Articles

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Maternal transfer of androgens in eggs is affected by food supplementation but not by predation risk

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Mothers may affect the future success of their offspring by varying allocation to eggs and embryos. Allocation may be adaptive based on the environmental conditions perceived during early breeding. We investigated the effects of food supplementation and predation risk on yolk hormone transfer in the pied flycatcher *Ficedula hypoleuca*. In a food supplementation experiment, females were food-supplemented prior to and during egg-laying and androgen concentrations were measured throughout the laying order. Predation risk was investigated in three different studies combining both correlative data, where flycatchers bred in close proximity to two different predator species that prey upon adult flycatchers (either Tengmalm's owl *Aegolius funereus* or pygmy owl *Glaucidium passerinum*), and an experimental manipulation, where flycatchers were exposed to cues of a nest predator (least weasel *Mustela nivalis*). Females receiving food supplementation laid eggs with lower concentrations of androstenedione (A4) than females not receiving food supplements. Yolk testosterone (T) concentration showed the same pattern but the difference was not statically significant. Testosterone (but not A4) concentration increased within clutches, from the first to the last egg, independently of the food supplementation. Females breeding under high predation risk did not differ from control females in their yolk androgen levels (A4, T or progesterone). However, concentrations of A4 tended to be lower in the proximity of pygmy owls, which could indirectly increase offspring survival after fledging. Food supplementation during egg-laying seems to have a stronger impact on maternal transfer of androgens than predation risk. Food availability and predation risk could differentially affect the trade-offs of androgen allocation for the offspring when raised in good vs. dangerous environments.

Parents may impact the probability of survival and the phenotype of their offspring not only through genetic factors, but also through the resources allocated to embryos and provided to young (Bernardo 1996, Mousseau and Fox 1998, Metcalfe and Monaghan 2001). These maternal effects are especially detectable in oviparous species, where the nutrients transferred in the eggs are the only source of nourishment for the embryo until hatching (Groothuis et al. 2005).

The transfer of androgen hormones in eggs is particularly important as it may alter the condition and fitness of offspring (review by Groothuis et al. 2005, Gil 2008). In birds, yolk androgens may enhance (Schwabl 1993, Eising et al. 2001) or reduce (Ruuskanen and Laaksonen 2013) the growth of young (review by Groothuis et al. 2005), and may increase their competitive ability (Schwabl 1993), begging behaviour (Eising and Groothuis 2003, Ruuskanen and Laaksonen 2013) and their survival under food-shortage (Pilz et al. 2004). In contrast, yolk androgens also have potentially negative effects on the immune function of young (Groothuis et al. 2005, Sandell et al. 2009). In the long-term, androgens may have positive consequences on offspring reproductive success (Schwabl 1993, Strasser and Schwabl 2004, Eising et al. 2006), but alter antipredator activity pattern (Ruuskanen and Laaksonen 2010) and basal metabolic rate (Ruuskanen et al. 2013).

Shortage of food and predation risk are well known physiological stressors that may affect animal condition. These stressors can also indirectly affect offspring growth and survival (Metcalfe and Monaghan 2001, Clinchy et al. 2004, Gasparini et al. 2007, Sheriff et al. 2009, Coslovsky and Richner 2011, Sheriff and Love 2013). Indeed both food availability and perceived predation risk may affect egg composition and the size of eggs and offspring in many vertebrates (Bian et al. 2005, Saino et al. 2005, Thomson et al. 2006, Sheriff et al. 2009, Coslovsky and Richner 2011, Giesing et al. 2011, Zanette et al. 2011, Coslovsky et al. 2012, Morosinotto et al. 2013). Therefore, to optimize maternal fitness and offspring survival, we can expect that maternal allocation of resources to the offspring will vary according to food availability and predation risk at the breeding site (Marshall and Uller 2007).

Hormone transfer in avian eggs can be affected by food availability through its influences on female body condition (review by Groothuis et al. 2005), but the mechanisms behind this transfer are still unclear. Few food supplementation experiments suggested that fed females produced eggs with less androgens compared to control females (Verboven et al. 2003, Gasparini et al. 2007). Females in good condition may transfer less androgens in the eggs, thus reducing the long-term costs of androgens for the offspring, while compensating for this variation in egg composition by providing good quality post-hatching parental care; thus reducing the benefits of androgen transfer. However, also the opposite pattern has been suggested, with females in good condition allocating more androgens to their eggs (review by Gil 2004). This could occur if high levels of androgens are actually beneficial for the offspring but are costly for the laying female and thus only females in good condition could allocate more androgens to the eggs.

The transfer of maternal androgens could also be potentially affected by the predation risk perceived during egg-formation, but the impacts of this stressor have not been thoroughly studied. Experiments have shown that females perceiving high predation risk during laying transferred higher levels of testosterone (in great tit *Parus major*, under adult/post fledging predation risk; Coslovsky et al. 2012), higher concentrations of corticosterone (in barn swallow *Hirundo rustica*, under nest predation risk; Saino et al. 2005) and more progesterone but less androstenedione (in Japanese quail *Coturnix japonica*, exposed to human disturbance; Bertin et al. 2008) to eggs. Under high predation risk, fast growth could benefit young by permitting earlier fledging, but for example, high begging rate caused by elevated androgen levels (Eising and Groothuis 2003) could be dangerous in the presence of predators, as it may increase the risk of nest detection. A comparative study suggested that passerines that are more susceptible to high nest predation risk evolved allocation of higher androgen levels to eggs, promoting faster body growth and potentially increasing the probability of nestling survival (Schwabl et al. 2007).

The transfer of androgens to eggs, their environmental plasticity, and their short- and long-term effects for offspring will vary across species and depend on the species' life-history strategy (Gil et al. 2007, Ruuskanen 2015). Investigating the impact of different ecological factors, such as food availability and predation risk, on maternal effects in the same species is thus essential to understand whether, and in which conditions, the transfer of androgens in the eggs may be adaptive in terms of offspring survival. We present the first study combining investigations on the effects of food and predation on maternal transfer of androgens in the same species, and in similar habitat conditions.

We examined, through several independent experiments and correlative studies, whether yolk hormone transfer in the pied flycatcher *Ficedula hypoleuca* is affected by female nutrition and perceived predation risk. In the first experiment, we measured the impact of food supplementation on androgen concentrations in eggs by giving daily food supplements to the females prior to and during egg-laying. We measured hormone levels in full clutches to investigate the effects of laying order on androgen transfer, as it may change throughout the clutch (review by Groothuis et al. 2005). This could be a strategy to produce phenotypically variable offspring, which might be a risk-spreading strategy in variable environments (the offspring diversity hypothesis: Laaksonen 2004). Decreasing androgen concentrations with laying may also facilitate brood reduction under poor food conditions (Schwabl et al. 1997), or increasing concentration may compensate for the competitive disadvantage of the later hatching young in asynchronous broods (Schwabl 1993). It is therefore of particular interest to examine whether food supplementation affects a potential laying order effect on hormone concentrations.

In the second set of studies, we measured how the transfer of androgens in eggs is affected by predation risk. First, under the risk imposed by breeding in forest patches occupied by breeding avian predators; second, under risk imposed by cues of nest predators. Studying animal responses to multiple predators is essential to understand the evolution of antipredator strategies in natural environments towards different predators of varying threats (Lima 1992, Caro 2005). The benefits of maternal transfer of androgens in the eggs may thus vary depending on the risk perceived by the mother during egg-formation.

Maternal androgens are deposited in the middle layers of the yolk through direct transfer from the theca and granulosa cells surrounding the follicle while the yolk is forming; blood circulating androgens from female vessels seem to have little impact on yolk concentrations (Hackl et al. 2003). The three androgens here considered are androstenedione, testosterone and progesterone. Androstenedione can serve as a source of biologically potent androgens for embryos, since it is a precursor of testosterone (Groothuis and Schwabl 2002), and testosterone is the most studied androgen in birds with well-known biological functions. Progesterone is a precursor of androgens and, although its potential influence on embryonic growth and development is still unknown, this hormone is largely found in in the yolk of avian eggs (Möstl et al. 2001, Hackl et al. 2003).

Our aim was to study the effects of both food supplementation and predation risk on the transfer of these three androgens, ultimately to investigate which of these factors has a stronger impact on maternal effects. In the food supplementation experiment we predicted that 1) if increased egg androgen levels are advantageous in poor growth conditions for offspring (Pilz et al. 2004, Groothuis et al. 2005), supplementary fed females should lay eggs with lower hormone levels because females supplemented with food should perceive favorable conditions for their offspring, with low risk of starvation. Lower hormone allocation would reduce the costs of androgens for the offspring. An alternative prediction is that, if yolk androgen transfer is costly for the females, females receiving food supplementation should be able to transfer higher levels of hormones in the eggs. This seems unlikely in our study system, however, since in pied flycatchers females naturally in good condition seem to transfer lower levels of androgens in eggs (Tobler et al. 2007). 2) We further predicted that the within-clutch hormone transfer pattern would be affected by food supplementation: fed females may allocate more androgens to the last egg to counteract hatching asynchrony, thus increasing the survival probability of the later hatched chicks. Alternatively, females could deposit

less hormones to later eggs, thus reducing competition with early hatchlings.

In predator experiments we expected that if androgen transfer in eggs evolved to increase offspring survival, 1) females exposed to higher predation risk could transfer higher androgen levels resulting in faster offspring growth; or females could transfer less hormone reducing potential costs to offspring immunity. Finally, 2) we expected that androgen allocation may be differently affected depending on the type of predation pressure exerted. If directed at nestlings, we expect higher concentrations of egg androgens to enhance growth and decrease time in the nest (perceived to be under risk). If directed at post-fledgling/adult periods we expect lower concentrations of egg androgens to prolong the nestling period. A longer nestling period would result in more developed offspring (better at flying) with higher chances to avoid predators. But a longer nestling period could also be advantageous for the parents, because feeding slow growing chicks would be less demanding and parents could be more vigilant towards the predators present in the area.

Methods

Study area and study species

The supplementary feeding experiment was conducted in Ruissalo, Turku, south-western Finland (60°N, 22°E) in 2004. Two-hundred nest boxes (surface area 12 × 12 cm, height 25 cm, diameter of entrance hole 32 mm) for small passerines were settled prior to the experiment in three forest patches dominated by oaks and pines. The predator studies were conducted in the Kauhava region, western Finland (63°N, 23°E) in similar nest-boxes during 2006, 2007 and 2009. Nest boxes were settled in May of each year, prior the arrival of pied flycatchers to the breeding sites, in groups of 2 to 4 boxes, ≥ 80 m apart, in forest patches that were located at least 500 m apart from each other. The forest patches used for the adult predation studies were either occupied by breeding avian predators or were patches without avian predators. Forest patches with and without breeding avian predators (see below) reflect the habitat preferences of the predators considered, mainly conifer dominated forests. The forest patches used for the nest predation experiment included both conifer-dominated and mixed conifer-birch forests. The slight variation in the habitat composition between the food supplementation experiment and the three predator studies could partly account for variation in the transfer of androgens within different years or studies.

Pied flycatchers are small migratory passerines that arrive in Finland in May; females start nest building one or two days after arrival, nest building last approximately 7–8 d (range 6–11 d), and usually lay 6 to 8 eggs (Lundberg and Alatalo 1992, Morosinotto et al. 2010, 2013). Pied flycatchers breed in natural cavities or nest boxes. Nest predation rates are low in our nest boxes with small entrance hole (diameter 32 mm; Morosinotto et al. 2012) but historically the species may have experienced significant nest losses in natural cavities (Walankiewicz 2002).

In the predator studies we considered two breeding predator species, either the Tengmalm's owl *Aegolius funereus* or the pygmy owl *Glaucidium passerinum*, or we used urine and hairs of least weasel *Mustela nivalis* (hereafter weasel) as cues of nest predator presence. These three predator species are all common in Finnish forests and predate upon adult and juvenile pied flycatchers (pygmy and Tengmalm's owl: Kellomäki 1977, Korpimäki and Hakkarainen 2012) or on eggs and nestlings (weasel: Korpimäki et al. 1991). The predation risk imposed by these three predators varies annually depending on the phase of the three-year population cycle of their main prey, voles of genera *Microtus* and *Myodes* (decreasing, low and increasing population phase: Korpimäki et al. 2004). We conducted the predator experiments in two decreasing (2006 and 2009) and one low vole year (2007), representing high ambient predation risk for passerines, an alternative prey for owls and weasels in years of low vole abundance in the field.

Experimental design

We collected eggs from flycatcher nests that received either supplementary feeding or were exposed to high predation risk prior to egg-laying, and from respective control/low risk nests. Egg formation in pied flycatchers takes approximately five days (Ojanen 1983). In all the studies the birds were exposed to the treatments for approximately 10–14 d, from the initiation of nest building until the end of egg-laying (food supplementation experiment), or until the collection of the 4th laid eggs (weasel experiment). In the high risk sites for the two owl species, Tengmalm's and pygmy owls were breeding in the area and thus were present in the forest patch throughout the breeding phase of the flycatchers (from territory choice to fledgling). Therefore, if there was an effect of food supplementation or perceived predation risk on maternal transfer to eggs, our studies should reveal it.

Food supplementation

Nest boxes were monitored every third day, to detect nest initiation. The first initiated nest was randomly assigned to either food supplementation treatment or control by flipping a coin. Thereafter, every subsequent initiated nest was assigned an opposite treatment. We began the supplementation only after nest-building had started to prevent individuals from choosing nest-sites due to the food-supplementation. This procedure randomized individual quality and environmental conditions among food-supplemented and control nests. Only females build nests, and to target the food supplements at the female only, the food was placed in a feeder inside the nest box (it is however possible that some of the food was eaten by males; Moreno et al. 2006). Food supplementation at treatment nests should therefore result in better condition of the laying female and the perception of a high quality nest site. We provided 5 g of live mealworms *Tenebrio molitor* daily, and approximately all the mealworms were consumed within the day. In the control nests we inserted an empty feeder and visited daily to ensure similar levels of disturbance. The food-supplementation began in average 4 d before egg-laying (mean 4.3 ± 1.71 SD) and continued until clutch completion. Pied flycatchers lay one egg per day, and the daily visits enabled us to determine the laying order of the eggs. Most eggs (71 of the 73 eggs

collected) were individually marked with a non-toxic marker pen on the day of appearance.

We collected the clutches when they were completed (on the day the 6th egg was laid) and no later than one day after incubation had started (determined by female presence or egg temperature). Collected clutches were replaced by plasticine eggs, similar in color and size to natural pied flycatcher eggs, to ensure that all eggs were laid. Eggs laid after clutch collection, in clutches larger than 6 eggs, were collected daily. All females accepted dummy eggs and continued incubating (or laying). The eggs were weighed and then stored at –18°C until hormone analyses.

Overall we collected 21 clutches, 10 control nests and 11 food supplemented nests. When considering only the 4th egg (for comparison with the predator experiment; see Predator studies and Statistical analyses sections) the sample size for the feeding experiment was 9 eggs collected in control and 11 in treatment sites, since for one nest the exact laying of the 4th egg was not known.

Predator studies

We performed three different predator studies. In the first two correlative studies we collected eggs from flycatcher nests in patches occupied by either breeding Tengmalm's or pygmy owls (hereafter high risk sites) and from patches without breeding avian predators, so where the owl species bred in the previous year but not in the study year (hereafter low risk sites, see methods in Morosinotto et al. 2010 and 2013). Both owl species bred in nest-boxes and their nests were checked weekly throughout this study to ensure ongoing hunting activity during flycatcher settlement and egg-laying.

In the third predator study, we collected eggs from flycatcher nests that were either exposed to predator cues or to control cues. The entrance hole of nest-boxes assigned to predator treatment was sprayed every second day with a mixture of water and urine of weasels, obtained by diluting dirty weasel bedding for 24–48 h. In addition, hair from a least weasel were glued on the entrance of the nest-box during the first or second spraying. Control boxes were sprayed with water and a drop of glue was added on the entrance (see Methods in Morosinotto et al. 2013). The spraying started in all the nest-boxes when nest building was initiated and lasted until laying of the first egg. The disturbance at the nest, due to human presence, was the same in control and treatment nests. When the nest building was completed but before egg laying, or latest on the day of laying of the first egg, a mounted stoat *Mustela erminea* (treatment nests) or a mounted chaffinch *Fringilla coelebs* (control nests) was presented for five minutes on top of the nest box.

Pied flycatcher females have been found to modify their breeding time and reproductive investment both when breeding in proximity to avian predators (delayed laying date and smaller clutches and/or offspring size: close to sparrowhawk *Accipiter gentilis* nests: Thomson et al. 2006; and close to pygmy owl nests: Morosinotto et al. 2010) and when exposed to weasel odor cues (earlier laying date and slightly larger clutches, Mönkkönen et al. 2009). In addition, breeding pied flycatchers exposed to the same predator treatments as used in this study transferred higher levels of immunoglobulins in their eggs (Morosinotto et al. 2013).

Nests were checked in all forest patches every three days until egg-laying was detected. The first three eggs were marked with a non-toxic pen marker and we collected the 4th unmarked egg and replaced it with a plasticine egg (similar in size and color to natural flycatcher eggs). We collected the 4th egg due to its midway position in the laying of the clutch (clutches generally include 6–8 eggs). In addition, by comparing the hormone concentrations in the 4th egg and the average levels of full clutches from the feeding experiment, we found that the hormone levels in the 4th egg are highly correlated to the average of the clutch (Pearson correlation $r = 0.90$, $p < 0.0001$ for both testosterone and androstenedione; between clutch repeatability of yolk androgen levels was 0.97 for both testosterone and androstenedione; Lessells and Boag 1987).

In the pygmy owl study we collected 52 eggs from nests in high risk sites (1 egg per nest), distributed in a total of 25 pygmy owl nesting sites (15 eggs collected in 2006, 16 eggs in 2007 and 21 eggs in 2009), and 89 eggs from nests in low risk sites, distributed in 29 forest patches (44 eggs collected in 2006, 31 eggs in 2007 and 14 eggs in 2009). One pygmy owl nest failed in 2006 during the pied flycatcher settlement phase. We excluded that single flycatcher egg from the analyses because presence and hunting activity of pygmy owls in the patch could not be confirmed after its nest failure. In the Tengmalm's owl study (year 2006) we collected 41 eggs from nests in 14 Tengmalm's owl nesting sites and 35 eggs from nests in 12 low risk forest patches. In the weasel experiment (year 2009) we collected 14 eggs from treatment nests and 21 eggs from control nests, from 14 and 13 forest patches respectively. All the eggs collected were laid by different females, except for one female that bred in a Tengmalm's owl low risk site in 2006 and in a high risk pygmy owl site in 2007.

Individual quality may have contrasting influences in the different predator studies. With the owl predators, pied flycatcher individuals chose their territories and initiated breeding after the owls had settled in the patch. In contrast, the weasel predator treatment started after individual territory choice and nest building had been initiated. This difference in the flycatcher breeding territory choice being pre- or post-predator settlement or appearance of predator cues should result in individual quality/condition being lower in owl high vs. low risk sites, since high quality individuals may avoid breeding near owls (Morosinotto et al. 2010). In the weasel experiment, individual quality should instead be random between treatment and control.

Female body condition

In the feeding and owl studies we measured female body condition, to control for the impact of individual quality on reproductive investment. All the females were caught at the nest either during the laying period (feeding experiment) or during incubation (predator studies). The birds were ringed, weighed (to the nearest 0.1 g), and their wing length was measured. Blood samples were taken from the brachial vein and kept refrigerated 6 to 8 h before measuring hematocrit. Before the measurements, the samples were centrifuged for

10 min at 10 000 rpm and the hematocrit was calculated as the ratio between red blood cells and plasma.

We have body mass, wing size and hematocrit data for 21 females in the feeding experiment (10 controls and 11 treatments). In the predator studies females were caught in 2006 and 2007. We have body measures for 49 females in the Tengmalm's owl study (28 females breeding in low risk and 21 in high risk sites) and for 93 females in the pygmy owl study (65 breeding in low risk and 28 in high risk sites). We have hematocrit measures for 44 females in the Tengmalm's owl (26 low risk and 18 high risk) and for 90 females in the pygmy owl studies (63 low risk and 27 high risk). The sample size for hematocrit is smaller due to blood sampling difficulties.

Egg and hormone analyses

All the eggs were weighed in the field and then frozen at –18°C until analyzed. The yolk and albumen of each egg was separated in the laboratory. Yolk was weighed (~ 0.1 mg) and homogenized and then all the samples were frozen again until hormonal assays were performed. For the eggs collected in 2004 and 2006, the entire yolk was analyzed for hormone content whereas only 3/4 of the yolk of eggs collected in 2007 and 2009 was used for these analyses. The remaining yolk was used for carotenoid and immunoglobulin analyses for a different study (Morosinotto et al. 2013). Yolk and albumen samples were frozen at –18°C and hormone analyses were conducted respectively in 2005, 2008 and 2011 in the laboratory at the Univ. of Veterinary Medicine in Vienna, Austria.

We analyzed the concentrations of androstenedione (hereafter A4) and testosterone (hereafter T) from all eggs, and the concentration of progesterone from the eggs collected in 2009 from pygmy owl and weasel experiments.

The protocol for analyses of yolk T and A4 was as in Ruuskanen et al. 2011 (using a modified protocol from Hackl et al. 2003, Bertin et al. 2008), whereas the analyses of yolk progesterone followed the protocol described in Bertin et al. (2008). All the protocols were slightly modified in the amount of water/methanol added depending on the amount of yolk used in the analyses. Briefly, we thawed the yolk and suspended each sample in distilled water and methanol, and vortexed twice for 30 s. After storing the samples overnight at 4°C they were vortexed again. The suspension was then diluted with 1:5 assay buffer, vortexed for 30 min and stored overnight at –20°C to precipitate the apolar lipids. The samples were then centrifuged (–15°C, 2500 g, 10 min) and part of the supernatant was used for the enzyme immunoassays (% cross-reactions of the antibody at 50% binding for testosterone: 4-Androsten-17β-ol-3-one (testosterone) 100%, 5α-Androstan-17β-ol-3-one (5α-DHT) 23.7%, 5-Androstan-17β-ol-3-one (5-DHT) 12.3%, 4-Androsten-3β, 17β-diol 7.6% and all the other were below < 5; see Hirschenhauser et al. 1999 for further explanations on the antibodies used and validation). The values presented in the paper are all concentrations (pg mg^{-1}). The inter-assay variation for the analyses of the samples from 2004, 2006 and 2007 was 9.9% and 5.5% for T and 12.9% and 9.3% for A4, while intra-assay variation was 7.9% and 10.1% respectively (Ruuskanen et al. 2011). The inter-assay variation for 2009 samples was 19.5% for T and 16.4 for A4 whereas the intra-assay variation was 8.5% and 4.2% respectively. The intra-assay variation for the progesterone (2009) was 9.2% whereas we have no inter-assay variation value because the analyses were run at once.

Statistical analyses

We tested the effects of feeding treatment and predator presence on yolk concentrations of T, A4 and, when available, progesterone using a generalized linear mixed model (GLMM, Proc Glimmix SAS 9.3). In all the studies both T and A4 were log-transformed to achieve normal distribution, whereas progesterone was normally distributed.

The initial model for the feeding experiment included treatment, laying order, i.e. continuous variable with eggs identified as first, middle (from the 2nd laid to the penultimate) and last, and the interaction laying order × treatment. The quadratic effect $(\text{laying order})^2$ was also tested to detect possible parabolic interactions but, if not significant, only statistics for the linear interaction is presented. In addition, yolk mass and laying date were initially included, since they are known to affect androgen deposition (Michl et al. 2005). Nest ID was included as a random effect; laying order was included as the slope of the random effect to take into account not only variation between individuals in the mean, but also in the slopes of the potential laying-order effect (Schielzeth and Forstmeier 2009).

The analyses for the predator studies were run separate for each predator, since the data were collected with different methods. In the pygmy owl study the initial model for T and A4 concentration included year (2006, 2007, 2009), predator presence (high vs low risk sites) and the interaction year × predator presence; the model for progesterone concentration included only predator presence (since data were collected only in 2009). The initial models for hormones concentration in the Tengmalm's owl and weasel studies included only predator presence/treatment, since data were collected only in one year (A4 and T concentration in 2006 for Tengmalm's owl study and A4, T and progesterone in 2009 for weasel experiment). Yolk mass and laying date were also included in all initial models. Forest patch was used as random factor in all predator studies to avoid spatial pseudo-replication.

Kenward–Rogers and default degree of freedom methods were used in models with and without random effect respectively and NOBOUND function was used to allow negative variance of the random effect (SAS Inst. 2008). The variable 'predator presence/treatment' was kept in all final models since it represented our main study question, while all the other factors were deleted from the final model if not statistically significant ($p \leq 0.05$).

Female body mass and hematocrit levels were analyzed in the supplementary feeding experiment (using a linear model; Proc GLM, SAS 9.3) and in the pygmy owl and Tengmalm's owl studies (using a linear mixed model with forest patch as random factor; Proc MIXED, SAS 9.3). Both female body mass and hematocrit were normally distributed. All initial models for the body mass included 'treatment' (food supplementation or predator presence/treatment), wing length and laying date, whereas those of hematocrit levels included 'treatment' (food supplementation or predator presence/

treatment), laying date and female mass. For the pygmy owl study, the year (2006 and 2007) and the interaction year × predator presence were also included.

Comparison between studies

Hormone concentrations in the feeding experiment were also compared considering only the 4th laid eggs, to replicate the data available for the predator studies. The model used replicated those of the predator studies but without adding forest patch as random effect, since there were no spatial pseudoreplication issues in the feeding experiment. The effect size of the variation in hormone concentration in the 4th laid egg in all the studies was calculated as *d* Hedges's coefficient (Table 2). This coefficient is one of the most used methods to measure the effects size between the standardized differences among two groups' means ('control' vs 'treatment'). It is not affected by unequal sampling variance in the paired groups and includes a correction for small sample sizes (Koricheva et al. 2013).

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.dc30t> (Morosinotto et al. 2016).

Results

Feeding experiment

Eggs produced after receiving supplementary feeding showed lower A4 concentrations compared to controls (Table 1). A4 allocation did not vary according to laying order and the interaction between laying order and treatment was not significant (Table 1, Fig. 1). T concentration did not differ significantly between food supplementation and control groups, although the tendency was to differ in the same direction as A4 (lower in food supplementation group; Table 1). T concentration increased with laying order, but there was no interaction between treatment and laying order (Table 1; Fig. 1). If considering only the 4th egg laid (for a comparison with the predation risk studies) we found lower concentrations of both A4 ($F = 20.37$, $DF = 1$, $p = 0.0003$; Fig. 2a) and T ($F = 8.51$, $DF = 1$, $p = 0.009$; Fig. 2b) in supplementary-fed nests than in control nests (see Table 2 for effect sizes).

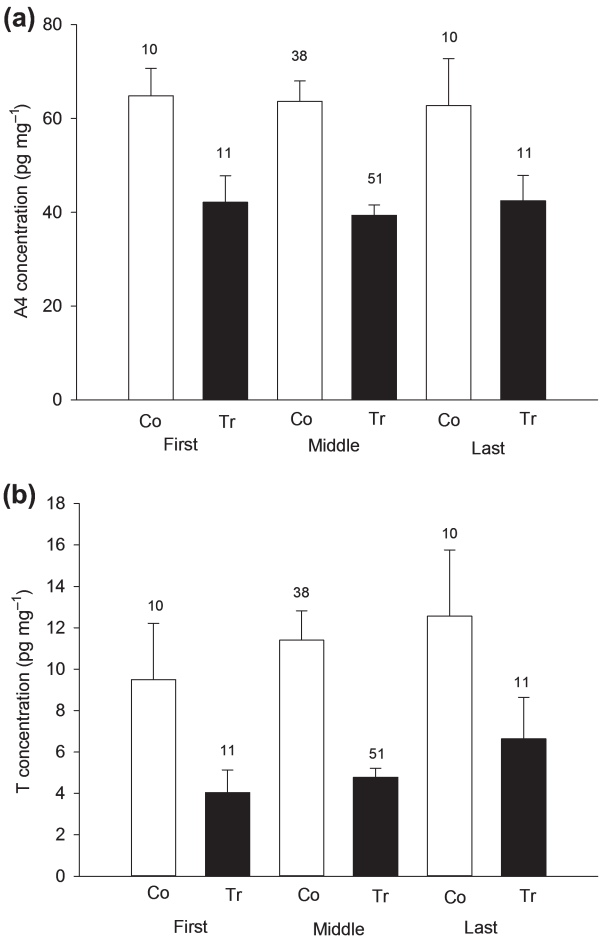

Figure 1. Androstenedione (A4; (a)) and testosterone (T; (b)) concentrations (mean ± SE) in the supplementary feeding experiment, according to laying order (first, middle or last eggs laid). White bars correspond to control nests (Co) whereas black bars correspond to nests that received supplementary feeding (treatment, Tr). Numbers on top of the bars correspond to the number of eggs collected per laying order class (first, middle, last) per treatment.

Table 1. Results of the GLMM for androstenedione (A4) and testosterone (T) concentrations for the supplementary feeding experiment. The nest ID was included as a random effect, while laying order (first, middle, last egg) was considered as the slope of the random effect. Factors in parentheses were not included in the final models due to non-significance, but reflect statistics of these individual terms entered into the final models. Slope ± standard error (SE) is presented for all continuous variables. Only denominator DF is presented since numerator DF is always 1.

Hormone	Variables	Slope ± SE	DF	F	p
A4	Treatment		18.8	8.20	**0.01**
	(Laying order)	–0.009 ± 0.02	20.0	0.17	0.68
	(Yolk mass)	0.08 ± 0.34	123.1	0.06	0.81
	(Laying date)	–0.003 ± 0.005	17.8	0.38	0.54
	(Treatment × laying order)		19.0	0.49	0.49
T	Treatment		19.0	3.40	0.08
	Laying order	0.09 ± 0.029	20.0	10.09	**0.005**
	(Laying date)	0.012 ± 0.012	18.0	1.04	0.32
	(Yolk mass)	0.88 ± 0.52	112.1	2.85	0.09
	(Treatment × laying order)		19.0	0.62	0.44

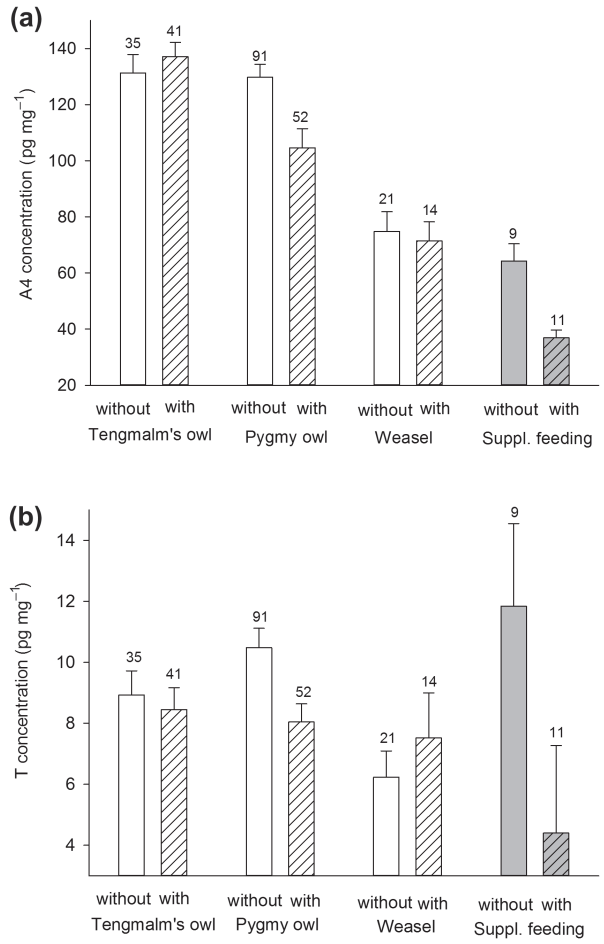

Figure 2. Mean ± SE of androstenedione (A4; (a)) and testosterone (T; (b)) in the 4th egg collected from the three predator studies (Tengmalm's owl, pygmy owl and weasel; white bars) and in the supplementary feeding experiment (grey bars; see Table 2 for the effect size of each treatment). Plain bars represent eggs collected in sites 'without' breeding predators/cues or without supplementary feeding (control/low risk sites) while hatched bars represent sites 'with' breeding predators or cues or receiving food supplementation (treatment/high risk sites). Numbers on top the bars correspond to the number of nests per treatment.

Predator studies

Close proximity to breeding predators or the presence of nest predator cues at nest sites did not affect maternal transfer of T or progesterone in the eggs (Table 2 and 3, Fig. 2). In the pygmy owl study there was however a tendency for allocating less A4 in high than low risk patches (Table 2 and 3; Fig. 2).

Table 2. Hedges *d* effect size for the mean hormone concentration on the 4th egg between 'control' and 'treatment' respectively in each study, both predators and feeding studies. Values are classified as small if $d \leq 0.2$, medium if *d* is around 0.5 and large if $d \geq 0.8$; the larger is the value the stronger is the effect.

	Testosterone (*d*)	A4 (*d*)
Supplementary feeding	1.23	1.86
Pygmy owl study	0.40	0.51
Tengmalm's owl study	0.10	0.16
Weasel treatment	0.27	0.11

In the pygmy owl study, yolk androgen concentration varied between years, both for A4 (mean ± SE: 2006: 136.94 ± 5.28 pg mg^{-1}, 2007: 136.97 ± 5.23 pg mg^{-1}, 2009: 70.09 ± 5.75 pg mg^{-1}; Table 3) and T (mean ± SE: 2006: 10.19 ± 0.69 pg mg^{-1}, 2007: 11.89 ± 0.82 pg mg^{-1}, 2009: 5.44 ± 0.59 pg mg^{-1}; Table 3).

Progesterone concentration was not influenced by perceived predation risk, neither in the pygmy owl study (mean ± SE: low risk sites 632.54 ± 63.6 pg mg^{-1}, high risk sites 567.39 ± 45.02 pg mg^{-1}; Table 3), nor the weasel experiment (treatment: mean ± SE: control 613.28 ± 51.81 pg mg^{-1}, treatment 646.31 ± 65.18 pg mg^{-1}; Table 3).

A comparison of hormone levels in the 4th collected egg from feeding and predator studies showed that the relative difference in 'treatment' effect was higher in the feeding experiments than in the predator studies (Table 2, Fig. 2).

Female condition

Supplementary feeding positively impacted female mass during egg-laying (mean ± SE: control 15.56 ± 0.3 g, treatment 17.01 ± 0.3 g; Table 4). Supplementary feeding had no obvious effect on female hematocrit (Table 4). In the Tengmalm's owl study, females were heavier in high risk sites compared to low risk (mean ± SE: low risk: 14.91 ± 0.14, high risk: 15.45 ± 0.78; Table 3). Female hematocrit was not affected by owl presence (Table 4).

In the pygmy owl study, female mass was unaffected by predator presence (Table 4). Hematocrit tended to be higher when breeding in active pygmy owls territories compared to low risk sites (mean ± SE: low risk 0.47 ± 0.004, high risk sites 0.48 ± 0.007; Table 4).

Discussion

Yolk androgen levels in eggs were affected by food supplementation whereas they were not affected by perceived adult or nest predation risk. A lower transfer of hormones in eggs after receiving food supplementation was consistent for both androgens considered. Testosterone transfer was also affected by laying order, increasing in consecutive eggs laid and being higher in the last laid egg.

Food supplementation

We found a lower androstenedione (A4) concentration, and a tendency of lower testosterone (T) levels, in eggs laid by food-supplemented pied flycatcher females compared to controls. We suggest that females in good body condition transfer less A4 to eggs to reduce the costs of androgens for offspring (such as increased metabolic rates and immunesuppression: Andersson et al. 2004, Groothuis et al. 2005, Sandell et al. 2009, Ruuskanen et al. 2013). Females in good condition may be able to provide enough food for optimal growth of their offspring, thus reducing the potential benefits of androgens (such as increased begging and competitive ability: Schwabl 1993, Eising and Groothuis 2003, Ruuskanen and Laaksonen 2013). Pied flycatcher females in good body condition, albeit not-experimentally manipulated, also

Table 3. Results of GLMMs for the yolk androgen concentrations in the 4th laid egg according to predator presence. Hormones here considered are androstenedione (A4), testosterone (T) and progesterone (P). Numerator DF is always equal to 1, except for the variable Year (and its interactions) when it is always equal to 2; therefore in the table only the denominator DF is presented. Forest patch was included as a random effect. Progesterone was measured only in 2009 in the pygmy owl and weasel studies. Factors in parentheses were not included in the final models due to non-significance, but reflect statistics of these individual terms entered into the final models. Slope ± SE is presented for all continuous variables. The interaction was re-entered in the final model only if both the terms included were part of the final model. Statistically significant values are presented in bold.

Predator	Hormone	Variables	Slope ± SE	DF	F	P
Pygmy owl	A4	Yolk mass	0.001 ± 0.0004	138.00	7.50	**0.007**
		Year		76.20	49.43	**<0.0001**
		Predator presence		54.72	3.55	0.06
		(Laying date)	–0.003 ± 0.004	134.30	0.52	0.47
		(Year × predator presence)		61.96	0.82	0.45
	T	Laying date	–0.01 ± 0.005	71.40	5.75	**0.02**
		Year		62.40	30.39	**<0.0001**
		Predator presence		44.30	0.07	0.79
		(Yolk mass)	0.00006 ± 0.0005	129.30	0.01	0.91
		(Year × predator presence)		75.14	0.75	0.48
	P	Laying date	–36.84 ± 8.65	30.58	18.14	**0.0002**
		Yolk mass	3.8 ± 0.62	27.96	37.92	**<0.0001**
		Predator presence		16.33	0.30	0.59
Tengmalm's owl	A4	Laying date	–0.01 ± 0.004	49.10	7.37	**0.009**
		Predator presence		11.39	1.55	0.24
		(Yolk mass)	0.00005 ± 0.0005	40.59	0.01	0.93
	T	Predator presence		23.94	0.21	0.65
		(Laying date)	0.002 ± 0.01	72.98	0.02	0.88
		(Yolk mass)	0.002 ± 0.001	71.86	2.7	0.10
Weasel	A4	Yolk mass	0.002 ± 0.001	31.81	3.77	0.06
		Treatment		27.23	0.02	0.90
		(Laying date)	–0.02 ± 0.01	31.00	2.39	0.13
	T	Laying date	–0.07 ± 0.02	30.40	14.06	**0.0007**
		Yolk mass	0.004 ± 0.002	30.46	5.17	**0.03**
		Treatment		20.48	0.17	0.68
	P	Laying date	–41.35 ± 11.75	24.29	12.39	**0.002**
		Yolk mass	3.40 ± 1.02	29.09	11.01	**0.002**
		Treatment		25.11	0.04	0.85

Table 4. Results of GLM for the supplementary feeding experiment and GLMM for the predator studies on female condition. Only denominator DF is presented since numerator DF is always 1. In the predator studies forest patch was always included as random effect. Factors in parentheses were not included in the final models due to non-significance, but reflect statistics of these individual terms entered into the final models. Statistically significant values are presented in bold.

Variables		Slope ± SE	DF	F	P
Supplementary feeding experiment					
Female mass	Treatment		1.0	11.40	**0.003**
	(Female wing)	0.13 ± 0.14	1.0	0.89	0.36
	(Laying date)	–0.12 ± 0.03	1.0	0.14	0.72
Hematocrit	Treatment		1.0	0.05	0.83
	(Female mass)	–0.016 ± 0.008	1.0	3.68	0.07
	(Laying date)	–0.0006 ± 0.001	1.0	0.18	0.67
Tengmalm's owl study					
Female mass	Predator presence		47.0	5.94	**0.019**
	(Female wing)	0.12 ± 0.07	46.0	3.27	0.08
	(Laying date)	0.002 ± 0.03	46.0	0.00	0.95
Hematocrit	Predator presence		12.5	1.32	0.27
	(Female mass)	–0.013 ± 0.009	36.8	2.21	0.15
	(Laying date)	0.0008 ± 0.002	39.7	0.14	0.70
Pygmy owl study					
Female mass	Predator presence		90.1	2.32	0.13
	Female wing	0.20 ± 0.06	90.1	10.76	**0.002**
	(Laying date)	–0.016 ± 0.03	84.5	0.05	0.83
	(Year)		89.0	0.57	0.45
Hematocrit	Predator presence		88.0	3.54	0.06
	(Female mass)	–0.0017 ± 0.004	87.0	0.23	0.63
	(Laying date)	0.01 ± 0.001	87.0	0.91	0.34
	(Year)		87.0	3.17	0.08

transferred less androgens in their eggs than females in poor condition (Tobler et al. 2007). Previous studies have also observed low androgen levels in eggs laid by supplementary fed females. Food supplementation in black-legged kittiwakes *Rissa tridactyla* had no obvious effects on A4 transfer in the first clutch (Gasparini et al. 2007, Benowitz-Fredericks et al. 2013) but fed females decreased the amount of androgens and antibodies allocated in second clutches (Gasparini et al. 2007). Androgen deposition is however not always consistent between studies. For example, in great tit food supplementation did not affect androgen transfer to the eggs (Ruuskanen et al. 2016) whereas in lesser black-backed gulls *Larus fuscus*, supplementary fed females allocated lower levels of androgens in eggs in one study (Verboven et al. 2003) but showed no variation in others (Verboven et al. 2010).

Our results and previous studies thus seem to suggest that females in good condition transfer less androgens to the eggs and this is expected to be beneficial. A recent study however highlighted that the balance between the costs and benefits of androgen transfer to eggs largely depends on the concentration of hormones in the yolk, and this relationship may not be linear. Nestling growth rate has often been found to increase as a result of high levels of androgens in the yolk but at really high concentrations the benefits disappear and nestling body size decreases (Muriel et al. 2015).

T concentration in egg yolk increased with laying order, being highest in the last laid egg. Our results are consistent with earlier work on androgen levels in eggs that showed laying order effects in many bird species (Schwabl 1993, Eising et al. 2001, Pilz et al. 2003, Benowitz-Fredericks et al. 2013). Within clutch variation in androgen levels across laying order can either reduce (counteract) or increase (promote) the size differences among offspring and hatching asynchrony, since high levels of T in the eggs promote faster growth (Schwabl 1993, Schwabl et al. 1997, Eising et al. 2001). Pied flycatchers have semi-asynchronous hatching, with hatching being spread over 1.25 d on average (Slagsvold 1986). Synchronous broods fledge more young than asynchronous broods (Hillström and Olsson 1994), therefore higher T levels in later laid eggs might be adaptive as this may counter asynchrony.

The laying order effect in T levels was not affected by supplementary feeding. This suggests the benefits of counteracting hatching asynchrony are higher than the fitness costs of androgen allocation, making it advantageous for females in both good and bad condition to show similar testosterone transfer patterns over the lay sequence. Variation in T transfer within the clutch may also contribute to producing offspring with diverse phenotypes. Resource availability in the environment varies in time and space and in this scenario different phenotypes may vary in their survival and reproductive success. It may thus be advantageous for the parents to produce offspring with various phenotypes per brood to increase the probability of survival of at least some young in unpredictable environments (Laaksonen 2004, Olofsson et al. 2009). Indeed, previous food supplementary studies show contrasting results with respect to laying order effects, depending for example on the quality of food provided (Sandell et al. 2007, Vergauwen et al. 2012) or on the natural food availability (Benowitz-Fredericks et al. 2013).

Finally, it has recently become evident that A4 and T are not equivalent in their concentration in the yolk, on the factors affecting their transfer to the eggs, or on their effects on offspring (Tschirren et al. 2014). In our results A4 was affected by food while T was unaffected but changed significantly within laying order. In collared flycatchers, females laying eggs with higher yolk A4 concentration were shown to recruit more young, suggesting natural selection favors higher concentrations of A4 and lower concentration of T (Tschirren et al. 2014). However, these hormones are generally correlated within the eggs, which suggests that females are limited in reaching the optimal balance of androgens in the eggs. Since all the hormone manipulation experiments in flycatchers so far considered A4 and T combined, it is difficult to evaluate the effects of each hormone individually, especially since embryos may be able to actively synthetize T from the allocated A4 (Ruuskanen 2015).

Predation risk

Increased predation risk did not explain variation in egg hormone levels. This lack of effect for all the three hormones considered (A4, T and progesterone) was consistent for both nest (weasel) and adult predators (either pygmy owl or Tengmalm's owl).

The lack of effect by nest predation risk on hormone transfer was unexpected. Higher androgen levels promote growth (Eising et al. 2001) that may allow offspring to fledge faster, which should be beneficial when the predators in the area represent a risk only for the nestlings and not for fledglings or adults. A comparative study has indeed shown that passerine species more vulnerable to nest predation risk allocate more T in their eggs than species that breed in safer nests (Schwabl et al. 2007). Birds are known to respond to nest predator feces and urine when making habitat and nest site selection decisions and to reduce their reproductive investment accordingly (Amo et al. 2008, Mönkkönen et al. 2009, Forsman et al. 2013), we thus expected females to modify their egg composition according to the perceived risk (Ibáñez-Álamo et al. 2015), but this was not observed.

Nest predation may be difficult to predict for the laying females (Ibáñez-Álamo et al. 2015) and thus it is possible that an adaptive transfer of hormones has not evolved. If this is the case, then the transfer of hormones to the eggs could be consequence of a passive transfer from the circulating levels in the females to the eggs. Pied flycatcher males do not seem to alter their circulating T levels after exposure of a weasel to the nest (but they do so when exposed to woodpeckers; Silverin 1998). If females also do not alter the levels of androgens in their blood when exposed to cue of nest predators, like weasel, then this could explain the lack of variation in yolk hormone levels, observed in this study. However, if the cues available to the laying female are predictive of the territory quality during the nestling phase, then hormone transfer to eggs should be adaptive in terms of increased survival for the offspring and/or higher maternal fitness (Marshall and Uller 2007, Ruuskanen 2015).

A lack of response due to methodological flaws in the weasel experiment is unlikely, since the same treatment elicited higher immunoglobulin transfer in the eggs of pied flycatchers in a previous study (Morosinotto et al. 2013)

and a similar methodology affected also flycatcher reproductive investment (Mönkkönen et al. 2009). The mechanisms behind the transfer of both immunoglobulins and hormones are however still unclear, and thus females could use different environmental cues when allocating these factors to the eggs.

Eggs laid in proximity to pygmy owl nests tended to have less A4 (Fig. 2a). We did not find any difference depending on the presence of Tengmalm's owl in the area. This difference with pygmy owl presence is however not entirely surprising since pied flycatcher are known to avoid areas with breeding pygmy owls and, when breeding there, to delay the start of egg-laying and have low reproductive investment, whereas they do not seem to respond to Tengmalm's owl presence (Morosinotto et al. 2010). Also female condition seem to differ between flycatchers breeding in proximity of these two predators (see below).

Lower levels of A4 in proximity to pygmy owl nests is in contrast with results from Coslovsky and colleagues (2012), showing that great tit females exposed to playback calls of sparrowhawks *Accipiter nisus* transferred higher concentrations of T to eggs. Our finding of lower androgen concentrations suggests that when the risk is directed at juveniles post-fledging, the benefits to offspring of androgen transfer are reduced. Fast growth and short nestling period may be disadvantageous for nestlings breeding in environments with high post-fledgling predation risk. The contradiction between our results and that of Coslovsky and colleagues (2012), where the playback of the predator used was also an adult and post-fledging predator like pygmy owls, may be due to differences in the life-history of the two study species or due to the experimental methodology. Indeed the responses to a live wild predator breeding in the area probably induced different responses than indirect cues of predators, like the playback of territorial calls used by Coslovsky et al. (2012).

The predator studies showed high annual variation in egg androgen concentrations, with the concentrations in 2009 being low compared to 2006–2007. Yearly variation was independent of the predator presence and the interaction between year and predator presence was never significant. We therefore conclude that the variation between years does not affect the interpretation of the results. Possible explanations for the low concentrations of hormones in 2009 could either be methodological (eggs from different years were analyzed after different time periods) or due to some unknown environmental variable that affected the hormone transfer. For example, although both the food supplementation and the predator studies were conducted in Finland the habitat composition between different forest patches was different, being either more conifer or more birch dominated, which could have affected the different androgens concentrations observed among different years and different experiments and correlative studies (Fig. 2).

Abundant evidence indicates that passerines breeding under high predation risk show behavioral adaptations in parental care to reduce the probability that the nests will be detected by predators (Caro 2005, Lima 2009, Zanette et al. 2011). But, how predation risk impacts the growing embryos via the hormones, proteins and immune factors transferred to eggs is still poorly known, and our results suggest that food availability will have a stronger influence on maternal allocation in eggs than predation risk.

Female body condition

We found that food supplemented females were heavier than control females. Higher female mass was expected and shows that females responded to the supplementary feeding. Hematocrit levels were not influenced by supplemental feeding, an unexpected result because increasing hematocrit levels were suggested to be caused by improved female body condition (Hoi-Leitner et al. 2001, Clinchy et al. 2004). However, hematocrit levels may be affected by several factors, including diet (Fair et al. 2007) and the food source used in our experiment could have different effects on hematocrit compared to previous studies.

Predator treatments or presence affected female body condition. Females breeding near Tengmalm's owls were heavier than those in low risk sites, but there was no difference in females in pygmy owl high risk sites compared to low risk ones. This could have been caused by habitat preference of pied flycatchers in good condition for Tengmalm's owl sites, which are less threat to passerines than pygmy owls. This could also partly explain the lack of response in egg composition. Habitat preference towards sites with breeding Tengmalm's owls could also be beneficial if they provide protection from other smaller predators, including pygmy owls and least weasels (protective nesting association: Quinn and Ueta 2008).

Female hematocrit levels in pygmy owl high risk sites, but not in Tengmalm's owl sites, were slightly higher compared to low risk sites. This was surprising as pygmy owls are efficient predators of small passerine birds (Kellomäki 1977) and high hematocrit is often linked to good body condition (Hoi-Leitner et al. 2001). In the presence of predation risk however, both low (Boonstra et al. 1998, Sheriff et al. 2011) and high hematocrit (Clinchy et al. 2004) have been linked to chronic stress. Clinchy et al. (2004) found higher hematocrit in song sparrows *Melospiza melodia* breeding in high risk sites, and especially when not receiving food supplementation. Higher hematocrit in risky areas is in line with the tendency we observed. Females breeding in proximity to pygmy owls may be more stressed than females breeding in low risk sites, even if this does not necessarily transfer to changes in egg composition. However, although hematocrit can reflect changes in the condition of the birds, we need to take into account that hematocrit may not accurately represent the physiological condition of the bird unless there is extreme variation from normal condition (Fair et al. 2007).

Conclusions

Our results show lower yolk androgen transfer to eggs in food supplemented individuals and increasing testosterone concentration across laying order but independently of food supplementation. In pied flycatchers androgens allocation may have evolved to counteract hatching asynchrony, independently of female body condition, and could also be used as a strategy to increase offspring phenotype variability. We found no clear allocation differences in response to perceived predation risk, suggesting that food supplementation has a

stronger impact on maternal androgen transfer than predation risk. This could be in line with the hypothesis that maternal allocation in the eggs is adaptive when the quality of the breeding environment during egg-laying is a good predictor of habitat quality during the nestling phase (Marshall and Uller 2007). In this context, food availability is more predictable than predation risk because the spatial and temporal variation in predation risk, according to movements of predators in the landscape, makes the quality of breeding sites rather uncertain (review by Lima 2009, Ibáñez-Álamo et al. 2015). Among the different predators the predation risk imposed by birds of prey in the surrounding of their nest should be the most predictable, since their presence in the territory is persistent throughout the breeding season of flycatchers. This could partially explain why the only evidences of variation in androgen transfer to the eggs, this study and Coslovsky et al. (2012), were found after exposure to acoustic cues or in proximity to nests of birds of prey. It remains unclear whether maternal transfer of androgens to eggs is adaptive, in terms of offspring survival or maternal fitness, and which environmental factors are used as cues by females to determine egg composition.

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