

Hormone levels in the outer layer of domestic goose (*Anser anser domesticus*) eggs change over the laying season

John P. Kent^{a*}, Kenneth J. Murphy^{a,b,d}, Attila Salamon^{a,b}, Thomas J. Hayden^b and Erich Möstl^c

^aBallyrichard House, Arklow, Co. Wicklow, Ireland

^bSchool of Biology and Environmental Science, University College Dublin, Belfield, Dublin 4, Ireland

^cDepartment of Biomedical Sciences/Biochemistry, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria

^dPresent address: Kings College London, Department of Forensic and Neurodevelopmental Science, Institute of Psychiatry, De Crespigny Park, London, SE5 8AF, UK

*E-mail: john.kent@ucd.ie

ABSTRACT

In general, avian species produce clutches of more than one egg. Differential investment in egg weight and hormone levels is possible and their effects on development and behaviour can be measured. Here we investigated changes in three hormones (testosterone, T; androstenedione, A₄ and progesterone, P₄) in the outer layer of yolks of infertile domestic goose eggs over the course of a laying season. There was a significant change in the concentration of all three hormones: T and P₄ increased in a linear manner across the season whilst A₄ increased until mid season and then steadily decreased. The correlation between T and P₄ ($r = 0.613$, $P < 0.001$), and between T and A₄ ($r = 0.746$, $P < 0.001$) were both positive, but the relationship between A₄ and P₄, while positive was not strong ($r = 0.379$, $P < 0.001$), although still significant. The increase in P₄ over the laying season is consistent with the transition from a laying to a broody state. The increase in T is consistent with a trade-off for the seasonal decline in egg weight.

Keywords: androstenedione; eggs; geese; maternal investment; progesterone; testosterone

1. INTRODUCTION

The maternal interuterine environment in mammals influences offspring development. In house mice (*Mus musculus*), females occupying an interuterine environment between two males had first oestrus at a later age and had longer oestrus cycles, a shorter reproductive life, and were more aggressive, than females positioned *in utero* between two other female embryos (vom Saal 1984; for review see Clark and Galef, 1998). From these findings a role for pre-natal hormones on post-natal development started to become apparent.

The finding that egg yolk was a source of maternal testosterone (T) and that yolk T influenced postnatal development in birds (Schwabl, 1993; 1996a) further stimulated interest in what are known as maternal effects (Kirkpatrick and Lande, 1989; Groothuis and Schwabl, 2008). The eggs of oviparous species are a perfect model for this since their development largely takes place outside the female's body and therefore facilitates experimental manipulation in a controlled manner.

In the egg yolk of black-headed gulls (*Larus ridibundus*; Eising *et al.*, 2001; Groothuis and Schwabl, 2002; Eising and Groothuis, 2003; Müller *et al.*, 2004), lesser black-backed gulls (*Larus fuscus*; Royle *et al.*, 2001; Verboven *et*

al., 2003) and common terns (*Sterna hirundo*; French Jr *et al.*, 2001) T levels increase with laying order. This increase in T in later laid eggs is thought to accelerate embryonic development and therefore plays a compensatory role in the context of hatching asynchrony since the later laid eggs may receive less incubation time (Müller *et al.*, 2004); incubation starts during laying in Canada geese (*Branta canadensis*; Cooper, 1978) and barnacle geese (*Branta leucopsis*; Hübner *et al.*, 2002). Hegyi and Schwabl (2010) found that T reduced early structural growth and mass gain post-natally in Japanese quail (*Coturnix japonica*), while Hegyi *et al.* (2011) showed that collared flycatcher (*Ficedula albicollis*) eggs with relatively higher yolk T levels were less likely to hatch. However, Boonstra *et al.* (2009) found that T decreased with laying order in Canada geese, but T was measured at the centre of the egg yolk (see Discussion). There was no relationship between T and laying order in Japanese quail (for review see Groothuis *et al.*, 2005 and Gil, 2008). Japanese quail are noted for their synchronised hatching that is facilitated by pre-hatching social facilitation mechanisms (see Freeman and Vince, 1974) and while T has no apparent role in hatching synchrony it was found to influence early behavioural development (Daisley *et al.* 2005; Okuliarová *et al.* 2007).

Between clutch variations in T, androstenedione (A_4), and/or dihydrotestosterone (DHT) have been found to be at least as variable as within clutch variation in black-headed gulls (Groothuis and Schwabl, 2002; Müller *et al.*, 2004). Low levels of androgens in late-laid clutches in canaries (*Serinus canaria*; Schwabl, 1996b) and sparrows (*Passer domesticus*; Schwabl, 1997; Mazuc *et al.*, 2003) may be attributed to reduced social interaction, since social competition is associated with high T early in the breeding season when territories are being formed (in keeping with the challenge hypothesis, see Wingfield *et al.*, 1990). Black-headed gulls at the periphery of the colony laid eggs containing more T and A_4 , and were more aggressive and had relatively larger territories than did those at the colony centre due to higher predation rates at the periphery (Groothuis and Schwabl, 2002). This is in accordance with the findings of Kent *et al.* (2009) showing that challenges at mating in domestic fowl (*Gallus domesticus*) were followed with increased faecal T in males and a corresponding increase in inter-individual distances. Thus T levels are sensitive to social factors which need to be acknowledged in the pre-natal context.

Groothuis and Schwabl (2002) pointed out that the eggs of semi-altricial and precocial birds contain much higher levels of A_4 than T, when compared to altricial birds. Mothers deposit the A_4 , a precursor of T (Kawata, 1995), in the egg as a reservoir, that can be transformed subsequently to the biologically more potent T by the embryo when necessary (see Groothuis and Schwabl, 2008). The finding that A_4 is the dominant androgen in eggs of precocial birds suggests that it has a more important role in development than does T (Hegyí and Schwabl, 2010; Hegyí *et al.*, 2011). Hegyí *et al.* (2011) found that in collared flycatchers, eggs containing higher A_4 levels within a clutch produced larger hatchlings, presumably due to their enhanced embryonic development, but these hatchlings had a slower growth rate to reach fledging weight.

Relative changes in maternal egg hormones over the laying season have the potential to give insight into hormone function. Plasma progesterone (P_4) levels increased during the laying phase in bantams (Sharp *et al.*, 1979) and faecal P_4 levels were higher during laying phase in canaries (Sockman and Schwabl, 1999). Within the hen egg, Möstl *et al.* (2001) found higher P_4 levels in the surface layer with the concentration decreasing towards the centre, similar to that found in quail eggs (Hackl *et al.*, 2003). The thecal layer of a maturing follicle in the domestic hen increases production of P_4 just before ovulation (Bahr *et al.*, 1983), explaining the higher P_4 at the surface layer of the yolk (Möstl *et al.* 2001; Hackl *et al.* 2003).

In the present study, changes over the laying season in three yolk hormones: T, A_4 and P_4 were measured in the outer layer of the egg yolks of domestic geese (late March to late May) and their relative changes are examined in

the light of their potential function in the reproductive phase of the goose.

2. MATERIALS AND METHODS

2.1. Subjects

Eggs were collected from later March to early May from a population of 245 female domestic geese with ganders (Legarth strain) made up of 10 flocks housed separately, the flocks ranging in size from 10 to 64 individuals and in age from one to 10 years. They were maintained at Ballyrichard, 72 km south of Dublin, Ireland (52.83°N, 6.13°W) as described by Kent and Murphy (2003). Eggs were collected on bird release in the morning at 10:00 and a number of times during the day. They were washed and then stored in constant temperature below 14 °C and away from direct sunlight, until setting at 14-day intervals.

2.2. Yolk sample collection

Eggs were incubated (WESTERN incubator) for 10 days, candled and the infertile eggs removed and selected and then weighed (see Table 1) so that they were uniform in size and shell characteristics with a variety of egg weights to avoid taking eggs from the same geese. Yolk samples taken after weighing using a 10 mL vacuum glass tube with a 1.25 mm wide and 25 mm long needle inserted into the egg in the middle from the side perpendicular to the long axis to puncture the yolk and extract yolk from the outer layer of the yolk. Care was taken to extract yolk sample from the outer 5 mm layer of the yolk. Samples containing albumen were discarded. The samples were then centrifuged and then stored at -20 °C until hormone extraction. Thus, freezing took place immediately after the yolk samples were collected from the batch of eggs, while the hormone extraction took place at a later date.

The use of infertile eggs avoided the contaminating effects of embryo development on hormone levels as found in domestic chicks (Elf and Fivizzani, 2002). Using infertile eggs is a useful non-invasive way to examine yolk hormone changes over the season in a controlled manner, free of contaminating developmental influences including paternal effects, even though they have been incubated for 10 days in this study. This approach parallels work using faecal samples, also a non-invasive technique (Goymann, 2005).

2.3. Yolk extraction

Yolk extraction was carried out at the University of Veterinary Medicine in Vienna according to the methodology of Möstl *et al.* (2001). Yolk (about 2 g) was weighed in glass scintillation vials, 10 mL distilled water

Table 1 Descriptive statistics

Date of setting	20/03/04	31/03/04	10/04/04	21/04/04	01/05/04	13/05/04	22/05/04
Day of year (from March 10)	11	22	32	43	53	65	74
No. of eggs set	860	904	851	732	689	776	520
No. of infertile eggs	114	106	102	97	73	120	102
% infertile	13.3	11.7	12	13.3	10.6	15.5	19.6
Sample size (<i>n</i>)	16	14	14	12	16	17	16
Egg weight day 10 (g)							
Mean	161.7	161.5	157.3	161.2	155.5	151.9	149.5
SD	33.2	27.2	26.8	30.2	27.8	37.6	19.5
T (ng g ⁻¹ yolk)							
First quartile	8.01	9.16	9.79	12.7	13.45	15.45	15.11
Median	10.46	11.05	14.27	15.25	16.95	20.86	19.87
Third quartile	13.8	16.63	17.17	18.4	25.68	22.6	26.17
A4 (ng g ⁻¹ yolk)							
First quartile	456.3	534.6	545	766	753.2	708	643.5
Median	511.8	710.2	803.9	942	964.2	893.6	786.5
Third quartile	582.2	898.7	1002.7	1237	1263.8	1201.6	1286.1
P4 (ng g ⁻¹ yolk)							
First quartile	70	1	435	824	966	1183	901
Median	364	1	878	1562	1620	2348	1778
Third quartile	1434	625	1730	2675	3365	3045	3057

T, Testosterone; A4, androstenedione; P₄, progesterone concentrations.

was added and the mixture exhaustively vortexed. 1 mL of the homogenated yolk–water mixture was transferred into a new tube and 4 mL of methanol was added. The sample was vortexed for 30 min, placed at –20 °C overnight and centrifuged on the next day at –10 °C (2500 g, 10 min). 1 mL of the supernatant was evaporated in a stream of nitrogen and re-dissolved in 500 µL assay buffer. Enzyme immunoassays for androgens and P₄ were used to measure hormone concentrations. For the androgen assays, 100 µL of the samples were diluted with 400 µL assay buffer, with 10 µL of the final solution used in the analysis. In the case of P₄, a further 1+9 dilution step was done. The initial weight of the samples was considered when calculating the results. The test validity was in accordance with Möstl *et al.* (2001).

2.4. Statistical analysis

Regression analysis using Minitab 16 was employed to test whether egg weight changed over the study period. Then backwards stepwise regression analyses were employed to determine the roles of day of year and egg weight on maternal yolk hormone levels. Egg weight, yolk T and A₄ concentrations were normally distributed. As P₄ concentration data was not normally distributed it was square root transformed (sqrt). Pearson correlation analysis was employed between the three hormones.

3. RESULTS

Day of year did not have a significant effect on egg weight ($F=2.23$, $P=0.138$), though a slight decline in egg weight in the egg sample used over the season was noted (see Table 1) in keeping with the findings of a larger study (Salamon and Kent, in preparation). There was no relationship between egg weight and T levels ($T=-0.82$, $P=0.416$). A significant linear increase in T over the season was found when the factor egg weight was removed ($Y=8.66+0.173x$, $P<0.001$, adjusted $R^2=34.1\%$; Figure 1a). Changes in T, A₄ and P₄ are presented in Table 1.

Egg weight did not affect the concentration of A₄ ($T=-0.52$, $P=0.602$). A₄ concentration increased until late April, and then declined steadily consistent with a quadratic trend (with the egg weight factor removed, $Y=252.2+26.9x-0.245x^2$, $P<0.001$, adjusted $R^2=21.1\%$; Figure 1b).

There was again no effect of egg weight on P₄ levels ($T=-0.41$, $P=0.683$). P₄ concentration increased in a linear manner over the season (sqrt $Y=15.65+0.446x$, $P<0.001$, adjusted $R^2=23.3\%$; see Figure 1c). Here with P₄ we were using square root transformed data for the analysis of change over time, as the data was not normally distributed (see Statistical analysis). A strong significant correlation was found between T and P₄ ($r=0.613$, $P<0.001$) and between T and A₄ ($r=0.746$, $P<0.001$). The relationship between A₄ and P₄ at $r=0.379$ ($P<0.001$) was not so strong, although still significant.

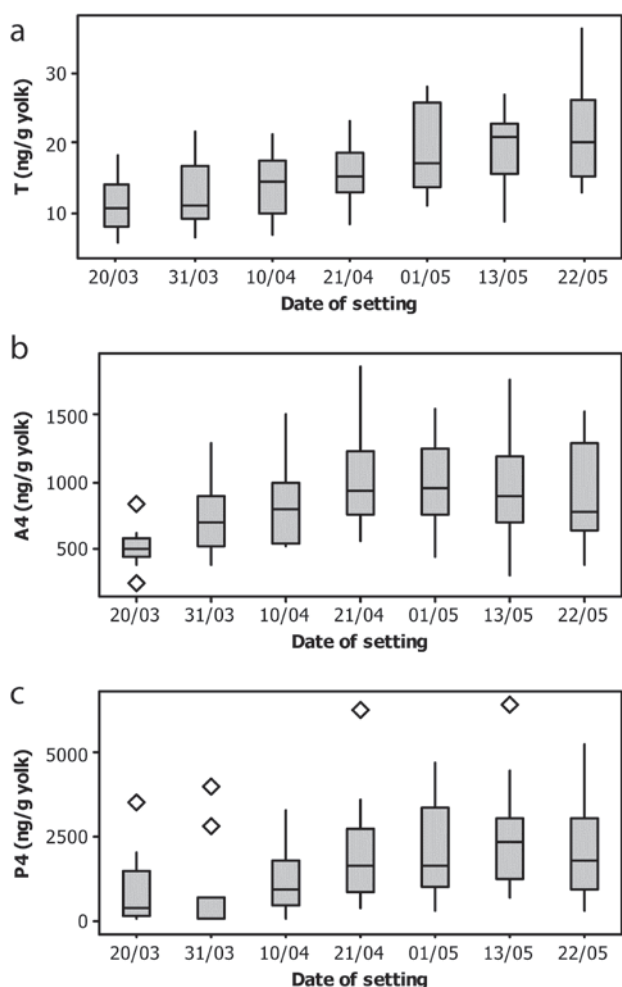


Figure 1 Changes in yolk: (a) testosterone, T; (b) androstenedione, A_4 ; and (c) progesterone, P_4 (ng g^{-1} yolk) over the laying season in goose eggs, using original and not transformed data. Box plots present interquartile values with the middle line showing the median (see Table 1) and the lines extending to the minimum and maximum data points except for outliers that are shown by the symbols (\diamond). Eggs were set on the date shown and were laid during the previous 14 days.

4. DISCUSSION

The T increase over the season is consistent with findings showing a T increase with laying order in precocial species such as the black-headed gull (Eising *et al.*, 2001; Groothuis and Schwabl, 2002; Eising and Groothuis, 2003; Müller *et al.*, 2004), lesser black-backed gull (Royle *et al.*, 2001; Verboven *et al.*, 2003), common tern (French Jr. *et al.*, 2001) and in keeping with the increase in T over the laying season in black-headed gulls (Müller *et al.*, 2004). Increasing T can act to compensate for hatching asynchronies in which incubation of late laid eggs can start before clutch completion (Müller *et al.*, 2004; see Groothuis *et al.*, 2005). Such a mechanism might occur in geese, as nest attentiveness increased before the last egg was laid in giant Canada geese (Cooper, 1978),

while incubation started before clutch completion in barnacle geese (Hübner *et al.*, 2002). Salamon and Kent (in preparation) found a decline in goose egg weight over the laying season (although not seen in the present study because we were randomly selecting eggs to be uniform in size and shell characteristics) and the increasing T found here may serve to compensate for the expected lower levels of investment in egg weight as the season progresses. Interestingly Boonstra *et al.* (2009) showed a decreasing T pattern in clutches of Canada goose eggs, where egg weight declined over the laying sequence, suggesting that females allocate less T to the later laid eggs in the laying sequence. The difference between the increasing T found here and the decreasing T found by Boonstra *et al.* (2009) may be attributed to sampling methodology. Here, samples were taken from the outer yolk layer. Boonstra *et al.* (2009) inserted the needle 20 mm into the egg. We, using goose eggs of comparable weight to Boonstra *et al.* (2009), which we hard boiled and sliced, found that a 20 mm insertion would extract a sample from the centre of the yolk. Möstl *et al.* (2001) showed that T, A_4 and P_4 concentrations differ in the various layers of hen egg yolk: sampling the outer yolk layer describes seasonal hormonal change more reliably, as the rapid yolk growth phase occurs 6–11 days prior to ovulation, when increasing levels of protein and lipids are deposited in the maturing follicle. The centre of the follicle may have developed several months before ovulation (Johnson, 2000).

There was no significant relationship between egg weight and hormone levels in our present study, which can be attributed to the sampling method (see Materials and Methods; and Taborsky, 2010). Eggs had to meet strict selection criteria for incubation, therefore only top quality, uniform eggs were chosen for incubation and from these only the infertile eggs provided the material for this study.

Here, the increases in T and A_4 were positively correlated in keeping with the findings of Groothuis and Schwabl (2002). However, A_4 rose from the beginning of the laying season, peaking at mid lay then declined, while T levels continued to rise. The concentration of A_4 was much higher than T, similar to findings in black-headed gulls (Eising *et al.*, 2001; Groothuis and Schwabl, 2002; Müller *et al.*, 2004), Japanese quails (Hackl *et al.*, 2003) and collared flycatchers (Hegyi *et al.*, 2011). These findings suggest that the high levels of A_4 function as a precursor for T. Hegyi *et al.* (2011) found that A_4 has a more important role than T in precocial birds by enhancing embryonic development.

P_4 and T increased over the laying season in parallel. The rise in yolk P_4 is consistent with the increase in plasma P_4 during the laying phase in bantams prior to the onset of incubation at which point they decline rapidly (Sharp *et al.*, 1979). In canaries, faecal P_4 increased and remained high during laying, but then returned to baseline levels during incubation (Sockman and Schwabl,

1999). Experimental studies with ring doves (*Streptopelia risoria*) showed that isolated birds injected with P_4 , sat on eggs almost immediately when paired in a test cage suggesting that increasing P_4 is preparing birds for the broody state (Lehrman, 1958; 1965). Studies using plasma and faecal samples, as well as the present work using egg yolk, are consistent with a P_4 increase associated with broodiness (see Wingfield *et al.*, 1990) and consistent with endocrinological mechanisms underlying the transition to broodiness.

Groothuis and Schwabl (2008) reviewed three alternative mechanisms for steroid hormone accumulation in eggs. One is the physiological epiphenomenon hypothesis (PEH), which predicts a positive correlation between concentrations of maternal hormones in the mother and the egg (Groothuis and Schwabl, 2008; for review see Gil, 2008). The PEH would explain the increased P_4 levels at the end of the laying season, since P_4 levels increase in the plasma when preparing for broodiness (Sharp *et al.*, 1979; see Wingfield *et al.*, 1990); however, Groothuis and Schwabl (2008) noted that the mechanisms regulating yolk and plasma hormone concentrations by the mother are still unclear.

We suggest that the use of infertile eggs is a useful non-invasive technique for studying maternal hormone investment in egg yolks as it is free of developmental and paternal influences.

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