A physiological marker for quantifying differential reproductive investment between the sexes in Yellow-legged gulls (*Larus michahellis*)

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Abstract

Asymmetry between males and females in the energy they invest initially in reproduction has resulted in the evolution of differing reproductive strategies (caring females vs. competitive males). However, parental care in many birds is shared by both sexes suggesting that male energy expenditure in agonistic behaviors and courtship feeding might compensate female costs of clutch production. Here, we tested the hypothesis that initial investment in reproduction by both sexes in the Yellow-legged Gull (Larus michahellis), a species with biparental care, is similar from a physiological perspective. In this income breeder, female and male reproductive investment during early breeding can be ultimately related to muscular activity (local foraging effort required for clutch production in females and courtship feeding and agonistic behaviors in the case of males). Thus, we evaluated sex-specific patterns of creatine kinase (CK, IU/L) levels in plasma, an indicator of physical effort associated with muscular activity dependent behaviors, through incubation as a reflection of the physiological response of both sexes to the reproductive investment they made up to clutch completion. Raw levels of CK were related to plasma levels of total proteins (TP, g/dL) to account for the differential physiological state of individuals when sampled (i.e. differential dehydratation). Female costs of clutch production were associated with post-laying levels of CK/TP. We grouped females according to their relative investment in clutch production: <15.8%, 15.8 to 17.3% and >17.3% of Field Metabolic Rate; which showed increasing values of CK/TP (24.6, 53.1 and 66.0IU/g, respectively). Moreover, we found similar CK/TP trends throughout incubation for both sexes (CK/TP=50.2-[3.3 × days from laying]) suggesting similar physiological responses to reproductive effort and, therefore, analogous sex-specific initial investment. Thus, male investment in agonistic behaviors and courtship feeding apparently equaled female investment in
clutch production. The use of CK measurements is revealed as a useful approach to investigating overall reproductive investment in reproduction for both sexes, providing further insights into our comprehension of reproductive strategies in seabirds.

Key words: Creatine kinase, clutch production, muscular activity, reproductive strategies, sex allocation, total plasma protein.
1. Introduction

Energy invested early in the reproductive cycle is an important component of the reproductive strategy followed by each sex (Patterson et al., 1980). According to sex allocation theory (Trivers, 1972), the initial asymmetry in the sex-specific energy invested in gametogenesis results in differing reproductive strategies between the sexes. Because of high energy invested by females in gametogenesis, they usually invest heavily in parental care since it is more profitable to continue with, rather than abandon, a costly activity (Dawkins and Carlisle, 1976). In contrast, low energy requirements for gametogenesis in males make competitiveness and extra-pair fertilizations a more profitable strategy for them (but see Kokko and Jennions, 2008). Sex-specific differences in initial reproductive investment are particularly evident in birds because of female costs of clutch production. Under this scenario, biparental care typical of many bird species (Lack, 1968) contrasts with classical sex allocation theory and suggests that male energy expenditure before clutch completion in behaviors such as male-male competition for mates, mate guarding or courtship feeding, might compensate female costs of clutch production (Gladstone, 1979; Burger, 1981; Giudici et al., 2010). As proposed by Baylis (1981), theory related to sex allocation strategies should be framed in terms of total energy costs associated with a mating event, rather than the production of individual gametes.

Male contribution to parental care is particularly important in the case of seabirds (Furness and Monaghan, 1987). Although qualitative sexual differences in reproductive investment patterns have been reported among gulls (Laridae), it is generally accepted that parental care (including incubation and chick rearing) does not quantitatively differ between the sexes (e.g. Butler and Janes-Butler, 1983; Burger, 1984; Stenhouse et al.,
2004). On the other hand, gulls have been described as income breeders, i.e. they mainly adjust their food intake concurrently with breeding, without reliance on stores (e.g. Hiom et al., 1991; Pons, 1992; Oro et al., 1995, 1996; Hobson et al., 1997; Hobson, 2006; Saino et al., 2010). Thus, female reproductive investment early in the reproductive cycle should be closely related to local foraging effort in order to supply the requirements of clutch production. In contrast, male reproductive investment during this period should be mainly devoted to courtship feeding and agonistic behaviors directed to compete for mates or to defense of their mates and nests (Burger, 1984, and references therein). Despite the qualitative differences in sex roles, both male and female reproductive investments are ultimately related to muscular activity, although parents remaining motionless on their nests are also serving an important role of protecting their nesting territories (see Burger, 1984). In this regard, exercise-induced muscle damage is characterized by the release of muscle-specific proteins such as creatine kinase (CK) to plasma (Knuth and Chaplin, 1994; Smith et al., 2004) and measurements of CK levels have been previously used as an indicator of physical effort associated with muscular activity dependent behaviors such as migration or foraging (Guglielmo et al., 2001; Navarro et al., 2008).

Here, we investigated patterns of CK in plasma through incubation in a seabird species with biparental care, the Yellow-legged Gull (Larus michahellis, Naumann 1840) as a reflection of the physiological response of individuals to the reproductive investment made up to clutch completion. As incubation constitutes a rest phase that allows individuals to recover their physiological state after the high energy demanding periods of pre-laying and laying (Alonso-Alvarez et al., 2002), recovery patterns in their CK levels should be expected. We first evaluated the relationship between costs associated
with clutch production, as estimated from Field Metabolic Rate (FMR), and CK patterns in females. If clutch production and local foraging effort are closely related processes, as we expect for income breeders, we predicted that females producing larger eggs would have higher CK levels due to greater foraging efforts. Secondly, we tested the hypothesis of comparable sex-specific investment before clutch completion by comparing CK trends for both sexes throughout incubation. We predicted that similar reproductive investment would result in similar CK recovery patterns.

2. Materials and methods

2.1. Model species and field methods

The Yellow-legged Gull was selected as an appropriate model species for this study since they are iteroparous, long-lived and sexual maturity is not reached until the 4th summer. These traits make this species particularly susceptible to trade-offs between current breeding and future reproductive output according to life-history theory (Williams, 1966) and, therefore, to selective pressures responsible for the differential allocation in reproduction between the sexes.

In gulls, females typically show modal clutches truncated to three eggs (Reid, 1987), although different clutch sizes can also be observed within population (e.g. two-egg clutches represent about 20% of overall clutches, Bosch et al., 2000). Incubation, which is shared by both sexes (Burger, 1984; Alonso-Alvarez et al., 2002), is gradually established as successive eggs are added to the clutch (Parsons, 1972). Due to the impossibility of sampling non-incubating birds and with the aim of standardizing the inclusion of individuals in the study (due to differing clutch sizes), bird sampling was performed after clutch completion. In particular, during peak laying of the breeding
season of 2008 (from 2-13 April), nests of Yellow-legged gulls breeding at the Peninsula de la Banya (40°40’N, 0°45’E; within the Ebro Delta Natural Park, Spain) were tagged when the first egg was laid, and inspected daily until clutch completion, with the aim of determining laying order, final clutch size and end of the laying period. Fresh, newly-laid eggs (< 24h after laying) were collected and replaced by dummy eggs, kept refrigerated and transported to the laboratory where they were frozen until processing. From those nests with a modal clutch of three eggs, 53 adults were caught during incubation using traps set at nests. Adults were captured early in the morning to avoid variation in the individuals’ physiological state throughout the day and the time elapsed between capture and sampling was similar for all individuals (about 15 minutes) in order to reduce the variability in levels of analyzed biochemical parameters associated to sampling procedure. Adult mass was measured using a dynamometer to the nearest mg. Two mls of blood were taken from the tarsal vein; 1.5 ml was placed in a vial with EDTA to later extract the plasma for the biochemical parameter determinations and the rest was preserved in a neutral vial for molecular identification of sex. All samples were frozen at -23 °C until sample analysis.

2.2. Laboratory procedures and data processing

Sex of gulls was determined using polymerase chain reaction (PCR) amplification of the CHD genes (Ellegren, 1996; Griffiths et al., 1998). To extract DNA, blood was boiled in 100mM NaOH for 10 minutes at 100°C before being added to the PCR reaction. PCR protocols were modified from Fridolfsson and Ellegren (1999) (see Genovart et al., 2003) using the primer set 2550F-2718R, and PCR products were visualized on a 3% agarose gel stained with ethidium bromide scoring a single band in males and two bands in females.
Plasma biochemical analyses of total protein (TP) and CK were determined using a Cobas 6000 automated analyzer (Roche Diagnostics, Mannheim, Germany). Manufacturer’s guidelines were followed for all methods. TP (g/dL) was analyzed by biuret assay (Weichselbaum, 1946), whereas CK (IU/L) was determined by kinetic methods following recommendations of IFCC (Bergmeyer et al., 1986; Schumann et al., 2002). Two individuals, one male and one female, were excluded from subsequent analyses since they showed CK values more than four times higher than male and female average concentrations. Occasional dehydration of animals, due to long foraging trips or exhausting exercise, is usually associated with hemoconcentration, leading to overestimates of plasma concentration. To reduce the variability of CK values due to undesirable factors such as the differential physiological state of individuals when sampled (i.e. differential dehydration), raw levels of this parameter were related to TP, which is widely accepted as a more conservative and well regulated parameter sensitive to dehydration status. In this way, we obtained standardized levels of CK related to grams of total plasma proteins (CK/TP) which were analyzed throughout.

To evaluate the influence of clutch production on female CK values, costs associated with clutch synthesis were estimated following Ruiz et al. (2000). Eggs were initially boiled to allow separation of different egg components (i.e. albumen and yolk). Each component was then freeze dried to constant mass and yolks were subjected to a lipid extraction with several rinses of 2:1 chloroform-methanol. In each step, sub-samples were weighed in order to ascertain protein and lipid content of each egg component. Clutch energy content (hereafter energy value) was then estimated by using appropriate conversion factors accounting for 39.75 kJ/g for lipids and 23.64 kJ/g for proteins...
(Ricklefs, 1974). Estimated energy values were refined by adjusting them to account for energy costs derived from absorption, metabolism and deposition of rebuilt materials. Average costs for lipids and proteins in birds were considered to be about 22% and 45% of estimated energy values, respectively (Blaxter, 1989). In this way, we obtained estimates of total amount of energy invested during egg synthesis (hereafter energy investment). As a measure of female costs of clutch production we calculated the ratio between energy investment in clutch synthesis and FMR (kJ/d) during clutch production. Following Ruiz et al. (2000), we assumed modal clutch (three eggs) production to last 16 days (10 days for the rapid yolk deposition, 2 days for the deposition of albumen, membranes and shells and 2 days for the follicle triggering interval). FMR was considered to be 3.29 times the Basal Metabolic Rate (BMR, Birtfriesen et al., 1989) and BMR was estimated from the relationship $BMR = 2.1857 \cdot mass^{0.7347}$ (Furness and Monaghan, 1987).

2.3. Statistical analyses

Linear models (LMs) were used to evaluate the relationship between female costs of clutch production and their CK/TP values. The number of days elapsed between clutch completion and capture of individuals (hereafter elapsed time) as well as the relevant interactions were included in this model to take into account any temporal trend in this parameter through the incubation period. LM was also applied to evaluate temporal trends of CK/TP throughout incubation. Here, the effect of elapsed time on the biochemical parameter was established. Sex and relevant interactions involving this factor were also included in these models in order to detect and quantify differences in temporal trends according gender. Statistical analyses were done using SPSS 15.0 (SPSS Inc., Chicago, USA).
3. Results

Of the 53 adults analyzed, 35 individuals were females and 18 were males. Biochemical parameters for both sexes are summarized in Table 1. When evaluating the influence of female costs of clutch synthesis on CK levels, the model indicated a significant effect on the CK/TP temporal trend ($F_{1,30}=5.03$, $p=0.03$ for the interaction between costs of clutch production and elapsed time). In order to explore this interaction, females were grouped according to tertiles of female costs of clutch production: low costs (<15.8% of FMR), high costs (>17.3%) and medium costs (15.8 to 17.3%). Higher costs of clutch production resulted in higher initial levels of CK/TP and a more pronounced decrease in these values along the incubation period (Fig. 1).

The absence of the interaction sex*elapsed time ($F_{1,47}=0.001$, $p=0.98$) indicated CK/TP slopes did not significantly differ between sexes. This pattern consisted of a gradual decrease of CK/TP values through the incubation period ($F_{1,48}=44.75$, $p<0.001$, 95% CI for the estimated slope=-4.3 to -2.3). Moreover, we did not detect a significant effect of sex, suggesting similar average values of CK/TP for both sexes ($F_{1,48}=0.124$, $p=0.73$, 95% CI for the estimated differences between males and females=-8.79 to 6.17; Fig. 2).

4. Discussion

Previous studies aimed at quantifying reproductive investment and, therefore, reproductive costs have been focused largely on trade-offs between investment in incubation or chick rearing and parental survival and fecundity (Navarro and Gonzalez-Solis, 2007, and references therein). However, to fully understand the evolution of reproductive strategies followed by each sex, all reproductive costs, including clutch...
production in the case of females and agonistic behaviors and courtship feeding in the case of males, need to be considered (Gladstone, 1979; Baylis, 1981; Burger, 1981; Nager et al., 2001). The initial asymmetry in reproductive investment between the sexes proposed by classical sex allocation theory (Trivers, 1972) has been previously assumed but never measured. This is due, in part, to the fact that traditional approaches would require a unique measure that integrates all the energy demanding processes related to reproduction. In this regard, reported results suggested that CK activity in plasma is a reliable indicator of the physiological responses to increased muscular activity to which males and females are subjected during this period (i.e. initial reproductive investment) and provide, therefore, a new and excellent method to examine a central component of the reproductive strategy followed by each sex. In particular, this physiological assay has revealed as a reliable proxy to female investment in clutch production and suggests that initial reproductive investment of males, including agonistic behaviors and courtship-feeding, apparently counterbalance female investment in clutch synthesis.

4.1. Female investment in clutch production

As expected for an income breeder, the relationship between female costs of clutch production and plasma CK values agrees with an initial reproductive investment by females that is closely related to local foraging effort. Females that invested heavily in clutch synthesis corresponded to those with higher muscular damage (i.e. higher CK levels) after clutch completion, indicating greater muscular activity probably due to greater foraging efforts. In other words, greater foraging efforts, as indirectly indicated by higher levels of CK in plasma, allowed females to produce larger and more energetically expensive eggs, probably enhancing egg hatchability and chick survival.
and, therefore, females’ current reproductive success (Parsons, 1970; Ruiz et al., 1998; Christians, 2002). However, the life-history theory (Williams 1966) predicts that increasing current reproductive efforts may reduce females’ future reproductive output by reducing their fecundity or survival until future breeding seasons.

Whenever initial female reproductive investment is closely related to local foraging effort, reproductive effort before clutch completion will depend on females’ physical condition and foraging efficiency: the fittest and most experienced females will require lower efforts to deal with clutch production. Indeed, several female intrinsic traits such as body condition, age or experience have been related to the amount of resources allocated to egg synthesis (as indicated by egg size, see Christians 2002). Such female intrinsic traits may also influence their physiological response to reproductive investment: the fittest females would show smaller physiological responses to similar reproductive requirements (see Guglielmo et al., 2001 for the relationship between birds’ trained state and plasma CK activity). Rather than direct estimations of resources allocated to egg synthesis (e.g. egg size or mass), this physiological assay is therefore proposed as a better and more realistic approach to investigate among-female relative reproductive investment before clutch completion.

4.2. Sex-specific reproductive investment

Methodological limitations to sampling gulls (see Material and methods) forced us to investigate the recovery patterns of CK in plasma through incubation and so we speculated about sex-specific physiological responses to the reproductive investment they were subjected to up to clutch completion. In this regard, similar recovery trends of CK for both sexes suggested similar recovery patterns of muscular damage and,
therefore, comparable reproductive investments before clutch completion. Male investment, including agonistic behaviors and courtship feeding during pre-laying and laying, apparently counterbalance female investment in clutch production.

Although speculative, our interpretation is in agreement with Burger (1984) who reported higher frequencies of agonistic behaviors for males of Herring Gull (*Larus argentatus*) before clutch completion, whereas both sexes contributed about equally to incubation, brooding and feeding. Additionally, results of a physiological approach for Cory's shearwaters (*Calonectris diomedea*), another seabird species with biparental care, revealed that males were in poorer condition than females before clutch completion probably due to reproductive investment of males in agonistic behaviors and courtship feeding (Giudici et al., 2010). In these iteroparous and long-lived species, parents are particularly limited in how they can increase effort in current reproductive investment in order to moderate the impact on future reproduction attempts (Williams, 1966; Croxall, 1982; Chaurand and Weimerskirch, 1994; Tveraa et al., 1997; Velando and Alonso-Alvarez, 2003; Navarro and Gonzalez-Solis, 2007). In this context, selective pressures directed at making investment in reproduction by both sexes equal should be expected.

5. Conclusions

Despite qualitative differences in sex roles of Yellow-legged gulls, CK levels in plasma are shown to be a reliable proxy to the overall energy investment that both sexes are subjected to up to clutch completion. This allowed quantitative comparisons of sex-specific reproductive investment during pre-laying and laying and suggested that agonistic behaviors and courtship feeding in the case of males may compensate for
females’ costs of clutch production. This physiological approach is expected to assist in understanding the selective pressures that have modulated sex roles in reproduction since provide information about a central component of the reproductive strategy followed by each sex.

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References


The female reproductive effort in gametogenesis, i.e. the energy investment to clutch production relative to the Field Metabolic Rate (FMR) during the clutch production period (16 days), have been also summarized.

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FIGURES

Figure 1.- CK/TP patterns through incubation (Elapsed time: number of days elapsed between clutch completion and capture of individuals) for females grouped according to three differential costs of clutch production (energy investment in clutch synthesis respect Field Metabolic Rate FMR, see Material and Methods): low costs (<15.8% of FMR), high costs (>17.3%) and medium costs (15.8 to 17.3%)

Figure 2.- Temporal trends of CK/TP through incubation (Elapsed time: number of days elapsed between clutch completion and capture of individuals) for each sex. Solid line represents regression line predicted by the model (see Material and Methods and Results).
Figure 1.

Costs of clutch production:
- High
- Medium
- Low

Elapsed time (days)

CK / TP (IU/g)