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EGG COMPONENTS VARY INDEPENDENTLY OF EACH OTHER IN THE FACULTATIVE SIBLICIDAL BLACK-LEGGED KITTIWAKE

--Manuscript Draft--

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Abstract:	Egg composition varies both within and between clutches and mothers are expected to alter their deposition of resources to the egg depending on environmental conditions and breeding strategies. Within-clutch variation in egg composition has been proposed to reflect an adaptive maternal strategy influencing sibling competition. In species with brood reduction, mothers should reinforce brood hierarchies due to hatching asynchrony and favour senior chicks by making first-laid eggs larger, richer in nutrients, with higher testosterone and carotenoid levels and lower corticosterone concentrations than last-laid eggs (parental favouritism hypothesis (PFH)). Moreover, mothers that are of better quality and/or experience better feeding conditions during laying are expected to increase their deposition of resources to the egg, resulting in differences between clutches (investment hypothesis (IH)). Several components may act together to provide an optimal reproductive strategy, but studies of variation in different egg components in the same egg are relatively rare. Here we analysed egg size, testosterone and corticosterone concentrations and carotenoids measured as yolk colour, between and within clutches for the facultative siblicidal Black-legged Kittiwake Rissa tridactyla. First-laid eggs were larger, contained lower testosterone, higher yolk colour score, and similar corticosterone levels than last-laid eggs. Thus only differences in egg size and yolk colour supported the PFH. We used within-clutch egg size dimorphism as an indicator of the quality of the mother or the feeding conditions during laying. In support of the IH, we found that mothers of better quality or that experienced better feeding conditions deposited more corticosterone into their eggs. High corticosterone levels may benefit nestlings when there is no brood reduction but high sibling competition is present. We found no support for the hypothesis that egg components are mutually adjusted to each other and we discuss the possible reasons for this.

Introduction

An individual's phenotype can be determined not only by its genotype and the environment which it experiences during development, but also by non-genetic maternal effects (Mousseau and Fox 1998). Maternal effects can be shaped by natural selection to act as a mechanism for adaptive phenotypic responses in offspring and have long-lasting effects on individual phenotype and fitness (Mousseau and Fox 1998; Lindstrom 1999; Metcalfe and Monaghan 2001) and the capability of accelerate or impede the evolutionary response to selection (Kirkpatrick and Lande 1989).

Egg size and composition provide powerful mechanisms through which maternal effects can influence offspring development and phenotype (Nager 2006). The investment hypothesis states that variation in egg composition between clutches exists as a response to the environment mothers experience during egg formation, maternal quality or both (e.g. Pilz et al. 2003; Gil et al. 2006). Mothers can also vary the deposition of resources within a clutch and divide them unequally across egg order. This might be the result of mothers adopting a bet-hedging approach (Crean and Marshall 2009) or reflect adaptive maternal strategies by influencing competitive hierarchies in offspring phenotype in order to maximise parents' reproductive success (parental favouritism hypothesis, Mock and Parker 1997). Females may deposit fewer resources into eggs with poor survival prospects to eliminate the weakest offspring (brood reduction strategy, Lack 1947; Clark and Wilson 1981; Schwabl et al. 1997) or deposit more resources into eggs with poor survival prospects in order to offset their developmental disadvantage (brood survival strategy, Slagsvold et al. 1984; Schwabl 1993; Lipar and Ketterson 2000).

Variation in egg size reflects differences in macronutrient contents (lipids, protein and water). Chicks hatched from larger eggs are bigger and have better chances of survival and higher growth rates than chicks from smaller eggs (Williams 1994; Krist 2011). Variation in absolute egg size and within-clutch egg size dimorphism can be associated with differences in maternal quality, environment during egg formation or both (Kilpi et al. 1996; Christians 2002; Nager 2006). Moreover, having a relatively small last-laid egg may facilitate efficient elimination of the youngest, weakest offspring (brood reduction strategy) whereas having a relative large last-laid egg may offset the developmental disadvantage of the last-hatched young (brood survival strategy, Slagsvold et al. 1984).

Other than macronutrients, mothers also deposit steroid hormones and carotenoids into their eggs. Among the steroid hormones are androgens that are deposited in the yolk primarily from the follicular wall that surrounds the growing oocyte (reviewed in Groothuis and Schwabl 2008; Okuliarova et al. 2010). Androgens can show marked variation with egg order and enhance growth and competitive abilities of nestlings but can decrease their immune function, although these effects appear to be species- and sex-specific (reviewed in Pitala et al. 2009). It had been suggested that another steroid hormone deposited in the egg is corticosterone, the main plasma glucocorticoid in birds (Hayward and Wingfield 2004, but see Rettenbacher et al. 2009). Corticosterone is not produced in the follicles but secreted by adrenal glands and is assumed to get into the egg by passive diffusion from the maternal blood circulation (reviewed in Groothuis and Schwabl 2008). Egg corticosterone levels can vary between clutches of females in relation to maternal body condition as well as within-clutches with respect to egg order (Love et al. 2005; Saino et al. 2005; Love and Williams 2008; Love et al. 2009; Poisbleau et al. 2009; Kozlowski and Ricklefs 2010). Elevated egg corticosterone levels have been shown to reduce offspring survival, growth, begging and immune function (reviewed in Gil 2008) but to increase post-fledging flight performance (Chin et al. 2009) and HPA-activity in response to stressful situations that favour survival in unfavourable circumstances (Angelier et al. 2010). Within-clutch variation in egg steroid hormones has been suggested to allow mothers to maximise reproductive success depending on the species' life history strategy by optimising brood hierarchies (Love et al. 2005, 2009; Kozlowski and Ricklefs 2010). Thus elevated androgen deposition into eggs with the highest survival prospect and elevated corticosterone levels into eggs with the lowest survival prospect could reinforce the brood hierarchy favouring the elimination of the weakest offspring (brood reduction strategy, Schwabl 1993; Lipar and Ketterson 2000). In contrast, elevated androgen and low corticosterone deposition into eggs with the poorest survival prospect could offset their developmental disadvantage (parental favouritism hypothesis, Mock and Parker 1997; Schwabl et al. 1997).

Other important egg components are carotenoids. Carotenoid deposition in yolk depends directly from its availability in the diet, therefore their concentration will signal females' capacity of feeding from good quality sources. As egg carotenoid levels positively correlate with embryonic growth (Biard et al. 2007; Romano et al. 2008) and play an important immune enhancing and detoxification role during embryo and neonatal development (Blount et al. 2000; Blount et al. 2002) differentially depositing carotenoids into eggs with respect to egg order is another possibility to manipulate brood hierarchies. Reduced deposition of carotenoids to the competitively inferior offspring could reduce its growth rate, retard its development and make it more prone to diseases (Saino et al. 2000; McGraw et al. 2005; Groothuis et al. 2005a; Rubolini et al. 2006). Thus a decrease in carotenoid levels across the laying sequence could make junior chicks less successful in sibling competition and therefore reinforce brood hierarchies.

Here we examined between and within-clutch variation in egg composition for a reproductive strategy, facultative brood reduction, which had only been rarely studied so far (Schwabl et al. 1997; Dentressangle et al. 2008; Drummond et al. 2008). We studied a colony of Black-legged Kittiwakes *Rissa tridactyla* breeding in the North Sea. The Kittiwake is a facultativly brood reducer species with a clear dominance-subordinate competitive hierarchy between siblings with the last-hatched chick unlikely to survive in poor environmental breeding conditions (Braun and Hunt 1984; Drummond 2006). The North Sea Kittiwake population had encountered poor environmental breeding conditions in recent years (Frederiksen et al. 2004) that favour a brood reduction strategy. As mothers may potentially vary all these egg components simultaneously and each egg component might require a balance with other components (Royle et al. 2001; Sockman et al. 2006; Navara et al. 2006a; Török et al. 2007; Hargitai et al. 2010) we measured size, testosterone, corticosterone and carotenoids in the same egg and how these different egg components were related to each other to test whether egg components are mutually adjusted. We predict that according to the investment hypothesis, egg composition is related to the environmental conditions during egg formation, maternal quality or both. According to the parental favouritism hypothesis we predict that, in order to reinforce brood hierarchies based on hatching asynchrony, mothers customise their within-clutch pattern of egg composition by decreasing testosterone and carotenoids and increasing corticosterone levels with laying order.

Methods

Field work was carried out on Kittiwakes breeding on the Isle of May (Firth of Forth, Scotland, 56°11N, 2°33W) in 2004, a year with poor breeding success (0.29 fledglings per nest; lower and upper 95% confidence interval of the long-term breeding success = 0.39 – 0.79 fledglings per nest; Vallarino 2009). Laying was synchronised over a period of around 14 days and 85% of females laid two-egg clutches with an average laying interval of 1.7 ± 0.4 days (n = 246) between eggs. Chicks hatch asynchronously with first-laid eggs (A-eggs) hatching 1-2 days before second-laid eggs (B-eggs). Soon after hatching the dominant first-hatched offspring may eject the junior chick from the nest (Braun and Hunt 1984; Vallarino 2009).

Accessible nests from throughout the colony were monitored daily from before the first egg was laid until all clutches were complete (116 clutches). Freshly laid eggs were individually marked with water-proof marker pens and measured. The eggs' length (l) and breadth (b) were measured to the nearest 0.1 mm with callipers and then returned to their nests. Egg volume was calculated using the formula $V=0.4866*b^2*l$ (Coulson 1963). The within-clutch egg size difference was calculated as $\log_{10}$(volume of A-egg/volume of B-egg) and used as an index of dimorphism (Greenwood 2003). This is a generally accepted dimorphism index among ecologists that is independent of the absolute values; it is symmetric around a neutral zero (monomorphy), positive when the A-egg is larger and negative when the B-egg is larger, and serves as an indicator of environmental conditions during egg formation, maternal quality or both.

To compare the composition of A- and B-eggs, complete two-egg clutches were collected in 2004 under license from Scottish Natural Heritage. Nests were checked every day from before the start of laying to establish the exact day each egg was laid. A-eggs were taken from the nest on the day they were laid and replaced with a dummy egg, a boiled chicken egg similar in size to a Kittiwake egg and painted to mimic the colour and patterning of Kittiwake eggs. In all cases the dummy eggs were accepted, females continued laying in the normal way and did not desert the nest. On the following days, checks were made to determine the laying date of the B-egg, which was also removed on the day it was laid and replaced with another dummy egg. Two days after this, another nest visit was carried out to verify that no third egg was laid (which never occurred) and the dummy eggs were removed to allow females to lay a replacement clutch. After collection of eggs, they were wrapped in cling film and newspaper and frozen at -20°C for further egg composition analyses (see below).

Egg composition analysis

To measure egg composition, frozen eggs were thawed and separated into albumen and yolk. We took wet weight of yolk and albumen to the nearest 0.0001 g. The coloration of the wet yolk was scored on a scale from 1 (pale yellow) to 15 (dark orange) in steps of 0.5 under standardised light conditions in the laboratory using the Roche yolk colour fan, Hoffman-La Roche, Basel, Switzerland (Vuilleum 1969). Yolk colour can be used as a reliable measure of the total carotenoid concentration in the egg yolk (Nys 2000; Verboven et al. 2005; Karadas et al. 2006;). Entire wet yolks were homogenized to avoid variation in concentrations of egg components in different layers of the yolk (e.g. see Lipar et al. 1999 for testosterone), and mixed with an equal amount of water (1:1 w/w). A sub-sample of known weight from each yolk-water mixture was then set aside for steroid extraction (see below).

Protein and Lipids - Yolk and albumen were dried at 60 °C to weight constancy and then individually weighed. Lipids were extracted from the yolk using a Soxhlet extraction with petroleum ether as a solvent (Dobush et al. 1985). The lean lipid-free part of the yolk was then dried and weighed. We then followed common protocols that assume complete separation of lipid and protein and all dry albumen mass is protein (Carey 1996; Nager et al. 2000) and calculated the protein content of the yolk (lipid-free dry yolk mass), the total protein content of the egg (adding dry albumen mass to the lipid-free dry yolk mass) and lipid content of the egg (subtracting the lipid-free dry yolk mass from the dry yolk mass before lipid extraction).

Hormone assays - Steroid hormones are mainly found in the yolk (Gil 2008) and we therefore only analysed the yolk for obtaining testosterone and corticosterone levels. Both hormones were extracted from the yolk-water mixture with a two-step extraction procedure using methanol and C18 isolute columns (International Sorbent Technology, UK). Two ml of methanol (HPLC Grade Methanol, Rathburn Chemicals, Walkerburn, UK) were added to the samples, which were then vortexed for 40 minutes and centrifuged at -8 °C for 10 minutes. 300 µl of the supernatant was transferred to a new vial, diluted with 2700 µl water and the sample cleaned by application to pre-conditioned isolute C18 columns. These columns were then washed with 3 ml water and testosterone eluted with 3 ml of 70% methanol. Corticosterone was extracted in a similar manner except that the initial methanol extraction was completed with 2.5 ml of methanol and the tubes vortexed for 1 hour. 1500 µl of the supernatant was then mixed with 13500 µl of water before being applied to C18 isolute columns (200 mg, 3 ml C18-220-0020-B, Isolute, International Sorbent Technology, UK) and eluted with 3 ml of 80% methanol. Dried down samples were then re-suspended with 330 µl of assay buffer (Phosphate Buffered Saline with 0.25% BSA), and 100 µl transferred to plastic tubes ready for assay. Extraction efficiency for the yolk samples averaged 81.5 ± 2.6% for testosterone and 82.3 ± 1.9% for corticosterone.

Testosterone was measured in duplicates and corticosterone in triplicates using established double antibody radioimmunoassay. Testosterone concentrations were measured following the protocol of Verboven et al. (2005). Measurements of corticosterone concentrations followed the protocol of Robertson (2009) using antibody B3-163 (Esoterix Inc., Calabasas Hills, California, USA) which has low cross-reactivities (<4%, 0.04-0.6% for gestagens) with other hormones and hormone-metabolites (manufacturer's data sheet). All samples were assayed in one assay for each hormone. The intra-assay coefficients of variation were 11.5% for testosterone and 9.2% for corticosterone. The mean sensitivity was 2 pg/g and 0.11 ng/g for testosterone and corticosterone, respectively.

Statistical analyses

All measured variables met the assumption of normality (Kolmogorov-Smirnov test, $P > 0.05$). To compare size between A- and B-eggs in all clutches where eggs were measured, we used a general mixed model with clutch as a random effect (random intercept only) to account for the non-independence of the two eggs from the same clutch, egg order as factor and laying date as a covariate. To compare composition between A- and B-eggs in the sub-sample of eggs that were collected we used paired t-tests. We analysed egg hormone content using two different measurements: concentration (hormone amount/quantity of yolk) and total amount of hormone in the whole yolk (concentration x total yolk weight). For all contrasts we also calculated standardised mean difference effect size (d) using equation (1) from Nakagawa and Cuthill (2007).

To estimate the percentage of variance in yolk hormones and colour accounted by female identity we fitted simple mixed models (SAS, version 9) with female identity as a random factor (random intercept only) and egg order as a factor and divided the variance component of the random effect by the total of all variance components (sum of variance component estimate for residual and female identity).

To test for relationships between different egg components in the same egg, we built mixed models with egg component of interest as the dependent variable, female identity as a random effect (random intercept only), egg order as a factor to control for effects of the laying sequence, and other egg components as covariates. To test for a relationship between egg components and absolute egg size and within-clutch egg size dimorphism, we used the same mixed model, but used egg size and within-clutch egg size dimorphism as covariates instead. To calculate effect sizes for these relationships we converted the F-value of the relationship between dependent and covariate into a partial correlation coefficient r (Nakagawa and Cuthill 2007).

Full statistical models were simplified through backward stepwise procedures where first the least significant two-way interactions and then main effects were sequentially removed to obtain a minimal adequate model that only retained significant effects at $P < 0.05$ (Crawley 1993). We report only interactions that were statistically significant ($P < 0.05$). All tests were two-tailed, and we report means ± 1 S.E.

Results

Using all two-egg clutches measured ($n = 116$ clutches), A-eggs (44.91 ± 0.26 cm^3) were bigger than B-eggs (43.23 ± 0.24 cm^3; $d = 0.81$) independent of laying date (mixed model, egg order: $F_{1,115} = 76.15$, $P < 0.0001$; laying date: $F_{1,114} = 2.59$, $P = 0.11$). In the sub-sample of clutches collected for egg composition, we found a similar egg size difference between A- and B-eggs (Table 1). Across clutches, larger eggs had larger yolks and albumen and contained more protein and lipids (mixed models controlling for clutch identity and egg order, $n = 11$ two-egg clutches; $r > 0.72$, $P < 0.002$). Yet, within clutches, an A-egg did not contain significantly more dry matter (lipids and protein = 9.30 ± 0.26 g) than its B-egg (9.17 ± 0.21 g; $d = 0.17$, paired t-test, $t_{10} = 0.82$, P = 0.43) nor did A- and B-eggs differ in either lipid or protein content (Table 1). A-eggs had higher yolk colour scores, and lower concentrations of testosterone in their yolks than B-eggs, but yolk corticosterone concentrations did not differ (Table 1). The total amount of testosterone in the yolk was also lower in A-eggs (480.3 ± 56.5 pg) than in B-eggs ($683.\ 8 \pm 60.9$ pg; $d = 1.04$, paired t-test, $t_{10} = 2.43$, $P = 0.04$), but total amount of corticosterone did not differ between A- (305.5

± 51.9 ng) and B-eggs (273.3 ± 30.95 ng; *d* = 0.23, paired t-test, t_{10} = 0.81, *P* = 0.44). Clutch identity accounted for 57.9% of the variance in yolk corticosterone levels (random effect, *Z* = 1.58, *P* = 0.11), 16.7% of the variance in yolk colour (random effect, *Z* = 0.52, *P* = 0.60) and 2.3% of the variance in yolk testosterone levels (random effect, *Z* = 0.07, *P* = 0.94).

Controlling for egg order (factor) and clutch identity (random effect), yolk testosterone concentration was found to be independent of yolk colour and B-eggs had higher yolk testosterone levels than A-eggs (mixed model, partial correlation between yolk testosterone and yolk colour: *r* = 0.31, *P* = 0.19; egg order: $F_{1,10}$ = 7.02, *P* = 0.02; Fig. 1a). Yolk corticosterone concentration was also independent of yolk colour and did not differ with egg order (mixed model, partial correlation between yolk corticosterone and yolk colour: *r* = 0.29, *P* = 0.33; egg order: $F_{1,10}$ = 0.24, *P* = 0.64; Fig. 1b). Yolk testosterone concentration was also independent of yolk corticosterone concentration and lower in A- than in B-eggs (mixed model, partial correlation between yolk testosterone and yolk corticosterone: *r* = 0.10, *P* = 0.74; egg order: $F_{1,11.9}$ = 6.92, *P* = 0.03; Fig. 1c). Yolk hormone concentrations varied independently of lipid content (mixed model with egg order as a factor and clutch identity as random effects, yolk testosterone concentration: *r* = 0.03, *P* = 0.92; yolk corticosterone concentrations: *r* = 0.23, *P* = 0.43), whereas yolk colour scores increased with increasing yolk lipids levels, although the relationship was not statistically significant (*r* = 0.51, *P* = 0.08).

Finally, we tested variation in yolk hormone levels in relation to absolute egg size and within-clutch egg size dimorphism. Absolute egg size and within-clutch egg size dimorphism were not correlated (*r* = 0.24, *P* = 0.48, *n* = 11) and both were retained as independent covariates in subsequent analyses. Yolk corticosterone concentration was lower in clutches where B-eggs were smaller than A-eggs compared to clutches where the two eggs were of similar size (Fig. 2a), but not to absolute egg size. Both yolk testosterone levels and yolk colour were unrelated to egg size dimorphism and absolute egg size (Fig. 2b, 2c).

Discussion

The parental favouritism hypothesis predicts that facultative siblicidal species reinforce competitive hierarchies between asynchronously hatched siblings by laying a larger first-laid egg with more testosterone and carotenoids, but less corticosterone than last-laid eggs. In a year with poor environmental breeding conditions and pronounced brood reduction (Vallarino 2009), Kittiwakes laying two-egg clutches produced larger A- than B-eggs. A sub-sample of clutches that was representative of the population's within-clutch pattern in egg size were analysed for egg composition. A-eggs had less yolk testosterone and a higher yolk colour score (reflecting carotenoids) compared to the B-egg, but there was no difference in yolk corticosterone levels between the eggs. Thus observed within-clutch variation in egg composition supported only partially the predictions from the parental favouritism hypothesis. The investment hypothesis predicts that mothers that are of better quality, that experience better feeding conditions during laying or both are expected to increase their deposition of resources to the egg, resulting in differences between clutches. The data partially supported this prediction as females that produced clutches with two similar-sized eggs (indicative of parental quality, feeding conditions or both) had higher yolk corticosterone levels than females laying clutches with larger differences in egg size, but other egg components were not related to egg size. There was no evidence that mothers modified egg components in concert and therefore does not support the hypothesis that deposition of egg components is mutually adjusted.

The Parental Favouritism Hypothesis

The parental favouritism hypothesis was only supported with respect to egg size and yolk colour score (indicating carotenoids), but not with respect to the sex steroids. B-eggs were smaller than A-eggs which can reinforce the competitive hierarchy established by hatching asynchrony (Slagsvold et al. 1984). Chicks hatching from larger eggs are more successful (Williams 1994; Krist 2011) presumably because they contain more macronutrients (lipids and protein). Although B-eggs were on average 2.9% smaller than A- eggs, they contained on average only 1.4% less macronutrients than A-eggs, and our test may have lacked sufficient statistical power to detect such a small difference in macronutrient content. Alternatively, this could also be due to smaller last-laid eggs having lower water content than A-eggs. Unfortunately, it was not possible to measure fresh egg mass in the field and we can therefore not directly compare water content of eggs.

Last-laid Kittiwake eggs also had lower yolk colour scores than first-laid eggs. Yolk colour scores of gull eggs have been shown to be a reliable measure of total carotenoid concentration in the yolk (Verboven et al. 2005) where β-carotene, lutein and canthaxanthin are the most abundant carotenoids (Surai et al. 2001). Thus variation in yolk colour score in Kittiwakes is likely to reflect variation in total carotenoid concentration or in any of its most abundant carotenoids. It is possible that the lower carotenoid levels in B-eggs reinforce brood hierarchies by reducing growth rate, retarding development and making junior chicks more prone to diseases thus less successful in sibling competition (Saino et al. 2000; McGraw et al. 2005; Groothuis et al. 2005a; Rubolini et al. 2006).

In contrast, our data on yolk steroid hormones did not support the parental favouritism hypothesis. Yolk testosterone levels were predicted to be elevated in the A-egg (Schwabl et al. 1997) to enhance development, increase begging and promote competitiveness (Gil 2008) of the senior chick. This expected within-clutch pattern in androgens had been found in the facultatively brood reducing Cattle Egret *Bubulcus ibis* (Schwabl et al. 1997), although only absolute amounts of androgens were compared between eggs. However, subsequent studies of other species with a brood reduction strategy and siblicide did not confirm this pattern. In boobies there was either no change in testosterone with egg order (Drummond et al. 2008) or elevated levels of testosterone and androstenedione in last-laid eggs only in poor environmental breeding conditions (Dentressangle et al. 2008). Similarly, last-laid eggs of Kittiwakes breeding in Alaska had elevated androstenedione levels independently of the supplementary feeding treatment during laying (Gasparini et al. 2007). Here, we showed that Kittiwake B-eggs also had higher testosterone levels than A-eggs in a year where brood reduction was widespread. Thus, the majority of studies suggest that mothers deposit preferentially androgens in last-laid eggs. It is not possible to discard an alternative explanation that differential deposition of androgens across the laying sequence may just reflect changes in maternal physiology as she moves through the reproductive cycle (Groothuis and Schwabl 2008). Yet even if the within-clutch androgen pattern simply reflects changes in maternal physiology, it may still have effects on the offspring (Marshall and Uller 2007), possibly to offset deleterious effects of asynchronous hatching in the junior chick (brood survival strategy, Schwabl 1993; Lipar and Ketterson 2000; Eising et al. 2001; but see Maddox et al. 2008). This might allow the junior chick to survive if environmental conditions are good or perhaps to allow it to survive sufficiently long to serve as an insurance if the senior chick dies early in the nestling period. However, it is interesting to note that there might be some flexibility of the pattern of differential androgen deposition depending on environmental conditions (Sandell 2007; Dentressangle et al. 2008), and future studies could resolve whether these are individual responses or different phenotypes breeding in different environmental conditions.

It had also been suggested that elevated levels of corticosterone in last-laid eggs could reinforce brood hierarchies by corticosterone's effect to reduce competitive abilities, growth rate or both and thus handicapping the junior chick (Love et al. 2008, 2009; Kozlowski and Ricklefs 2010). Yet our results suggest that it is unlikely that there are systematic biologically meaningful differences in yolk corticosterone levels between the two eggs of Kittiwake clutches. This is in contrast with reported increases in corticosterone with laying order in other asynchronously hatching species (Black Guillemot *Cepphus grylle*, Love et al. 2009; Cockatiel *Nymphicus hollandicus*, Kozlowski and Ricklefs 2010).

Recently, there has been some discussions on whether birds deposit corticosterone into their eggs at all (Rettenbacher et al. 2009). Corticosterone maybe confounded by cross-reactions with other yolk steroids, in particular progesterone, which is present in much larger amounts than corticosterone (Groothuis et al. 2005b). However, we conclude that measured concentrations in yolk corticosterone are unlikely to be caused by progesterone cross-reactions. First, our observed levels of yolk corticosterone are within the range of previous studies (1.1 – 32.2 ng/g yolk, Love et al. 2008). To explain yolk corticosterone concentration in Kittiwakes (22.1 ± 3.25 ng/g yolk, n = 22 eggs) as a by-product of cross-reactivity, progesterone levels in Kittiwake eggs would need to be at least 3683 pg/g yolk (assuming 0.6% cross-reactivity of our antibody with progesterone, see methods). This is well above the range of progesterone levels recorded for wild birds so far (400-500 ng/g yolk, Lipar et al. 1999; Lipar 2001). Second, the within-clutch pattern in yolk corticosterone does not match the within-clutch pattern in yolk progesterone described elsewhere (Lipar 2001; Paitz et al. 2011). Thus, Kittiwakes are likely to deposit corticosterone into their eggs. Nevertheless, cross-reactivity with progesterone could increase the variability in corticosterone levels and decrease the power of statistical comparisons.

The Investment hypothesis

Individuals of better quality, with better feeding conditions or both are expected to lay larger eggs or clutches with similar-sized eggs (Kilpi et al. 1996; Hebert et al. 2002). When relating egg components with absolute and relative egg size, we found that between-clutch variation in yolk corticosterone levels was negatively related to within-clutch egg size dimorphism and thus partially support the investment hypothesis. Females that laid two eggs of similar size, and presumably were in better condition, laid eggs with higher levels of yolk corticosterone than females with large egg size dimorphism. This is in contrast to the observed intra-clutch pattern in European starlings (*Sturnus vulgaris*) where females in poorer condition deposit more corticosterone in their eggs than females in better condition (Love et al. 2005; Love and Williams 2008). The latter pattern is presumably because of yolk corticosterone levels passively reflect the higher baseline corticosterone levels in stressed mothers (Love et al. 2005; Saino et al. 2005). It is unlikely that the intra-clutch differences in corticosterone levels reflect a greater maternal stress of elevated egg production effort because corticosterone levels were independent of absolute egg size. Instead, deposition of yolk corticosterone may have fitness benefits by acting as a bet-hedging strategy in stochastic environments at the time of laying, thus conditions during chick rearing may be low and unpredictable (Love et al. 2005; Angelier et al. 2010). For example, high yolk corticosterone levels increase offspring flight performance (Chin et al. 2009) which might allow offspring to leave the nest site and forage by themselves earlier.

Mutually adjustment of egg components?

It has been suggested that egg components are mutually adjusted, giving rise to positive or negative associations between them. When statistically controlling for the effect of egg order, we did not find any associations between egg components in Kittiwakes. Few studies have found a positive association between yolk testosterone and carotenoids (Navara et al. 2006b; Török et al. 2007; Hargitai et al. 2010 for Great Reed Warbler *Acrocephalus arundinaceus*, estimated effect sizes *r* between 0.56 and 0.77) whereas others did not (Groothuis et al. 2006; Safran et al. 2008; Hargitai et al. 2010 for Cuckoos *Cuculus canorus*, estimated effect sizes *r* between 0.01 and 0.28). Our sample had a statistical power of 46 – 94% to detect an association with a similar effect size as studies that found a relationship, and our observed effect sizes were similar to studies that did not find the association. Moreover, in studies that demonstrated an association between testosterone and specific carotenoids, this was only found with β-carotenes rather than overall carotenoid levels as tested in our study. However, a general lack of correlation between egg components would be expected if deposition of components are influenced by environmental stimuli during short critical periods and the timing of critical periods for different components are likely to differ (Groothuis and Schwabl 2008; Okuliarová et al 2010).

Conclusions

Several traits will influence brood hierarchies and they are likely to differ in the strength of effects they can exert. Difference in age between siblings as a consequence of hatching asynchronously is thought to be the main factor influencing the magnitude and outcome of sibling rivalry (Stenning 1996; reviewed in Beissinger and Stoleson 1997). The within-clutch differences in egg size and composition may either reinforce or compensate the effects of asynchronous hatching (Muck and Nager 2006; Bogdanova and Nager 2008; Kim et al. 2010;). The effects of egg size and composition *per se* on the outcome of the sibling conflict, however, are difficult to separate from those from hatching asynchrony and may exert only a minor impact (Maddox and Weatherhead 2008; Forbes and Wiebe 2010). However, although working in concert, the role of some traits in modulating brood hierarchies is likely to be more important than others. In species with brood reduction, parental favouritism appears to work mostly through asynchronous hatching rather than through differential deposition of egg components (Mock and Parker 1997; Drummond et al. 2008) and our data on Kittiwakes agree with this. It is interesting that individual egg components either reinforce the effects of hatching asynchrony (egg size, carotenoids), compensate for its effects (testosterone) or are neutral (corticosterone). As the likely strength of their effects differ and may vary with environmental conditions, this variety of gradients may give the birds the necessary flexibility to cope with conditions during chick rearing that cannot yet be predicted at the time of egg laying (Groothuis et al. 2006).

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The authors declare that they have no conflict of interest.

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Table 1. Mean values (± 1 standard error) of egg volume and egg components (lipids, proteins, yolk colour, testosterone and corticosterone) for first-laid (A-eggs) and second-laid (B- eggs) from 11 clutches collected in 2004. Size and components of A- and B-eggs were compared using paired t-tests.

	A-egg	B-egg	Effect size *d*	Paired t-value (*df* = 10)	*P*-value
Egg volume (cm^3)	45.20 ± 1.05	43.89 ± 0.93	0.42	2.91	0.02
Lipids (g)	1.79 ± 0.09	1.69 ± 0.09	0.36	1.65	0.12
Proteins (g)	3.89 ± 0.06	3.87 ± 0.06	0.11	0.33	0.74
Yolk colour	13.8 ± 0.2	12.0 ± 0.2	2.86	6.50	<0.001
Testosterone (pg/mg yolk)	36.54 ± 3.79	52.89 ± 4.31	1.27	2.64	0.02
Corticosterone (ng/g yolk)	22.9 ± 4.02	21.37 ± 2.48	0.14	0.52	0.61

Figure 1

Fig. 1 Interrelationships between (a) yolk testosterone and yolk colour, (b) yolk corticosterone and yolk colour, and (c) yolk corticosterone and yolk testosterone. Closed symbols show A-eggs and open symbols show B-eggs. Lines connect the eggs of the same clutch

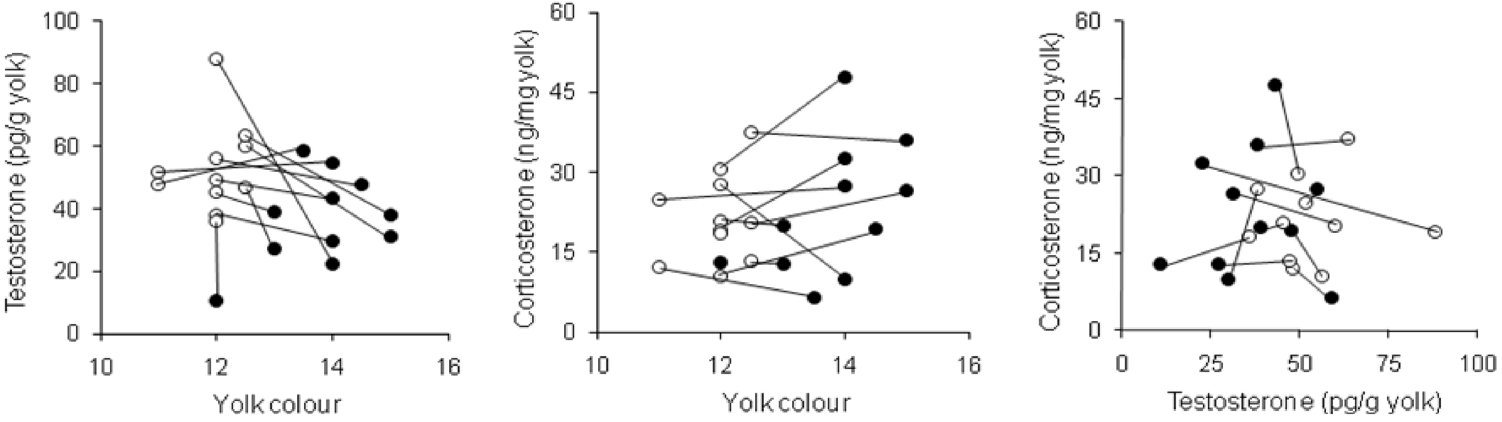

Figure 2

Fig. 2 Relationship between within-egg size dimorphism, as an indicator of female quality, feeding conditions during egg formation or both, and (a) yolk corticosterone levels, (b) yolk testosterone levels and (c) yolk colour. The data are shown for A- (closed symbols) and B-eggs (open symbols); the two eggs of the same clutch have the same value on the vertical axis. (a) With increasingly smaller B-eggs relative to the A-egg in the same clutch (increasing within-clutch egg size dimorphism) the eggs had decreasing yolk corticosterone levels (mixed model, within-clutch egg size dimorphism: $F_{1,9} = 8.64$, $P = 0.02$, $r = 0.70$; absolute egg size: $F_{1,9.89} = 0.20$, $P = 0.67$, $r = 0.04$; egg order: $F_{1,10} = 0.27$, $P = 0.61$). (b) Yolk testosterone level was not related to the within-clutch egg size dimorphism (mixed model, within-clutch egg size dimorphism: $F_{1,7.98} = 0.71$, $P = 0.42$, $r = 0.10$; absolute egg size: $F_{1,9.81} = 0.90$, $P = 0.37$, $r = 0.09$; egg order: $F_{1,10.6} = 7.37$, $P = 0.02$), nor was (c) yolk colour (within-clutch egg size dimorphism: $F_{1,7.88} = 3.17$, $P = 0.11$, $r = 0.17$; absolute egg size: $F_{1,9.96} = 1.34$, $P = 0.27$, $r = 0.10$; egg order: $F_{1,10} = 42.24$, $P < 0.001$)