Nest predation risk and deposition of yolk steroids in a cavity nesting songbird: an experimental test

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Summary Statement

Experimentally increased perceived nest predation risk did not affect yolk androgen deposition, but decreased yolk progesterone in a cavity nesting songbird.

Abstract

Maternal hormones can shape offspring development and increase survival when predation risk is elevated. In songbirds, yolk androgens influence offspring growth and begging behaviors which can help mitigate offspring predation risk in the nest. Other steroids may also be important for responding to nest predation risk, but non-androgen steroids have been poorly studied. We used a nest predator playback experiment and liquid chromatography with tandem mass spectrometry (LC-MS-MS) to assess whether nest predation risk influences deposition of 10 yolk steroids. We found no clear evidence that yolk androgen deposition changed when perception of nest predation risk was experimentally increased. However, elevated nest predation risk led to decreased yolk progesterone deposition. Overall, our results suggest yolk progesterone may be more important than yolk androgens in responses to offspring predation risk and highlight new avenues for research.

Introduction

Predation of dependant offspring is a major source of mortality across taxa (fish: Eckert 1987; mammals: Promislow and Harvey 1990; invertebrates: Gosselin and Qian 1997; birds: Martin 1992; Martin et al. 2017). When offspring predation risk is variable and the level of risk is detectable by parents, selection may favour the evolution of parental responses to predation risk that improve the chances that offspring survive to independence (Mosseau and Fox 1998; Uller 2008; Martin and Briskie 2009; Yin et al. 2019). For example, maternal hormone allocation can promote the development of phenotypic traits that reduce offspring exposure or susceptibility to predators (Schwabl et al 2007). Such hormone-mediated maternal effects may be especially

important for offspring survival early in life before offspring can directly detect the risk of predation themselves (Badyaev and Uller 2009). However, early exposure to hormones can have significant, long-term effects on offspring phenotypes that may yield fitness costs during later life stages (Gil 2008; Martin and Schwabl 2008; von Englehardt and Groothuis 2011; Mouton and Duckworth 2021). Moreover, variation in other parental care traits (e.g. oviposition site, brooding, provisioning) are often sufficient to alter offspring development in response to predation risk (Martin and Schwabl 2008; Martin and Briskie 2009; DuRant et al. 2013). The potential costs of pleiotropic effects and ability of parental care behaviors to modify risk leaves the importance of maternal hormones for mitigating offspring predation risk unclear.

In songbirds, higher predation rates on offspring in the nest are expected to favor faster offspring development in order to leave the risky nest environment sooner (Martin 1995; Martin and Briskie 2009). Elevated nest predation risk may also favour reduced parental provisioning and less conspicuous offspring solicitation behaviours to reduce the likelihood that predators find nests (Martin et al. 2000; Haff and McGrath 2011; Mouton and Martin 2019). Intriguingly, androgens, such as testosterone deposited into the egg yolk by mothers have been shown to influence both growth and begging behaviors in some species (Schwabl 1996; Pilz et al. 2004; reviewed in Gil 2008; von Englehardt and Groothuis 2011) . Consequently, mothers may be able to plastically alter the quantity of androgens they deposit in eggs to adaptively shape the phenotype of their offspring in a high nest predation risk environment. However, the effects of yolk androgens on growth and begging have been inconsistent among studies (Smiseth et al. 2011). Yolk androgens have also been associated with competitive interactions among siblings within a brood suggesting that depositioin of yolk androgens may respond to environmental cues other than nest predation risk (Muller and Groothuis 2013; Muriel et al. 2019). Moreover,

exposure to maternal androgens also influences traits that may yield costs for offspring including compromised immune function (Navara et al. 2005; Müller et al. 2005) and the impacts on traits, such as higher metabolism or aggression, that influence survival and reproductive success far into adulthood (Tobler et al. 2007; Partecke and Schwabl 2008; Duckworth et al. 2015). Selection on long-term traits like aggression expressed across numerous life stages may create fitness costs that limit androgen-mediated maternal effects in response to nest predation (Mouton and Duckworth 2021). Thus, the role of nest predation risk as a driver of plasticity in androgen-mediated maternal effects is unclear.

Parents also deposit substantial quantities of other steroid hormones in their eggs and non-androgen steroids may also respond to nest predation risk (Groothuis et al. 2019). For example, variation in estrogens or progestogens may also influence the development of offspring traits such as heart rate, neophobia, and fearfulness that are beneficial in a high nest predation risk environment (Bertin et al. 2009; de Haas et al. 2016; Herrington et al. 2016). However, the phenotypic effects of most hormones present in yolk on developing offspring are poorly understood (Groothuis et al. 2019; Merrill et al. 2019). Patterns of covariance or relative concentrations of multiple types of hormones may also be key for shaping offspring phenotypes if the effects of each hormone depend on the levels of other hormones (Roberts et al. 2007; Groothuis et al. 2019). While the effects of non-androgen steroids on offspring phenotype are still unclear, studies examining how environmental selection pressures, such as nest predation risk, influence yolk steroid deposition in a multivariate framework are needed to develop clear hypothesess about their function and evolution.

In this study, we manipulate perceived predation risk of mothers during egg production by broadcasting nest predator vocalizations near nests of the western bluebird (*Sialia mexicana)*. We then use LC-MS-MS, a technique that enables simultaneous and accurate detection and measurement of multiple yolk steroids (Merril et al 2019), to assess how cues about nest predation risk influenced the deposition of all detectable yolk steroids. Our goals were (1) to test the idea that yolk androgen deposition would vary with cues of nest predation risk and (2) to examine whether other, lesser studied yolk steroids might also plastically respond to nest predation risk.

Materials and Methods

Study species and data collection:

We studied Western Bluebirds (*Sialia mexicana*) breeding in the Coconino National Forest of central Arizona, USA (~34◦N) from April-July in 2015 and 2017. The weather during nest building and egg formation in 2015 was cooler and wetter with substantially more snowfall than 2017 (NOAA Climate Data Division). Our field site is 2350m in elevation and consists of mixed deciduous and coniferous forest (Martin et al. 2000). We collected eggs from experimental and control nests within a day of the completion of clutches and kept eggs frozen until we were able to analyze steroid concentrations. We recorded the mass of separated frozen whole yolks just prior to preparing them for steroid assays. All data were collected under the Montana IACUC no. 059-10TMMCWRU.

Nest predator playback experiment:

We used two distinct experimental protocols to increase the perceived risk of nest predation. In 2015, we designated a ~400x100m 'treatment plot' prior to nest building and broadcast red squirrel vocalizations (*Tamiasciurus hudsonicus*; a common nest predator at this site and that will chew their way into cavity nests) using speakers (Eco Extreme by Grace Digital, San Diego, CA, USA) and MP3 players (Sansa Clip by San Disk, Milpitas, CA, USA). Speakers were interspersed every ~50m throughout the plot and were placed in new locations within 20m of this central location every day. Nestboxes were spaced out relatively evenly across the plots and were roughly 20-40m apart. We did not begin experimental treatments until after territories had been established to prevent effects on settling or nest site choices. We monitored nest boxes on these plots to identify active nests and collected eggs once laying was complete. Due to logistical constraints, we slightly modified experimental protocols in 2017. In this year, we targeted experimental treatments at individual nests just after nest building began by placing a speaker 5-7m away from the nest in a new location everyday. The same procedure was used for control plots (in 2015) and control nests (in 2017) except that we broadcast vocalizations of the western tanager (*Piranga ludoviciana*; a common songbird species that is not a threat and does not compete with our study species). We alternated control and treatment plot assignments to spread treatments across the breeding season.

Our goal for both protocols was to create an effect that increased the number of predator vocalizations heard near a nest site while minimizing the likelihood that parents would become accustomed to the experiment. To achieve this effect, we started playback each day at around 0600 and stopped the playback 6-8 hours later, hid speakers in new locations every day, and alternated playback of vocalizations and silence on each speaker. Each MP3 player was loaded

with playlists of 1 minute tracks that played randomly such that the speakers played 4 minutes of silence for every minute of vocalizations. Each protocol increased the number of nest predator vocalizations heard by parents throughout their territories during egg formation to increase the perceived risk of nest predation compared to controls, as verified by similar experiments in other studies (e.g. LaManna and Martin 2016). Notably, speakers were always placed at distances from nests within typical home range sizes of red squirrels (Munroe et al. 2009) such that each broadcast vocalization indicates a predator that might encounter the nest during the breeding season. The distance between speakers and nests was more variable across days in 2015 (~5-30m) than 2017 (~5-7m), but mothers were generally exposed to playback from speakers in multiple parts of their territory. To account for potential differences in magnitude among protocols or other ecological variation among years (e.g. weather), we tested for interactions between treatment and year in all statistical analyses (see below) Overall, in both years combined we collected 16 eggs from 6 control (7 eggs from 3 nests in 2015; 9 eggs from 3 nests in 2017) and 26 eggs from 9 treatment nests (16 eggs from 5 nests in 2015; 10 eggs from 4 nests in 2017).

Yolk assays:

We extracted steroids from 0.5g of homogenized yolk samples by adding 2mL 100% methanol and vortexing for 60s. We removed neutral lipids by storing samples at -20°C overnight, then spinning at 2000rpm at 0°C for 20 mins. We then added another 2mL methanol, repeated the extraction process, and diluted the resulting 4mL of methanol up to 50 mL with MilliQ water (Millipore, Bedford, Massachussetts, USA). We used solid-phase extraction using C18 Sep-pak cartridges (Water, Ltd., Watford, UK) charged with 5mL methanol and rinsed with 5 mL water

prior to passing samples at a rate of ~2mL/min. We rinsed all cartridges with 5mL water and then eluted steroids with 5mL of diethyl ether and dried samples under nitrogen gas.

Steroids were quantified at the Metabolomics Lab of Roy J. Carver BiotechnologyCenter, University of Illinois at Urbana-Champaign.We quantified steroids at 5°C using LC-MS-MS with a 5500 QTRAP LC-MS-MS system (AB Sciex, Foster City, California, USA). We separated analytes using a Penomenex C6 Phenyl colum (2.0mm x 100mm, 3μm) with mobile phase A (0.1% aqueous formic acid) and mobile phase B (0.1% formic acid in acetonitrile). We used a 0.25mL/min flow rate and a linear gradient (0-1 min, 80%A; 10min, 65%A; 15min, 50%A; 20min, 40%A; 25min, 30%A; 30min, 20%A; 30.5-38min, 80%A). We acquired mass spectra under positive electrospray ionization with the ion spray voltage of 5500 V. The source temperature was 500 °C. The curtain gas, ion source gas 1, and ion source gas 2 were 36, 50, and 65 psi, respectively. We used multiple reaction monitoring to measure steroids and D9-progesterone (from m/z 324.1 to m/z 100.1) was used as an internal standard. We used standard curves ranging from 2 to 1000 pg to identify and quantify each steroid. Our method was capable of assessing up to 29 steroids but only 13 were present at detectable levels and we only consider the 10 steroids we were able to quantify across the majority of yolk samples (Table 1). Yolk assays were conducted blind to the experimental design or the treatment group for any sample.

Statistical Analyses:

To test whether nest predation risk influences co-variation among yolk steroid concentrations, we used redundancy analysis (RDA). RDA is a canonical ordination method which combines multiple regression with principal components analysis (PCA; Bourcard et al. 2011). It first computes 'constrained' RDA axes in multivariate response data that are best

explained by a set of explanatory variables. We included treatment, year and their interaction as explanatory variables to assess whether changes in the experimental protocol affected the perceived level of risk. RDA then computes 'unconstrained' PCA axes from the residual variation allowing examination of the covariation among hormones that is unrelated to our explanatory variables. We calculated adjusted R^2 using Ezekiel's formula (Ezekiel 1930) and obtained p-values using permutation tests (Bourcard et al. 2011). Because the interaction between treatment and year was significant (see results), we also ran separate analyses with only treatment as an explanatory variable for each year separately to interpret the interaction.

We also used univariate linear mixed models to assess whether perceived nest predation risk affected variation in the concentration of each individual yolk steroid. We included treatment and year and their interaction as fixed effects, but dropped the interaction term when it was not supported ($p > 0.1$, see electronic supplementary material). We included nest ID as a random effect, but this variance term was indistinguishable from zero in models for 3 hormones (Table S2). This suggests that random variation in hormone concentrations among nests was negligible. We dropped the random effect terms in these models and used mean values for each nest to minimize Type I error.

Yolk steroid concentrations may vary with yolk mass and lay order, so we repeated all analyses with total steroid amount (i.e. concentration x yolk mass). Yolk mass did not differ among experimental treatments or year (linear mixed model: Treatment: $\beta = -0.012$, $t = -0.368$, $p = 0.730$; Year: $\beta = -0.033$, $t = -0.952$, $p = 0.364$) and results from multivariate and univariate analyses based on total steroid amounts were very similar to analyses based on steroid concentrations (Table S2,S3). We did not know lay order for some of our samples which limited our ability to directly test hypotheses about lay order. However, we repeated all analyses

including an effect of lay order. Lay order did not differ among experimental treatments or year (linear model: Treatment: β = 0.371, t = 0.735, p = 0.468; Year: β = 0.095, t = 0.197, p = 0.845). Thus, accounting for lay order did not qualitatively change our results (Table S3). Below, we report results from steroid concentration analyses from the full dataset, but also mention models including lay order for comparison.

We conducted all analyses in program R (R Core team 2019) using the *vegan* package (Oksanen 2019) for multivariate analyses and the *lmerTest* package (Kuznetsova et al. 2017) for univariate analyses. All data were scaled and centered prior to analyses.

Results and discussion

Overall, we detected yolk steroid concentrations that were consistent with previous work in other species using LC-MS-MS (Table 1). Yolk testosterone and androstenedione concentrations were relatively low, especially compared to studies using immunoassay techniques, but were qualitatively similar to low levels detected using LC-MS-MS and in other cavity-nesting species (Table 1; Gil et al. 2007; Schwabl et al. 2007; Merrill et al. 2019).Yolk androgens may modulate the behavior, growth, and development of offspring traits in potentially adaptive ways under elevated nest predation risk (Schwabl 1996; Pilz et al. 2004; Schwabl et al. 2007; Gil 2008; Smiseth et al 2011; von Englehardt and Groothuis 2011). However, while our results suggest that nest predation risk consistently reduced yolk progesterone levels, it did not have a consistent influence on the deposition of yolk androgens.

In our multivariate analysis, the interaction between treatment and year was significant (permutation test: F = 2.956, p = 0.017; Table S1) and the model constraints (RDA 1-3) explained 24.3% of the variation in the data (permutation test: F = 4.074, p = 0.001). Separate

RDAs for 2015 (permutation test: F = 2.460, p = 0.037) and 2017 (permutation test: F = 2.531, p = 0.022) indicated significant treatment effects. Treatment was associated with lower progesterone in both years (Fig. 1). In 2015, treatment eggs had higher levels of etiocholanolone, but in 2017 the opposite pattern was observed. (Fig. 1). All other steroids showed relatively weak association with predation treatment or varied in their relationship to treatment across years (Fig. 1). Our univariate analyses generally matched patterns from the multivariate analysis (Fig. 2). Progesterone was reduced in treatment vs. control nests (β = -0.79, t = -2.232, p = 0.046; Fig. 2; Table S2). The interaction between year and treatment was not significant except in the etiocholanolone model (β = -2.441, t = -3.129, p = 0.009; Fig. 2; Table S2). Etiocholanolone was detected in higher concentrations from treatment nests in 2015, but lower concentrations in 2017 (Fig. 2; Table S3). Treatment was not significantly associated with concentrations of any other hormone (Fig. 2; Table S2).

Our results align with two recent studies showing that yolk androgen deposition did not differ with nest predation risk (Morosinotto et al. 2016; Possenti et al. 2018). However, our intraspecific results contrast with results of comparative studies that often find that average concentrations of yolk steroids, in particular androgens, differ among species that vary in nest predation rates (Schwabl et al. 2007; Merril et al. 2019). Notably, all species examined by intraspecific studies have been cavity-nesting or colonial breeders with low average nest predation rates. The low background risk for these taxa may reduce variation in predation risk and any benefits of plastic responses. Greater plasiticity in yolk steroids concentration in response to nest predation risk may be observed in species that face higher nest predation rates (e.g. open cup nesting songbirds; Martin and Briskie 2009), though experimental tests in such species are lacking.

Yolk testosterone and androstenedione are likely metabolized by the embryo into etiocholanolone (Paitz et al. 2011, Kumar et al. 2019; Campbell et al. 2020), which does not bind to the androgen receptor and occurred at marginally higher concentrations in high predation risk nests in 2015, but lower concentrations in 2017 (Fig. 1, 2). The cause of these inconsistent patterns in etiocholanolone in relation to nest predation risk is unclear and could be due to variation in some unknown environmental condition, experimental protocols, or small sample size. The effects of maternally-derived etiocholanolone on development of offspring phenotypes are poorly studied. One study demonstrated that avian embryos treated with etiocholanolone did not show faster growth rates (Campbell et al. 2020). Other studies suggest etiocholanolone may be involved in the production of red blood cells in both mammals and birds (erthypoesis, Paitz et al. 2011) and/or modulate neural activity in a wide range of vertebrates (von Englehardt et al. 2009; Mouton and Duckworth 2021). Overall, eticholanolone responses to nest predation risk has not been previously considered and, while this study is limited in sample size, the strong interaction in our data highlight areas for future research.

The lack of a consistent response in yolk androgens to nest predation risk may also reflect evolution of unrelated functions. Selection may shape yolk androgen reaction norms along other environmental gradients, such as food availability, parasitism, population density, or predation risk during later life stages (Tschriren et al. 2004; Coslovsky et al. 2012; Muller and Groothuis 2013; Bentz et al. 2016; Morosinotto et al. 2016). For example, in more northern populations of western bluebirds, yolk androgens have been implicated in adaptive responses in aggression and dispersal to the availability of nest sites and population density (Duckworth et al. 2015). Interestingly, we found the first residual axis (PC1) explained the most variation in the yolk steroid data (30.9%) and was heavily driven by the androgens testosterone and androstenedione

(Table S1). Thus, there is plenty of variation in these hormones among eggs and population density could explain variation in yolk androgens in this population as well, though we were unable to test this idea directly.

We found somewhat lower concentrations of progesterone relative to other species (Table 1; Merrill et al. 2019). However, in both years, eggs from high predation nests had lower levels of progesterone than eggs from control nests (Fig. 1,2, Table S2). In contrast, yolk progesterone did not differ with nest predation risk in pied flycatchers (*Ficedula hypoleuca;* Morosinotto et al. 2016) or perceived risk of adult predation in great tits (*Parus major*; Coslovsky et al. 2012). The effects of yolk progesterone on offspring development are not as well studied as the effects of androgens, but may also shape the development of offspring traits that are important in the context of nest predation. For example, increased yolk progesterone is associated with greater embryonic heart rate and improved auditory learning, greater baseline corticosterone levels, and neophobia after hatching (de Haas et al. 2016; Herrington et al. 2016*;* Jenni-Eiermann et al. 2020). Moreover, artificial selection for increased 'fearfulness' led to the evolution of reduced yolk progesterone in Japanese quail (Bertin et al. 2009). Thus, yolk progesterone could feasibly alter offspring metabolism and behavior in ways that might increase fitness in a high nest predation environmnent. Still, the role of maternal progesterone in adaptively shaping offspring phenotype requires more study.

Lower progesterone might also be expected in eggs from high risk nests if parents begin incubating sooner after lay (Paitz and Casto 2012; Kumar et al. 2018a,b). Early onset of incubation is expected with higher nest predation risk and could potentially cause lower progesterone by facilitating earlier embryonic conversion of progesterone to other substances (Clark and Wilson 1981; Martin and Briskie 2009; Paitz and Casto 2012; Kumar et al. 2018a,b).

However, incubation would likely influence the metabolism of other steroids as well (Paitz et al. 2011; Kumar et al. 2019; Campbell et al. 2020, but see Kumar et al. 2018b) and we did not detect a similar pattern in androgens, estrogens, or gluccocorticoids. Moreover, while several hormones were more concentrated in later laid eggs, accounting for earlier exposure to incubation (i.e. using lay order) in our models did not qualitatively affect any conclusions related to nest predation risk (Table S3). Notably, in some taxa, subtle variation in incubation temperature can independently yield major changes in offspring phenotypes that are also sensitive to yolk steroids including growth, behavior, immune function (DuRant et al. 2013). Thus, the interaction of temperature and maternal hormones may be important for interpreting plastic responses to environmental variation including nest predation risk.

We did not detect corticosterone in any samples, which is consistent with very low levels found in a growing number of liquid chromatography studies (e.g. Rettenbacher et al. 2011; Merrill et al. 2019). Corticosterone has been detected in yolk using immunoassay techniques, but issues with cross-reactivity suggest such estimates may largely reflect concentrations of progesterone instead (Rettenbacher et al. 2009). Experimentally increased yolk corticosterone has been linked to improved flight performance in offspring which may be adaptive in high predation environments (Chin et al. 2009). However, yolk corticosterone is thought to reflect maternal plasma concentrations (Groothuis and Schwabl 2008) and, unlike encounters with adult predators, nest predation risk does not seem to influence circulating levels in mothers (Silverin 1998; Fontaine et al. 2011). In any case, our results are consistent with limited transfer of cortiscosterone into egg yolks.

While maternal effects can adaptively shape offspring phenotypes across life stages (Yin et al. 2019), the evolution of specific plastic physiological or behavioral mechanisms may vary among species with unique life history strategies or ecological constraints (Bentz et al. 2016). Our results suggest that androgen-mediated maternal effects may not be a major part of plastic responses to offspring predation risk in a cavity-nesting bird. Still, we note that our sample size and year effects may have limited our power to detect subtle effects and encourage cautious interpretation of our negative results. Ultimately, examining interactions between environmental variation, parental care behaviors and the diversity of maternal steroids that females allocate to eggs promises to greatly enhance our understanding of adaptive maternal effects in variable environments.

Competing Interests

No competing interests declared.

Author Contributions

JCM conceptualized the study, conducted fieldwork, analyzed data, and wrote an initial draft of the manuscript. RAD and TEM acquired funding. RTP prepped and analyzed samples. All authors contributed to revisions

Data Availability

All data is freely available through Figshare (https://doi.org/10.6084/m9.figshare.19385777.v1)

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Figures and Table
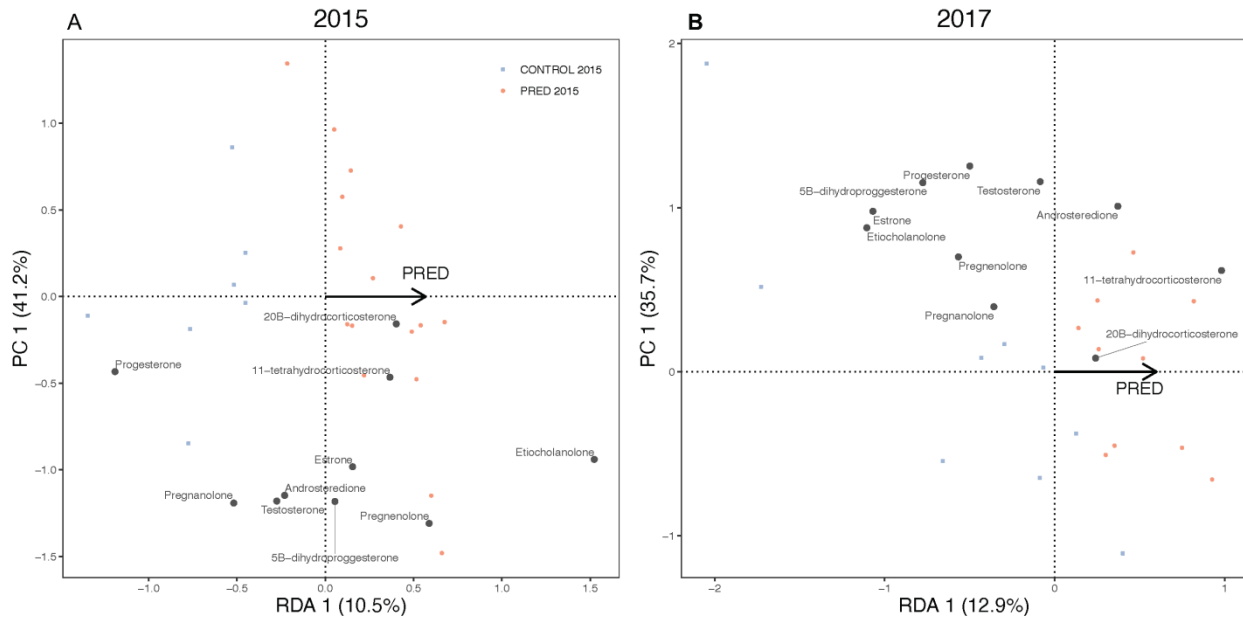
Figure 1: RDA correlation triplot decpicting relationships between predation risk treatment and 10 yolk steroids for (A) 2015 and (B) 2017. Predation treatment was associated with greater values of RDA1 than controls (arrow). The loadings of each steroid along RDA1 varied across years. The correlation between predation risk treatment and steroid concentrations is related to the angular difference between the arrow and each steroid (gray points and text; 180° = strong negative correlation; 90° = uncorrelated, 0° = strong positive correlation). Eggs from high nest predation risk treatment (red) vs. controls (blue) are depicted as points.
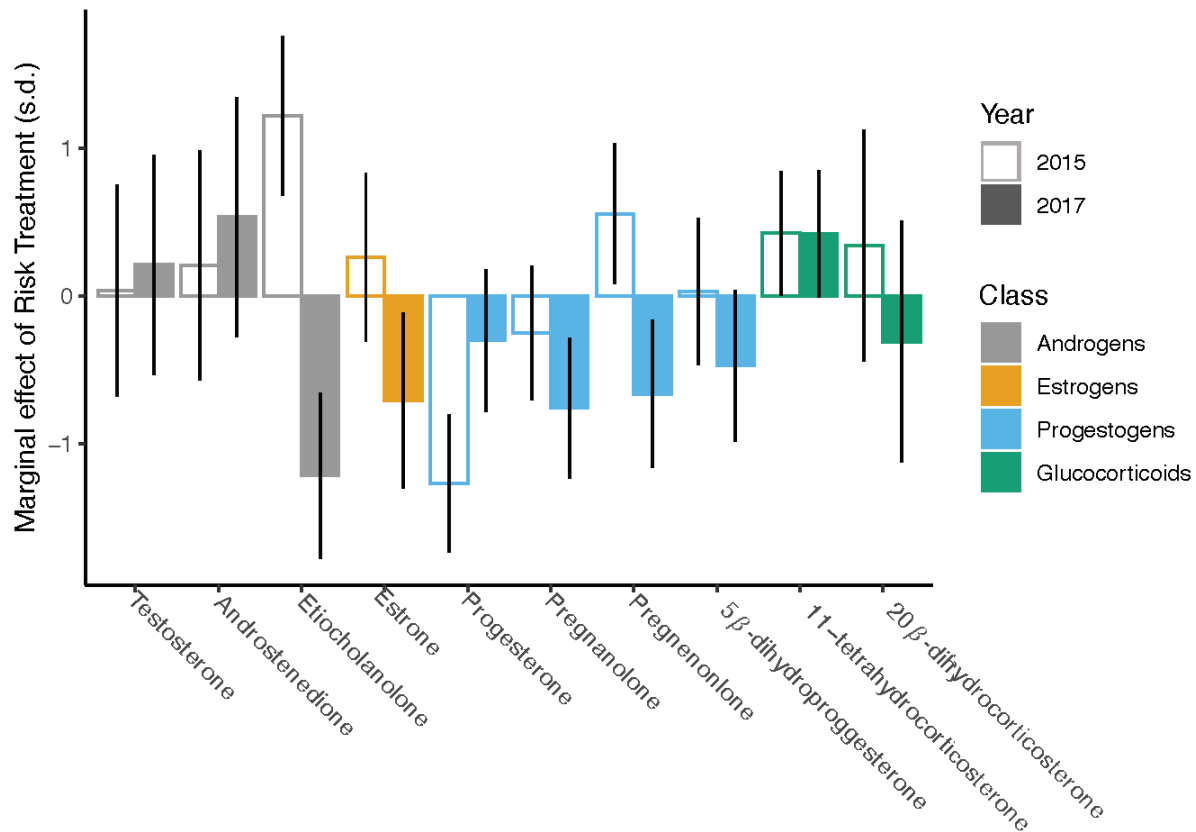
Figure 2: Results from univariate analyses testing the relationship of predation risk treatment with 10 yolk steroids. Marginal change in yolk steroid concentrations with increased perceived nest predation risk (marginal effects of risk treatment ± 1 s.e.m) in each year after accounting for shared nest effects in linear mixed models. Colors depict classes of steroids. Light shading indicates 2015 and dark shading indicates 2017.

Table 1: Variation in concentration of hormones across all eggs (pg/mg).

Hormone (# eggs)	Mean	Standard Error	CV
Estrone (42)	12.258	0.787	0.416
Androstenedione (42)	2.048	0.255	0.807
Testosterone (42)	3.497	0.340	0.630
Etiocholanolone (42)	15.38	1.257	0.530
Progesterone (42)	62.43	9.961	1.034
Pregnenolone (42)	405.15	35.33	0.565
11- dehydrotetrahydrocorticosterone (42)	1.209	0.097	0.518
5β-dihydroprogesterone (42)	2470.6	133.3	0.350
Pregnanolone (42)	1326.4	106.8	0.521
20β-dihydrocorticosterone (32)	0.536	0.084	1.013
17α-hydroxypregnenolone (3)	1.867	1.292	4.481
Cortisone (3)	0.019	0.011	3.669
Estradiol (2)	0.879	0.618	4.552
Dehydroepiandrosterone (0)	0	-	-
11-Ketotestoterone (0)	0	-	-
17α-hydroxyprogesterone (0)	0	-	-
Dihydroprogesterone (0)	0	-	-
11-Dexoycortisol (0)	0	-	-
5β-dihydrocortisone (0)	0	-	-
20β-DHP (0)	0	-	-
Cortisol (0)	0	-	-
5β-dihydrocortisol (0)	0	-	-
5β-tetrahydrocortisone (0)	0	-	-
β-cortolone (0)	0	-	-
B-cortol (0)	0	-	-
Corticosterone (0)	0	-	-
5β-tetrahydrocorticosterone (0)	0	-	-
5β-corticosterone (0)	0	-	-
Deoxycorticosterone (0)	0	-	-

Table S1. RDA results. Biplot scores represent the correlations between each axis and the explanatory variables. Species scores represent the correlation between each axis and the response variables (steroids).

	RDA1	RDA2	RDA3	PC1	PC2	PC3
a) Eigenvalues and their importance						
Eigenvalue	1.574	0.575	0.285	3.093	1.241	1.127
Proportion Explained	0.157	0.057	0.029	0.309	0.124	0.113
CumulativeProportion	0.157	0.215	0.243	0.553	0.677	0.789
b) Biplot scores for constraining factors:						
Predation Risk Treatment	-0.015	0.386	0.236	-	-	-
Year (2017)	0.242	0.433	-0.088	-	-	-
Predation Risk x Year	0.111	-0.618	0.149	-	-	-
c) Species scores:						
Estrone	-0.674	0.208	-0.244	-0.864	-0.240	-0.179
Androstenedione	-0.113	-0.205	0.083	-1.082	-0.405	0.618
Testosterone	0.110	-0.064	-0.121	-1.124	-0.703	0.353
Etiocholanolone	-0.164	0.817	-0.279	-0.800	0.269	0.062
Progesterone	-0.439	-0.535	-0.416	-0.627	-0.233	-0.272
Pregnenolone	-0.956	0.163	0.192	-0.756	0.491	-0.270
11-dehydrotetrahydrocorticosterone	-0.628	-0.023	0.387	-0.385	-0.275	-0.527
20β-dihydrocorticosterone	-0.222	0.143	0.220	-0.164	-0.497	-1.128
5β-dihydroprogesterone	-0.578	0.064	-0.083	-0.947	0.764	-0.082
Pregnanolone	-0.891	-0.262	-0.064	-0.636	0.722	-0.187

Table S2. Model results from univariate analyses based on (a) yolk steroid concentrations and (b) total amount of yolk steroids. Main effect coefficients and estimates of between nest variation are from models with interaction terms removed except the interaction was supported (bold; P<0.1). Results for fixed effects for models with 0 Nest ID variance are from li—near models using mean values for each nest.

a) Yolk steroid concentrations

Hormone	2017 (vs. 2015)			Risk Treatment (vs. Control)			Interaction (Year X Risk)			Nest ID
	Estimate	Std. Error	P	Estimate	Std. Error	P	Estimate	Std. Error	P	Var.
Estrone	**-1.1628**	**0.4127**	**0.015**	-0.198	0.420	0.646	-0.969	0.824	0.266	0.395
Androstenedione	-0.292	0.523	0.591	0.348	0.533	0.532	0.331	1.124	0.777	0.715
Testosterone	0.065	0.482	0.895	0.106	0.491	0.833	0.173	1.035	0.871	0.557
Etiocholanolone	**1.245**	**0.602**	**0.062**	**1.139**	**0.246**	**0.044**	**-2.441**	**0.780**	**0.009**	0.361
Progesterone	**-0.732**	**0.347**	**0.058**	**-0.790**	**0.354**	**0.047**	0.967	0.672	0.181	0.179
Pregnenolone	**-1.461**	**0.368**	**0.002**	-0.025	0.374	0.946	-1.221	0.691	0.105	0
11-dehydrotetrahydrocorticosterone	**-0.879**	**0.279**	**0.010**	0.420	0.287	0.171	-0.005	0.601	0.994	0.008
20β-dihydrocorticosterone	-0.496	0.541	0.377	0.032	0.551	0.955	-0.652	1.135	0.577	0
5β-dihydroprogesterone	**-0.822**	**0.343**	**0.032**	-0.213	0.350	0.554	-0.501	0.713	0.496	0.116
Pregnanolone	**-1.544**	**0.318**	**<0.001**	-0.492	0.324	0.155	-0.508	0.660	0.457	0

b) Total amount of yolk steroids

Hormone	2017 (vs. 2015)			Risk Treatment (vs. Control)			Interaction (Year X Risk)			Nest ID
	Estimate	Std. Error	P	Estimate	Std. Error	P	Estimate	Std. Error	P	Var.
Estrone	**-1.111**	**0.476**	**0.044**	-0.134	0.470	0.782	-0.975	0.947	0.334	0.468
Androstenedione	-0.419	0.574	0.493	0.251	0.567	0.673	0.137	1.244	0.916	0.722
Testosterone	-0.079	0.518	0.883	-0.018	0.511	0.973	-0.087	1.106	0.939	0.532
Etiocholanolone	**1.247**	**0.640**	**0.082**	**1.239**	**0.575**	**0.057**	**-2.232**	**0.890**	**0.034**	0.740
Progesterone	**-0.840**	**0.366**	**0.047**	**-0.874**	**0.365**	**0.039**	0.827	0.711	0.279	0.177
Pregnenolone	**-1.299**	**0.462**	**0.018**	0.216	0.451	0.642	-1.246	0.883	0.192	0
11-dehydrotetrahydrocorticosterone	**-0.867**	**0.297**	**0.018**	0.407	0.299	0.206	-0.022	0.637	0.973	0.015
20β-dihydrocorticosterone	-0.262	0.329	0.443	0.319	0.332	0.356	-0.464	0.677	0.509	0.008
5β-dihydroprogesterone	**-0.718**	**0.372**	**0.081**	-0.124	0.372	0.744	-0.341	0.774	0.670	0.126
Pregnanolone	**-1.347**	**0.265**	**0.001**	-0.269	0.267	0.343	0.321	0.544	0.575	0.022

Table S3. Model results from univariate analyses based on yolk steroid concentrations based on a reduced dataset to account for lay order. Main effect coefficients and estimates of between nest variation are from models with interaction terms removed except the interaction was supported (bold; P<0.1). Note: P-values from models with 0 Nest ID variance are based on greater degrees of freedom (n-p for n=42).

Hormone	2017 (vs. 2015)			Risk Treatment (vs. Control)			Lay Order			Interaction (Year X Risk)			Nest ID
	Estimate	Std. Error	P	Estimate	Std. Error	P	Estimate	Std. Error	P	Estimate	Std. Error	P	Var.
Estrone	-1.000	0.540	0.112	-0.544	0.575	0.380	**0.214**	**0.094**	**0.031**	-1.540	1.118	0.226	0.525
Androstenedione	-0.315	0.619	0.630	0.048	0.660	0.943	**0.364**	**0.088**	**<0.001**	0.483	1.479	0.758	0.760
Testosterone	0.164	0.514	0.7601	-0.464	0.547	0.427	**0.465**	**0.07**	**<0.001**	0.076	1.229	0.953	0.524
Etiocholanolone	**2.438**	**0.389**	**<0.001**	**1.074**	**0.313**	**0.002**	0.117	0.080	0.156	**-3.41**	**0.474**	**<0.001**	0
Progesterone	-0.485	0.384	0.248	**-1.116**	**0.408**	**0.030**	**0.331**	**0.092**	**0.001**	1.008	0.787	0.254	0.193
Pregnenolone	**-1.317**	**0.263**	**<0.001**	0.300	0.279	0.292	**0.1725**	**0.095**	**0.080**	-0.660	0.567	0.253	0
11-dehydrotetrahydrocorticosterone	**-0.779**	**0.346**	**0.065**	0.420	0.368	0.296	0.045	0.117	0.702	-0.023	0.819	0.978	0.036
20β-dihydrocorticosterone	-0.385	0.370	0.334	0.435	0.394	0.307	0.152	0.132	0.257	-0.245	0.864	0.787	0.008
5β-dihydroprogesterone	-0.710	0.414	0.131	-0.291	0.441	0.531	0.156	0.121	0.207	-0.697	0.933	0.485	0.137
Pregnanolone	**-1.237**	**0.284**	**<0.001**	-0.362	0.302	0.239	-0.065	0.103	0.531	0.239	0.624	0.704	0