing (1998a) and Hill (1999). It has been shown that increased dietary carotenoid supply (through supplemental feeding) resulted in an increased maternal capacity to produce high quality eggs (Blount *et al.* 2002 a,b; *Chapters 2, 3 & 5*), and in males, a sperm count index was positively correlated with carotenoid pigmentation of integument, which I have suggested could reflect carotenoid limitation of sperm quality in duller males (Blount *et al.* 2001; *Chapter 6*).

I have suggested throughout this thesis that the observed effects of carotenoid supplementation on egg production and egg quality were operating via effects of carotenoids on maternal condition, or carotenoid transfer directly into eggs. However, I

cannot rule out the alternative possibility that females increased their allocation of resources into egg production because their male mate became more attractive due to supplemental feeding. It has been hypothesised that males with brighter carotenoid pigmentation signal their superior resistance to parasites and diseases (Lozano 1994), oxidative stress (von Schantz *et al.* 1999) or sperm quality to prospective mates (Sheldon 1994; Blount *et al.* 2001, *Chapter 6*). It was clear that carotenoid supplementation at the nest resulted in increased male pigmentation (Blount *et al.* 2001, *Chapter 6*), just as in females (Blount *et al.* 2002a, *Chapter 2*; *Chapter 4*). It is not known whether more brightly pigmented male gulls are more sexually attractive, nor whether this could invoke increased maternal investment in offspring production. However, some studies of birds have shown that females invest more in egg production when paired to more attractive males (Burley 1988; Cunningham & Russell 2000; Gil *et al.* 1999). This possibility could be ruled out in future studies by working on a species where females and males could be supplemented independently, unlike gulls.

Interactions between immune function and reproduction: the role of carotenoid supply

I found no evidence to suggest that facing an immune challenge reduced the quality of the eggs that females laid. Chicks produced by immune challenged females had lower plasma carotenoid levels than controls, but there was no evidence that this influenced their immune function or survival (*Chapter 5*). However, *Chapter 4* shows that immune-challenge control-diet females produced relatively light foster broods in comparison with females of the other treatments, consistent with the explanation that there was a trade-off between immune function and maternal work rate which was

mediated by carotenoid supply. It is interesting that such a cost of reproduction, that became apparent during chick rearing, originated when females faced an immune challenge several weeks earlier at the time of egg production. It seems that immune-challenge females maintained a high level of investment in egg production, but this was at the expense of their own condition. These results, along with other recent evidence that costs incurred through egg production can translate into reduced parental performance later on (chick rearing: Monaghan *et al.* 1998; future fecundity: Nager *et al.* 2001), cast doubt on the traditional assumption that the costs of reproduction in birds arise principally through rearing offspring (Lack 1947; Lessells 1991). This assumption has directed the development of theoretical predictions of the optimum number of offspring per breeding attempt (Godfray *et al.* 1991; Stearns 1992). However, such theory predicts a larger optimal reproductive rate than that which is commonly observed in nature (Stearns 1992). There is increasing evidence to suggest that this discrepancy could arise because of a failure to take into account the full costs of producing offspring, including egg production, as first hypothesised by Partridge & Harvey (1985; and see Monaghan & Nager 1997 for a review).

The results of this thesis compliment those from other studies of birds in suggesting that females facing an immune challenge do not produce poorer quality eggs, and may even produce eggs of higher quality than controls. Two other studies have shown that females exposed to more parasites during the pre-laying period produce higher quality eggs, containing higher concentrations of immunoglobulins (kittiwakes, *Rissa tridactyla*; Gasparini *et al.* 2001), and giving rise to increased growth and survival rates in chicks, with no difference in the quality of parental care being observed in comparison with controls (great tits, *Parus major*; Heeb *et al.* 1998). In a study of European starlings,

Sturnus vulgaris, Williams *et al.* (1999) reported that injection of females with sheep red blood cells invoked an immune response, but there was no effect on egg size, chick growth or fledging success. One potential explanation for Williams *et al.*'s results is that immune-challenge females invested in producing high quality eggs, but consequently exhibited lower quality parental care in comparison with controls. However, this is not clear because the quality of parental care (e.g. nest visit rates) was not reported, nor eggs fostered to control parents. Interestingly, it has been shown that birds experimentally exposed to more parasites during the pre-laying period produce smaller clutches (Heeb *et al.* 1998), which, by the hypothesis that I have suggested, could potentially reflect a trade-off between egg quality and egg number.

Taken together, the results of *Chapters 4* and *5* could indicate that the production of high quality eggs is more important than parental condition during chick rearing in determining the success of reproduction. Consistent with this view, cross-fostering studies of a variety of avian species have shown that chick growth (e.g. Amundsen & Stokland 1990; Magrath 1992; Amundsen *et al.* 1996), and, less commonly, chick survival (Bolton 1991; Blomqvist *et al.* 1997; but see for example Magrath 1992; Amundsen *et al.* 1996) were related to egg size, independently of the quality of the rearing environment. Styrsky *et al.* (2000) reported that fledgling mass and size remained positively correlated with egg mass despite abundant free food during the rearing period in house wrens, *Troglodytes aedon*, suggesting that offspring growth and development were in part predetermined by some aspect of egg quality that covaried with egg size. Whether such results generally reflect the egg content of carotenoids, and (or) some other resource influenced by maternal carotenoid supply (e.g. passive immunity, lipids), deserves study.

Some implications for studies of sexual selection

Carotenoids clearly have a role in many different biological contexts. The nature of the information conveyed by carotenoid-based signals is currently a relatively contentious topic in evolutionary ecology (reviews by Olson & Owens 1998; Møller *et al.* 2000; Hill 1999; see *Chapter 1* for a review). However, the hypothesis that the most brightly pigmented individuals are preferred in mate choice because they are most likely to be in good health (Lozano 1994; von Schantz *et al.* 1999) is rendered more plausible by the findings that carotenoid supplementation resulted in increased antioxidant activity in adult females (Blount *et al.* 2002a; *Chapter 2*), an increased ability to cope with SRBC challenge (as revealed by relative chick-rearing performance among treatments; *Chapter 4*), and higher immune responses in chicks (*Chapter 5*). Similarly, the hypothesis that carotenoid pigmentation in female birds could reveal the capacity to produce high quality eggs (Blount *et al.* 2000, *Chapter 1*), is rendered more plausible by the observation that clutches exhibiting the steepest decline in yolk carotenoid levels over the laying sequence are produced by the dullest females (Blount *et al.* 2002a; *Chapter 2*). A recent study of two-spotted gobies has shown that experimentally increased redness in females, which largely reflects the carotenoid pigmentation of eggs visible through the body wall, is more attractive to males (Amundsen & Forsgren 2001). Investigations of interactions between carotenoid signals, health and (or) egg production capacity could use a similar approach to the one adopted in this thesis, using carotenoid supplementation and assays of antioxidant activity and immune responses, but focusing on study species for which carotenoid pigmentation is known to play a role in mate choice.

Some implications for studies of nutritional biochemistry

An improved understanding of the causes and consequences of variation in body levels of carotenoids in birds, and levels deposited into eggs, could lead to advances in the management of species that are of commercial or conservation importance. For example, in contrast to domestic hens (Hencken 1992), it has recently become apparent that β-carotene features highly in the yolk of several species of wild birds (Royle *et al.* 1999; Surai *et al.* 2001). The results of *Chapter 3* (Blount *et al.* 2002b) suggest that such a profile is not simply a reflection of a superabundance of β-carotene in the diet, at least not in gulls. Could the relatively poor ability of domestic hens to deposit β-carotene into yolk have arisen inadvertently, due to a genetic correlation with some other trait that has been the focus of selective breeding for enhanced productivity? Perhaps an investigation of yolk carotenoid profiles in the wild ancestor of the domestic hen, red jungle fowl, *Gallus gallus*, could help answer this question.

Attempts to improve disease resistance are a major focus of research in poultry science, but the emphasis is on genetics and inoculation programmes (Bacon *et al.* 2000). Nutritional modulation of disease susceptibility has traditionally been viewed as a relatively unprofitable solution from an economic perspective (Klasing 1998b). However, there is increasing consumer interest in the potential health benefits of eating carotenoid-rich eggs (Surai *et al.* 2000; Surai & Sparks 2001), and, interestingly, consumers tend to prefer more deeply pigmented yolks which they assume to be associated with good health of the layer (Hernandez 1998; Nys 2000). The results of this thesis provide some factual basis for that assumption, and moreover, emphasise that carotenoid supplies can be limiting for physiological performance. Such research could

provide a good basis for the marketing of carotenoid supplements for human and agricultural use.

Zoos and aviculturalists commonly provide carotenoid supplements in the diets of certain bird species in captivity to yield integument pigmentation that appears species-typical (e.g. flamingos, *Phoenicopterus* spp, Fox & McBeth 1970; bee-eaters, *Merops* spp, Dierenfeld & Sheppard 1996; review by Slifka *et al.* 1999). However, there has been no systematic review of the carotenoid requirements of captive birds for reproduction. The results of this thesis suggest that supplementation of captive birds' diets with carotenoids could result in enhanced reproductive performance (Blount *et al.* 2001, 2002 a,b; *Chapters 2, 3, 4, 5 & 6*). Further studies are now required to elucidate the specific types and amounts of carotenoids that are consumed by different bird species in nature. This may be particularly important to avoid imposing energetic costs, which could potentially arise through metabolic transformations of carotenoids (Hill 1996; *Chapter 3*), or prooxidant effects, which could potentially arise through excessive supplementation (e.g. Burton & Ingold 1984).

Future perspectives

Carotenoids are not the only resources that could underlie trade-offs between reproduction and immune function in wild birds. The role of many potentially limiting resources awaits detailed study, including vitamins A and E (effects on immune function, Gore & Qureshi 1997; Lessard *et al.* 1997; effects on sperm quality, Friedrichsen *et al.* 1980), hormones (effects on chick growth, Schwabl 1996b; Lipar & Ketterson 2000) and passive immunity (effects on chick growth and survival; Heeb *et al.* 1998). It is now becoming clear that correlations exist between the levels of such

resources in egg yolk (e.g. hormones and antioxidants, Royle *et al.* 2001; carotenoids and passive immunity, Blount *et al.* 2002a, *Chapter 2*). A major challenge that remains is to explore the functional significance of such patterns, both in terms of how parental experience of the environment translates into intercorrelations between resources responsible for maternal effects, and the consequences of such patterns for offspring. For example, could variation in photoperiod link hormones and antioxidants? It has been shown that photoperiod influences the maternal level of deposition of hormones into yolk (Schwabl 1996a), and similarly, photoperiod is an important factor influencing the de novo production of carotenoids in nature (Latscha 1990). Does an inverse correlation between yolk levels of hormones and antioxidants over the laying sequence in gull eggs comprise a mechanism to facilitate either facultative brood reduction or survival, depending on the prevailing environmental conditions post-hatching (see Royle *et al.* 2001). Finally, although maternal effects may primarily be expressed during the nestling period, there may be pervasive influences on adult phenotype and performance, which are largely unexplored in birds (Lindström 1999). We have discussed such a possibility with respect to sperm quality (*Chapter 6*), and there are similar potential avenues of investigation with respect to yolk carotenoids.

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