A supergene affects steroid metabolism during early ontogeny in a bird with alternative reproductive morphs

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Abstract

Reproductive phenotypes are shaped by genetic, physiological and environmental variation that an organism experiences during ontogeny. Steroid hormones play an integrative role in this process through both genomic and non-genomic pathways. Differences in steroid hormone metabolism may be rooted in genomic variation. Here we evaluate the influence of supergene variants underlying alternative reproductive tactics on sex steroid metabolism during ontogeny in ruffs (Calidris pugnax). Adult ruff males exhibit three male mating morphs called Independents, Faeders and Satellites, that differ prominently in circulating androgen (testosterone and androstenedione) concentrations. Across morphs and sexes chicks showed similar mean androgen concentrations during ontogenetic development. However, variances in circulating androgens showed the same pattern as corresponding variances previously observed in adults. HSD17B2 had been previously identified as a key gene for mediating differences in androgen levels between morphs as it encodes the enzyme that converts testosterone to androstenedione and is located within the supergene. Observed HSD17B2 expression in embryonic brain tissue was consistent with predictions based on genetic and endocrine differences. Taken together, the observed differences in circulating androgen concentrations and gene expression point to testosterone synthesis as a key mechanism that shapes developmental trajectories and differences in brain organization among morphs.

Title

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Abstract

Reproductive phenotypes are shaped by genetic, physiological and environmental variation that an organism experiences during ontogeny. Steroid hormones play an integrative role in this process through both genomic and non-genomic pathways. Differences in steroid hormone metabolism may be rooted in genomic variation. Here we evaluate the influence of supergene variants underlying alternative reproductive tactics on sex steroid metabolism during ontogeny in ruffs (*Calidris pugnax*). Adult ruff males exhibit three male mating morphs called Independents, Faeders and Satellites, that differ prominently in circulating androgen (testosterone and androstenedione) concentrations. Across morphs and sexes chicks showed similar mean androgen concentrations during ontogenetic development. However, variances in circulating androgens showed the same pattern as corresponding variances previously observed in adults. *HSD17B2*had been previously identified as a key gene for mediating differences in androgen levels between morphs as it encodes the enzyme that converts testosterone to androstenedione and is located within the supergene. Observed *HSD17B2* expression in embryonic brain tissue was consistent with predictions based on genetic and endocrine differences. Taken together, the observed differences in circulating androgen concentrations and gene expression point to testosterone synthesis as a key mechanism that shapes developmental trajectories and differences in brain organization among morphs.

Keywords

Androstenedione, *Calidris pugnax,* chromosomal inversion, gene expression, progesterone, testosterone

Introduction

Reproductive phenotypes are shaped by multiple processes and conditions experienced during development including gene expression, hormone secretions, environmental and social conditions and their interactions. The challenge is to disentangle the mechanisms by which phenotypic variation is shaped. In some species, discrete differences in reproductive phenotypes between and within sexes are based on discrete genotypes (Oliveira, Taborsky, & Brockmann, 2008). These species provide an excellent opportunity to investigate how genetic variation affects underlying proximate mechanisms of phenotypic variation beyond the dichotomy of males and females.

Much of our knowledge on how genes shape reproductive phenotypes, particularly behavioural phenotypes, is based on sexual differentiation studied in only a few model species. From these studies, two key mechanisms have been identified (Adkins-Regan, 2005; Arnold, 2004; McCarthy & Arnold, 2011). First, the genetic sex of brain cells can cause direct sex differences in brain phenotypes (Arnold, 2020; Chen, Grisham, & Arnold, 2009; Chen, McClusky, Itoh, Reue, & Arnold, 2013; De Vries et al., 2002; McCarthy & Arnold, 2011; McPhie-Lalmansingh, Tejada, Weaver, & Rissman, 2008). Second, sex genes determine the gonadal sex (Ayers, Sinclair, & Smith, 2013; Goodfellow & Lovell-Badge, 1993; Smith et al., 2009) and sexually differentiated gonads can escalate sex-biased gene expression through their effect on the hormonal milieu (Wittman, Robinson, McGlothlin, & Cox, 2021). The gonads are main producers of vertebrate sex steroids, such as testosterone, estrogens and progesterone (Adkins-Regan, 2005). Variation in sex steroid synthesis is pivotal in regulating behavioural variation, because steroids can cross the blood-brain barrier, target brain areas involved in social behaviour, and can alter gene expression through binding to intracellular receptors that act as transcription factors in the nucleus (Adkins-Regan, 2005; Cheng, 2002; De Vries & Simerly, 2002; Simerly, 1993). However, their synthesis is not restricted to the gonads; the brain also harbors the necessary enzymes for *de novo*sex steroid synthesis (Baulieu, 1991; Diotel et al., 2011; Hojo & Kawato, 2018; Jalabert,

Ma, & Soma, 2021; Schlinger, 2015; Tsutsui, 2011). For example, in mammals, the chromosomal sex (XX or XY) determines the gonadal sex via the *Sry* gene present on the Y chromosome (Goodfellow & Lovell-Badge, 1993). *Sry* leads, with other contributing genes, to the formation of the testes. The embryonic or neonatal testes then secrete testosterone that either directly, or via its transformation into estradiol, binds to neuronal receptors. Beside these stand-alone effects, genes from sex chromosomes can also interact with hormones to lead to further sexual differentiation in physiology and behaviour. Hence, direct genetic effects, hormonal effects and their interaction influence the development of sexually differentiated physiological or behavioural traits (Arnold, 2020; Chen et al., 2009; McCarthy & Arnold, 2011).

The timing in variation of sex steroid synthesis or gene expression in brain cells is crucial, because variation in phenotypes develops mainly during ontogeny. Phoenix et al. (1959) were the first to apply the concept of behavioural organization. Their concept states that gonadal sex steroids have permanent or organizational effects during development, as opposed to the transient or activational effects that occur later in life (Phoenix et al., 1959). This concept remains a fundamental principle of phenotypic differentiation of brain and behavior (Adkins-Regan, 2005; Arnold, 2020; De Vries & Simerly, 2002; McCarthy & Arnold, 2011; Zupanc & Lamprecht, 2000). Organizational effects occur particularly during early stages of brain development during embryonic and juvenile stages, and establish the necessary structures for future behavior through organization of neural networks (Court, Vandries, Balthazart, & Cornil, 2020; Juraska, Sisk, & Doncarlos, 2013; Sisk & Zehr, 2005). However, neural organization is not restricted to the effects of gonadal sex steroids as previously thought, also including non-gonadal steroids and signals that act directly on brain cells, e.g. gene products downstream from sex chromosome genes (Adkins-Regan, 2005; McCarthy, 2020; McCarthy & Arnold, 2011).

Within the sexes, chromosomal rearrangements, such as chromosomal inversions, often manifest in genetic variation that underlies behavioural variation (Fuller, Haynes, Richards, & Schaeffer, 2016; Huang, Dang, Chang, & Wang, 2018; Jay et al., 2021; Küpper et al., 2016; Lamichhaney et al., 2016; Lindtke et al., 2017). Chromosomal inversions can give rise to supergenes that maintain different adaptive allele combinations through suppression of recombination (Dobzhansky, 1950; Kim et al., 2017; Schwander, Libbrecht, & Keller, 2014; Thompson & Jiggins, 2014). Similar to sex chromosomes that encode the differences between males and females, supergenes provide the genomic substrate for the evolution of near-discrete behavioural variation within species, such as alternative reproductive tactics (Horton et al., 2014; Küpper et al., 2016; Lamichhaney et al., 2016; Mérot, Llaurens, Normandeau, Bernatchez, & Wellenreuther, 2020). Variation between alternative reproductive phenotypes may be produced by mechanisms similar to those that differentiate sexes (Oliveira, Canário, & Ros, 2008). When so, changes to genes that alter steroid regulation seem to be particularly important for the development of reproductive phenotypes (Maney & Küpper, 2022). In white-throated sparrows (*Zonotrichia albicollis*), a large autosomal inversion captures the estrogen receptor gene *ESR1* (Tuttle et al., 2016). The supergene determines two reproductive morphs that differ in plumage coloration, territorial and parental behavior (Falls & Kopachena, 2010; Horton et al., 2014; Maney & Küpper, 2022). Much of the behavioural variation observed between adult morphs can be explained by the expression of *ESR1* in brain cells. This morph difference in *ESR1* expression emerges already during ontogeny (Merritt et al., 2020). Little is known whether it is a general mechanism that supergenes underlying different reproductive phenotypes encode for variation in steroid metabolism also in other species than the white-throated sparrow. Sex steroids are thought to also underlie organizing effects for observed behavioural differences within sexes (Brockmann, 2001; Knapp, 2004; Rhen & Crews, 2002; Zupanc & Lamprecht, 2000). Hence, genetic variation influencing sex steroid metabolism during ontogeny could be a key mechanism mediating morph-specific behaviours through morph-specific brain organization (Adkins-Regan, 2005; Jasmine L Loveland, Giraldo-Deck, & Kelly, 2022; Rhen & Crews, 2002).

Most insights into the role of steroid metabolism and regulation in determining behavioural phenotypes come from experimental studies in well characterized model species that are easy to keep under laboratory settings and have a large number of offspring. However, whether such manipulation induced variation reflects natural variation in steroid concentrations during ontogeny often remains unknown. Longitudinal studies with repeated sampling of the same individuals can help to close this gap in our knowledge. However,

methodological challenges often prevent the evaluation of steroid variation during ontogeny in non-model species. First, variation in steroid levels may occur early in development when blood sampling is not feasible without killing the individual. Second, meaningful variation in steroid levels may pass unnoticed if it occurs in-between sampling events. Dense sampling can overcome this challenge, but sampling is often restricted by the size and blood volume of the subjects. Third, the period of variation in steroid levels may vary between sexes, morphs and individuals. In that case, the sampling may match the hormonal peaks in some individuals but not in others (Fig. 1).

Here we evaluate the influence of genetic variation induced by a prominent supergene on sex steroid metabolism during ontogeny in ruffs (*Calidris pugnax*). Ruffs have three well-described male mating morphs, Independents, Satellites and Faeders (Hogan-Warburg, 1966; Jukema & Piersma, 2006; Küpper et al., 2016; Widemo, 1998). These morphs are determined by recessive and dominant alleles of a supergene that arose through a chromosomal inversion (Küpper et al., 2016; Lamichhaney et al., 2016). The inversion region contains several genes including *HSD17B2* , *SDR42E1* , *CY5B5* that are involved in sex steroid synthesis and metabolism (Küpper et al., 2016; Lamichhaney et al., 2016). Independents are the ancestral morph, whereas Satellites and Faeders are derived morphs. The inversion resulted first in a cluster of alleles (Faeder allele) encoding the Faeder morph. Later, rare recombination events between the ancestral and the Faeder allele resulted in a cluster of alleles (Satellite allele) encoding the Satellite morph (Hill, Enbody, Bi, Lamichhaney, & Schwochow, 2022; Küpper et al., 2016; Lamichhaney et al., 2016). Independents are always homozygous for the ancestral non-inverted sequence (Independent allele) whereas Satellites and Faeders are always heterozygous, having one inversion-derived allele (either Satellite or Faeder allele) and one ancestral allele. Any zygotic combinations containing only inversion-derived alleles are lethal. Because the inversion is autosomal, the three morphs exist in both males and females, although the phenotypic differences are most pronounced in adult males during the breeding season.

Male Independents are the largest and most frequent morph, who display and defend small territories on leks (van Rhijn, 1991; Widemo, 1998). They are joined by Satellites who team up with certain Independents for co-display without defending their own territory (van Rhijn, 1991; Widemo, 1998). The third morph, Faeders, are the smallest and rarest morph, who mimic females and sneak copulations (Jukema & Piersma, 2006). The behavioural variation is tied to variation in circulating steroid concentrations during the breeding season (Küpper et al., 2016; J.L. Loveland, Giraldo-Deck, et al., 2021). Independents have higher levels of testosterone than the two other morphs. Conversely, Satellites and Faeders have higher levels of androstenedione (Küpper et al., 2016; J.L. Loveland, Giraldo-Deck, et al., 2021), a precursor of testosterone and less potent androgen (Moeller & Adamski, 2009), than Independent males. Manipulation experiments demonstrated that the behavioural differences persist even when the differences in circulating hormone levels are experimentally reduced, suggesting organizational variation between morphs (Morgan, 2010).

We examined how genetic variation harboured by supergene variants affects steroid metabolism during ontogeny in ruffs. We analyzed circulating hormone concentrations repeatedly during juvenile development and determined relative gene expression of candidate genes in embryonic brains (Fig. 2). Variation between groups is usually analyzed by comparing their means. However, circulating hormone concentrations are highly variable within individuals. Depending on the synchrony of hormonal fluctuations, morph variation could result either in differences among means or among variances and skewness (exemplified in Fig. 1). Therefore, to examine longitudinal variation between sexes and morphs, we compared means, variances and skewness of circulating testosterone, androstenedione and progesterone concentrations in juvenile ruffs between morphs and sexes sampled weekly from day 10 until day 30 past hatching. In contrast to other studies that started sampling around hatching (e.g. (Adkins-Regan, Abdelnabi, Mobarak, & Ottinger, 1990; Corbier, Dehennin, Auchere, & Roffi, 1992; Ottinger, Pitts, & Abdelnabi, 2001; Schumacher, Sulon, & Balthazart, 1988; Y. Tanabe, Saito, & Nakamura, 1986; Yuichi Tanabe, Yano, & Nakamura, 1983)), we started sampling only at an older age to allow for repeated measurements without compromising individual survival. Based on previous results from adult males, we predicted that androgen concentrations should vary between morphs with Independents having high circulating testosterone and low circulating androstenedione concentrations, whereas both derived morphs would show the opposite pattern (Küpper et al., 2016; J.L. Loveland,

Giraldo-Deck, et al., 2021). Because the inversion is autosomal, we predicted morph-specific variation to be similar between males and females. For circulating progesterone, no differences between adult morphs have been found (Küpper et al., 2016; J.L. Loveland, Giraldo-Deck, et al., 2021), but given its prominent role in the regulation of aggression in other vertebrates (Adreani, Goymann, & Mentesana, 2018; Goymann, Wittenzellner, Schwabl, & Makomba, 2008; Moore, Hews, & Knapp, 1998), and given that *SDR42E1* , a gene potentially involved with progesterone synthesis, is found in the inversion region (Lamichhaney et al., 2016), we included progesterone in our analyses.

To evaluate morph differences before hatching, we evaluated variation in gene expression in a macro-dissected brain region of 14-day-old embryos that contains the areas of the social behaviour network. For these samples not enough blood could be sampled to compare hormone concentrations. Instead, we measured morph-specific gene expression of candidate genes involved in androgen metabolism, particularly the inversion gene *HSD17B2* . This gene encodes the enzyme HSD17B2, which mainly converts testosterone to androstenedione (Baker, 2001; Küpper et al., 2016; Lamichhaney et al., 2016; J.L. Loveland, Lank, & Küpper, 2021; Miller & Auchus, 2011). In view of the relatively high levels of circulating androstenedione in inversion morphs compared to Independents (Küpper et al., 2016; J.L. Loveland, Giraldo-Deck, et al., 2021; Jasmine L Loveland et al., 2022), *HSD17B2* has been suggested as the candidate gene for explaining the observed hormonal difference among adult morphs (Küpper et al., 2016; Jasmine L Loveland et al., 2022). However, previous studies of adults failed to detect variation in testicular *HSD17B2* expression among morphs (J.L. Loveland, Lank, et al., 2021).

Materials and Methods

Captive breeding

We sampled 115 chicks (58 Independents, 39 Satellites and 18 Faeders) and 51 embryos (24 Independents, 13 Satellites and 14 Faeders) from ruffs housed at Simon Fraser University in Burnaby, British Columbia, Canada, between 2017 and 2019. This captive population consisted of approximately 300 individuals of all three morphs and was originally established from eggs collected near Oulu, Finland, in 1985, 1989, and 1990 (Lank, Farrell, Burke, Piersma, & McRae, 2013). We collected eggs several times per day during the breeding season from April to end of July and placed them in an incubator at 37.5°C and 55% humidity. We candled eggs regularly to confirm which embryos developed and were alive (Giraldo-Deck, Loveland, Goymann, Tschirren, et al., 2022). In chicken, which have a similar incubation period as ruffs, morphological differentiation of the gonads is completed after day 8.5 of incubation (Smith & Sinclair, 2004), and from then on steroid synthesis and the expression of their receptors increases (Woods, Simpson, & Moore, 1975). At very low expression levels, differences between sexes and morphs would be difficult to detect. To detect potential morph differences in enzymes or receptors involved in sex steroid metabolism, we sampled embryos after 14 days of incubation. We collected brains for gene expression analyses (Fig. 2) and took a blood sample (50-75µl) to determine genetically sex and morph (for details of sex and morph determination see (Giraldo-Deck et al., 2020)). The remaining eggs continued incubation. Hatched chicks were individually colour-ringed and hand-raised with other chicks of similar age in mixed-sex and mixed-morph groups under *ad libitum* food until an age of 30 days, when they were incorporated into the adult population. At ages 10, 17, 24 and 30 days post hatching, we sampled chicks with body weights within the normal range for their sex and age, indicating good health and normal development (Fig. 2). We chose these sampling ages, because chicks were large enough (>22.7 g) that sampling of the required blood sample (approximately 250µl) did not affect their survival. We collected blood samples from the wing vein, always between 8:30am to 10:00am. We immediately centrifuged the samples at 3000 rpm for 15 minutes, collected the plasma and stored it at -20°C until analysis. To determine circulating hormone concentrations, we used minimal amounts of 50µl, 40µl and 20µl of plasma for testosterone, androstenedione and progesterone, respectively. When less plasma was available, we prioritized the analysis for testosterone, followed by androstenedione and progesterone. We used the red blood cells for genetic sex and morph determination. All housing and procedures (permit

#1232B-17) were approved by the Animal Care Committee of Simon Fraser University operating under guidelines from the Canadian Council on Animal Care.

Hormone analyses

Plasma testosterone, androstenedione and progesterone levels were determined by radioimmunoassays (RIAs) following published protocols for extraction and measurement (Goymann, East, & Hofer, 2001; Goymann et al., 2006, 2008) that had been previously used to evaluate hormone concentrations in adult ruffs (J.L. Loveland, Giraldo-Deck, et al., 2021). For testosterone and androstenedione, we extracted plasma samples with dichloromethane (DCM) after overnight equilibration (4°C) of the plasma with 1500dpm of tritiated testosterone or androstenedione (Perkin Elmer, Rodgau, Germany). The organic phase was then separated from the aqueous phase by plunging the extraction tubes into a methanol-dry ice bath and decanting the dichloromethane phase into a new vial. This extraction step was repeated twice to increase extraction efficiency. Then, the DCM phase was dried under a stream of nitrogen at 40°C, dried samples were resuspended in 300µl phosphate buffered saline with 1% gelatine (PBSG) and left overnight at 4°C to equilibrate. Progesterone was extracted with ethyl ether after overnight equilibration (4°C) of the plasma with 1500dpm of tritiated progesterone (Perkin Elmer, Rodgau, Germany). Separation of the organic and aqueous phase was conducted similar to the procedure described for the other hormones. For all hormone extracts, an 80µl aliquot of the redissolved samples was transferred to scintillation vials, mixed with 4ml scintillation fluid (Packard Ultima Gold) and counted to an accuracy of 2–3% in a Beckman LS 6000 ß-counter to estimate individual extraction recoveries. The remainder was stored at -40degC until RIA was conducted. Mean +- sd extraction efficiency for plasma testosterone was 84.9 +- 5.8% (N = 342), for androstenedione 85.0 +- 6.5% (N = 289), and for progesterone 74.9 +- 8.7% (N = 290). For the RIA, a standard curve was set up in duplicates by serial dilution of stock standard testosterone and androstenedione ranging from 0.39–200pg, and progesterone from 1.95–1000pg. Testosterone, androstenedione or progesterone antisera (Esoterix Endocrinology, Calabasas, CA, USA) were added to the respective standard curve, the controls and to duplicates of each sample (2x100µl of the PBSG buffered sample). After 30min, the respective testosterone, androstenedione or progesterone labels were added and the assays incubated for 20h at 4°C. Bound and free fractions were then separated at 4°C by adding 0.5ml dextran-coated charcoal in PBSG assay buffer. After 14min incubation with charcoal, samples were spun at 3600g for 10min at 4°C and supernatants decanted into scintillation vials at 4°C. After adding 4ml of scintillation liquid (Packard Ultima Gold), vials were counted. Standard curves and sample concentrations were calculated with Immunofit 3.0 (Beckman Inc. Fullerton, CA), using a four parameter logistic curve fit. We analyzed data in seven assays for each hormone and determined the lower detection limits of the standard curves as the first value outside the 95% confidence intervals for the zero standard (Bmax), resulting in values of 0.29–0.55pg/ml for testosterone, 0.70–0.92pg/ml for androstenedione, and 1.98–3.84pg/ml for progesterone. The intra-assay coefficients of variation as determined by extracted chicken plasma pools were 4.0%, 5.4%, 9.7%, 12.5%, 2.7%, 11.5% and 3.4% for the testosterone assays, 7.2%, 5.8%, 4.7%, 3.3%, 2.8%, 7.8% and 5.0% for the androstenedione assays, and 4.9%, 3.3%, 0.9%, 5.3%, 10.1%, 6.1% and 3.7% for the progesterone assay. The inter-assay coefficients of variation as determined by extracted chicken plasma pools were 13.0% for the testosterone assays, 12.1% for the androstenedione assays, and 7.5% for the progesterone assays. Because the testosterone antibody used shows significant cross-reactions (44% according to the specification sheet provided by the manufacturer) with 5α-dihydrotestosterone, our testosterone measurement may include a fraction of 5α-dihydrotestosterone.

Tissue sampling

To analyze gene expression of candidate genes involved in steroid metabolism, we collected brains of 14-day-old embryos. After decapitation, we placed the head into a cube with embedding medium (Neg50) and froze it immediately on dry ice. To standardize the orientation of the head within the cube, we placed it so that the beak was vertical with its tip pointing upward and stored the cube at -80°C until sectioning on a cryostat.

Brain macrodissection

For sectioning, we cut heads into 150 µm thick slices using a cryostat and transferred those onto Superfrost slides. Slices were allowed to thaw briefly to adhere to the slide and then kept in a slide box on dry ice followed by storage at -80°C. One embryo head was sectioned at 20µm and stained with a standard cresyl violet protocol to serve as an initial reference for identifying major landmarks and brain nuclei. We aimed to ensure that all areas of the social behavior network (SBN) were included in our samples. The SBN for birds, as defined by Goodson (Goodson, 2005), consists of the nucleus taeniae and bed nucleus of the stria terminalis, preoptic area, lateral septum, anterior and ventromedial hypothalmus and substancia grisea centralis (i.e. central gray). As it was not possible to reliably dissect each area separately in ruff embryos, we collected all areas within a single sample per bird. Major landmarks were sufficient to ascertain that SBN areas were included in our sampling. We collected sections starting where the lateral ventricle of the telencephalon was visible and ended sample collection at the posterior end of the midbrain. We made a single "top" horizontal cut perpendicular to the lateral ventricle at the top of the lateral septum, i.e. the top border. To exclude the hindbrain, a single "bottom" horizontal cut was made where hindbrain was clearly present. If a section did not clearly contain hindbrain, all brain tissue was collected on that section. This method ensured that we collected the entire hypothalamus, nucleus taenia, bed nucleus of the stria terminalis and preoptic area. The landmark for ending collection was when left and right optic tectums were no longer connected along their midline, a sign that the bulk of the midbrain had passed. In this way, we excluded dorsal telencephalon, posterior optic tectum and hindbrain.

For the brain macrodissection, we thawed each slide by brief submersion first in ethanol (30s) and then in phosphate buffered saline (PBS) (5s) and followed by immediate collection of brain areas of interest under a dissection microscope. We placed the tissues in RNALater kept on dry ice.

RNA extraction, cDNA synthesis

We extracted RNA from sampled tissues using the RNeasy Minikit (Qiagen) following manufacturer protocols. We measured RNA concentration with a Nanodrop and used only samples with a 260/280 ratio of [?]1.8. We synthesized 1µg of RNA into cDNA using the iScript cDNA synthesis kit (Bio-Rad) in 20µl reactions according to manufacturer's instructions. We diluted cDNA six-fold before use as template in final qPCR assays (see details below).

Quantitative PCR (qPCR)

We conducted all qRT-PCR assays according to guidelines by Bustin et al. (Bustin et al., 2009). Initially, we set out to measure the expression of 10 target genes, which encode for following proteins: AR (Androgen receptor), ERα (Estrogen receptor alpha), ERβ (Estrogen receptor beta), PGR (Progesterone receptor), HSD17B2 (Hydroxysteroid 17-beta dehydrogenase 2), HD17B3 (Hydroxysteroid 17-beta dehydrogenase 3), STAR (Steroid acute regulatory protein), ARO (Aromatase), SRD5A1 (Steroid 5 alpha-reductase 1) and SRD5A2 (Steroid 5 alpha-reductase 1). As reference genes we used *HPRT1* (*Hypoxanthine Phosphoribosyltransferase 1*), *RPL32* (*Ribosomal Protein L32*) and *ACTB* (*Actin beta*) (primer sequences previously published in (J.L. Loveland, Giraldo-Deck, et al., 2021; J.L. Loveland, Lank, et al., 2021)). We ran assays on a LightCycler 480 II (Roche) using the SsoAdvanced Universal SYBR Green Super mix (Bio-Rad) in 384-well plates (Roche). Each reaction was run in duplicates with the following cycling conditions: pre-incubation step (95°C for 30s), 45 cycles (95°C for 10s, annealing and extension at 60°C for 30s) with acquisition at the end of each cycle, followed by a melt curve (95°C for 5s with 5 acquisitions per °C from 65°C to 97°C with a 0.11°C ramp rate). For final reactions, each well consisted of a 10µl reaction containing 1X SsoAdvanced Universal SYBR Green Super mix, 340nM of each primer and 12.5ng of cDNA template (i.e. 1.5µl of the 1:6 cDNA dilution; the estimate of template amount assumes a one-to-one correspondence between input RNA and synthesized cDNA). We performed qRT-PCR assays across five plates that were balanced for morph and sex for each tissue.

Using the same conditions, we ran a standard curve with serially diluted cDNA (1:4 to 1:64) from one Independent male embryo brain to calculate the amplification efficiency of each primer pair. Efficiency was calculated with the Absolute Quantification tool (Roche) and 2nd Derivative Maximum method, which uses

the formula [Efficiency = $10^{-1/\text{slope}}$] based on the quantification cycle (Cq, termed crossing point (Cp) in the software) and log concentration of template in each well. The theoretical efficiency of perfect amplification (i.e. exact doubling with each cycle) is 2. The efficiencies for all primer pairs are listed in Supplemental Table S1 and were within the range of 1.98–2.09). We used these efficiencies for further analyses for brain cDNAs.

For quality control, on every plate we confirmed that each primer pair produced a single melt curve peak in the presence of cDNA template and showed either no amplification when water was used as template or amplification that was clearly from primer-dimer. The genes *ESR1, ESR2, STAR* and *ACTB* did not satisfy these criteria or had efficiencies below or above an acceptable range (1.9–2.1) and hence were excluded.

Relative abundance calculation

We performed calculations from the raw amplification data in the LightCycler 480 Software (version 1.5.1.62) and used R for further statistical analyses. Reference genes were similarly expressed between sexes ($F_{(1,\ 44)}$ = 0.76; p =0.39) and morphs ($F_{(2,\ 43)}$ = 0.47; p =0.63). Hence, we used both reference genes to analyse variation in relative expression between sexes and morphs.

Statistics

Circulating hormone concentrations in juveniles

To examine whether morphs differed in mean circulating steroid concentrations, we modeled testosterone, androstenedione and progesterone concentrations of juveniles separately. For each hormone, we included all individuals with at least one measurement. We log-transformed hormone concentrations to normalize the residuals. We used linear mixed models to calculate mean concentrations and 95% credible intervals (95% CrI) in relation to sex, morph and age, including interactions between the three predictors. Additionally, we included individual ID and year as random factors to account for repeated measures of the same individual and between-year variance. We simulated 10,000 values from the joint posterior distribution of the model parameters using the function *sim* of the package *arm*(Gelman & Hill, 2006), and a flat prior distribution, to obtain model estimates with R 4.0.0 (R Core Team, 2020). The means of the simulated values from the joint posterior distribution of the model parameters were used as estimates, and the 2.5% and 97.5% quantiles as lower and upper limits of the 95% CrI. Instead of p -values, we provide the posterior probabilities for specific hypotheses, calculated as the proportion of simulated values that met the hypotheses. Probabilities higher than 0.975 or lower than 0.025 would be considered as significant ($p < 0.05$) to reject hypotheses according to frequentist statistics. We analyzed residual distributions graphically using normal quantile-quantile plots and by plotting residuals against leverage and against fitted values of each factor. We used the same residual analysis and the same simulation procedure in all other models. Additionally, we evaluated differences in variance and skewness between morphs and sexes, because individual variation in timing of morph- or sex-specific hormonal peaks may reflect in variation in variances and skewness between morphs or sexes (Fig. 1c). To evaluate differences in variance and to what extent these differences were driven by single individuals, a particular year or intrinsic morph differences, we modeled concentrations of each hormone for each morph separately. We included 'individual ID' and 'year' as random factors. We did not include variables 'sex' or 'age' as predictors, because in initial models these variables did not affect hormone concentrations (Fig. 3; Supplementary Table S2). We then extracted means and 95% CrI of the random variance and the variances explained by individual ID and sampling year. Similarly, we evaluated skewness for each morph separately without considering sex and age. For this, we used the function *skewness* of the package *moments* (Komsta & Novomestky, 2022). We evaluated differences in variance and skewness between sexes analogously to the analyses between morphs. For this, we excluded morph and age as predictors due to a limited sample size.

Because androstenedione is the direct precursor for testosterone (Moeller & Adamski, 2009) and the back-conversion from testosterone to androstenedione depends on *HSD17B2* , a gene located within the inversion, we used a linear mixed model to examine the association between the two steroid hormones. We used log-transformed androstenedione concentrations as dependent variable and log-transformed testosterone concentrations as independent variable and included 'individual ID' and 'year' as random factors.

Relative gene expression in embryonic brains and livers

To explore differences among morphs and between sexes in relative gene expression of candidate genes involved in steroid metabolism, we modeled relative gene expression separately for each gene. We used linear models to calculate mean concentrations and 95% CrI in relation to sex and morph and their interaction.

Results

Circulating steroid concentrations in juveniles

Mean circulating concentrations of testosterone, androstenedione and progesterone were similar between morphs, sexes and ages (10, 17, 24 and 30 days post hatching) (Fig.3, Supplementary Table S2). However, variances of androgen concentrations differed between the ancestral and both derived morphs in both sexes and across ages (Fig. 3, Table 1, Supplementary Table S3). In both sexes, the highest testosterone concentrations occurred only in Independents (Fig. 3; $\text{range}_{\text{Independents}}$=5-2830 pg/ml, $\text{skewness}_{\text{Independents}}$=8.35, $\text{range}_{\text{Satellites}}$=6-125 pg/ml, $\text{skewness}_{\text{Satellites}}$=2.09, $\text{range}_{\text{Faeders}}$=8-73 pg/ml, $\text{skewness}_{\text{Faeders}}$=0.71), whereas the highest androstenedione concentrations occurred only in Satellites and Faeders (Fig. 3; $\text{range}_{\text{Independents}}$=28-315 pg/ml, $\text{skewness}_{\text{Independents}}$=1.20, $\text{range}_{\text{Satellites}}$=32-3296 pg/ml, $\text{skewness}_{\text{Satellites}}$=5.10, $\text{range}_{\text{Faeders}}$=27-2034 pg/ml, $\text{skewness}_{\text{Faeders}}$=3.71). For testosterone, random variance in Independents was 2.66-times (95% CrI: 1.88; 3.66) the random variance of Satellites (P $(\sigma_{\text{Independents}}{}^2/\sigma_{\text{Satellites}}{}^2 > 1) > 0.99$) and 4.17-times (95% CrI: 2.60; 6.16) the random variance of Faeders P $(\sigma_{\text{Independents}}{}^2/\sigma_{\text{Faeders}}{}^2 > 1) > 0.99$). For androstenedione, random variance in Satellites was 3.75-times (95% CrI: 2.58; 5.33) the random variance of Independents (P $(\sigma_{\text{Satellites}}{}^2/\sigma_{\text{Independents}}{}^2 > 1) > 0.99$) and random variance in Faeders was 4.01-times (95% CrI: 2.46; 6.37) the random variance of Independents P $(\sigma_{\text{Faeders}}{}^2/\sigma_{\text{Independents}}{}^2 > 1) > 0.99$) (Fig. 3). Outlier individuals did not cause these differences in variance, because for testosterone, random variance in Independents was higher than the variances explained by differences between individuals or between years (Table 1). Conversely, for androstenedione, Satellite and Faeders of both sexes had higher random variances than the variances explained by the differences between individuals or years (Table 1). For progesterone, random variances were similar across morphs (Fig. 3 and Table 1) and sexes (Supplementary Table S3). In Satellites and Faeders, androstenedione concentrations positively correlated with testosterone concentrations, but in Independents androstenedione concentrations were independent of testosterone (Fig. 4a).

Relative gene expression in embryonic brains

Only the expression of *HSD17B2* differed among morphs (Supplementary Table S4). In both sexes, Satellites and Faeders had a higher relative *HSD17B2* expression and a higher variance compared to Independents. In females only, Faeders had also a higher relative*HSD17B2* expression than Satellites (Fig. 4b). Relative expression of all target genes were similar between sexes (Supplementary Table S4).

Discussion

Breeding ruffs show striking differences in behavior and steroid physiology across three morphs that genetically differ only in an autosomal supergene (Küpper et al., 2016; Lamichhaney et al., 2016). Here we analyzed how the supergene variants affect steroid metabolism during ontogeny. We found differences in circulating hormone concentrations and relative gene expression in a macro section of the brain that contains the social behaviour network of embryos. Mean hormone concentrations did not differ across morphs, but the variance and skewness of androgen levels of Satellite and Faeder juveniles, who both carry inversion-derived alleles differed from those of Independents. Specifically, high circulating testosterone concentrations occurred only in Independents and high androstenedione concentrations occurred only in the two derived morphs Faeders and Satellite. These differences in variance and skewness were present in males and females and were not

restricted to a specific age. Within a set of seven candidate genes involved in sex steroid metabolism, only *HSD17B2,*whose enzymatic product converts testosterone to androstenedione (Baker, 2001; Miller & Auchus, 2011), had a higher relative expression and a higher variance in brain areas including the social behaviour network of derived morphs compared to Independents.

Repeated sampling provides momentary glimpses into variation of hormonal levels during development. The three morphs differed in androgen variances and skewness, but not in their means at different sampling ages. Since we statistically controlled for ´individual ID´ and ´year´, differences in variances and skewness between morphs indicate that juveniles raised androgens from time to time asynchronously (Fig. 1c), suggesting that variation in androgen concentrations occurs throughout ontogeny when androgen synthesis is stimulated. Among Independents, juveniles only raised testosterone, whereas Satellites and Faeders only raised androstenedione (Fig. 3). Peak concentrations were similar to baseline concentrations reported for adults during the breeding season (J.L. Loveland, Giraldo-Deck, et al., 2021). The morph differences observed in juveniles mirror endocrine patterns of adult males, where Independents have high testosterone levels and Satellites and Faeders have high levels of androstenedione (Küpper et al., 2016; J.L. Loveland, Giraldo-Deck, et al., 2021; Morgan, 2010). This suggests that profound regulatory changes in steroid metabolism have been induced by the inversion. Results from an experimental challenge in adult males with gonadotropin-releasing hormone are consistent with this. (J.L. Loveland, Giraldo-Deck, et al., 2021).Stimulation of the hypothalamic-pituitary-gonadal axis induced a robust increase in androstenedione in Satellites and Faeders, but only a subdued increase in testosterone levels, whereas Independents showed the opposite pattern suggesting a disruption of testosterone synthesis in Satellites and Faeders (J.L. Loveland, Giraldo-Deck, et al., 2021). Our results indicate that the proposed mechanistic constraint of testosterone synthesis in derived morphs is not restricted to adult males, but present across earlier life stages in both sexes.

When comparing gene expression of steroid related genes between morphs and sexes in embryos, we found that both morphs carrying inversion haplotypes had an upregulation of *HSD17B2* in the social behaviour network of the brain, suggesting that androgen metabolism during embryonic development varies in this brain region. These differences were observed in both sexes. The inversion haplotypes of Faeders and Satellites contain several deletions at the surrounding sequences of*HSD17B2* that could affect its promoter, explaining the observed differences in expression (Küpper et al., 2016; Lamichhaney et al., 2016). *HSD17B2* converts testosterone back to androstenedione (Baker, 2001; Miller & Auchus, 2011) meaning that overexpression of*HSD17B2* will reduce testosterone and instead increase androstenedione concentrations in brain areas that include the social behaviour network. Furthermore, a single residue change in the HSD17B2 enzyme of inversion haplotypes coincides with a site that increases the catalytic rate of testosterone to androstenedione conversion up to 5-fold in the human ortholog (Jasmine L Loveland et al., 2022; Sager et al., 2021). The additive effects of increased expression and greater catalytic rate likely contribute to the elevated androstenedione levels of morphs with inversion haplotypes.

A previous study that evaluated relative gene expression in adult testis between morphs found no differences in *HSD17B2* expression (J.L. Loveland, Lank, et al., 2021). Different hypotheses could explain these contrasting results. First, different tissues and different life-stages were analyzed. Often, gene expression is tissue-specific and further depends on the developmental stage. The brain, in particular, requires a precise hormonal regulation (Bentz, Thomas, Rusch, & Rosvall, 2019; Khaitovich, Enard, Lachmann, & Pääbo, 2006) that could explain the observed morph-specific expression in neural tissue. Alternatively, variation in circulating androgen concentrations between morphs may mask genetically determined variation in *HSD17B2* expression.*HSD17B2* is expected to be expressed when testosterone needs to be converted to its less potent form, androstenedione, for example, when testosterone levels are high. In ruffs, high levels of testosterone are associated with Independents, but not with the inversion morphs, who instead have high androstenedione levels [32,53, this study].*HSD17B2* expression may not only depend on the genetically determined regulation, but also on the availability of the substrate (testosterone) and the requirement of the catalytic product (androstenedione). A lack of expression differences in *HSD17B2* in some tissues may simply be the result of balancing effects of different processes such as gene regulation and substrate availability/product requirement.

Intriguingly, the observed differences in androgen metabolism during early ontogeny might be involved in organizing morph-specific behaviour. Analogous to the proximate mechanisms of sexual differentiation (Adkins-Regan, 2005; Arnold, 2004, 2020; De Vries & Simerly, 2002; McCarthy & Arnold, 2011), the inversion-derived alleles may lead to morph differences in brain cells and affect circulating androgen concentrations during early stages of ontogeny. Our results suggest that*HSD17B2* , which is located in the inversion region, has a key role in mediating variation in androgen metabolism between morphs and may also affect estrogen metabolism either via aromatization of testosterone or because the enzymatic product of *HSD17B2* catalyzes also estradiol to estrone (Baker, 2001; Miller & Auchus, 2011). Interestingly, morphs but not sexes, differed in androgen metabolism. Therefore, variation in testosterone synthesis during ontogeny is likely to affect brain organization of morph-specific behaviour in both sexes. This is consistent with previous manipulation studies in ruffs. In adult females, exogenous testosterone activates typical morph-specific male display behaviour (Lank, Coupe, & Wynne-Edwards, 1999). However to link steroid variation among morphs to brain organization, further studies would need to evaluate how the observed differences in androgen metabolism influence the development of brain function and how they interact with the effects of sexual differentiation.

The consistent hormonal differences between morphs may have organizational effects underlying the profound differences in social behaviours between morphs. Variations in circulating androgen concentrations as well as in relative gene expression of *HSD17B2*were pronounced between Independent vs the two derived morphs, but absent between Satellites and Faeders. Similarly, aggression varies strikingly between Independents and derived morphs. Lekking Independents aggressively defend small courtship territories, whereas Satellites and Faeders lack this behavior (Hogan-Warburg, 1966; Jukema & Piersma, 2006; Widemo, 1998). Consistent with aggression being organized during development, exogenous androgens increase aggression levels only in Independents, but not in Satellites (Morgan, 2010). The experience of elevated testosterone concentrations in circulation or directly in the brain during ontogeny seems to be important to maintain testosterone sensitivity required for territorial aggression in the breeding context. Interestingly, some bird species that do not increase testosterone concentrations in response to simulated territorial intrusions are insensitive to testosterone implants (Lynn, 2008). Hence, the suggested link between the apparent inability of the two derived morphs Satellites and Faeders to raise testosterone, and their reported testosterone insensitivity, could point towards a more general mechanism among birds in the organization of aggression.

Supergenes underlying variation in reproductive phenotypes occasionally capture genes, which encode proteins involved in sex steroid metabolism (Maney & Küpper, 2022). This might not be a coincidence. Steroid related genes could be considered as "hotspots" for major evolutionary transitions of this kind for following reasons. Sexual selection is a main driver in the evolution of alternative reproductive tactics (Shuster & Wade, 2003) and the expression of traits targeted by sexual selection is often mediated by sex steroids (Adkins-Regan, 2005). For example, aggressive behaviour, the expression of secondary sex characters or muscle hypertrophy often increase with an increase in testosterone concentration (Hau, 2007). Hence, it seems plausible that genetic variation underlying different hormonal profiles within sexes is a key component in the evolution of distinct reproductive phenotypes within species. Divergent co-evolution of such distinct traits can be facilitated by hormonal pleiotropy (Cox, 2020; Cox, Hale, Wittman, Robinson, & Cox, 2022; Wittman et al., 2021) and by the fact that sex steroids mediate the investment into life-history trade-offs (Hau, 2007; Hau & Wingfield, 2011). Genetic and physiological regulatory axes are functionally linked to steroids (Cox, McGlothlin, & Bonier, 2016; Hau & Wingfield, 2011), thus chromosomal rearrangements that capture steroid-related genes can exert large pleiotropic effects while still maintaining phenotypic integrity.

We conclude that in ruffs inversion-derived alleles affected circulating steroid concentrations and steroid-related gene expression in the social behaviour network of the brain early during ontogeny. Variance and skewness, but not mean sex steroid concentrations differed between morphs, who had exhibited similar differences as reported from adult males. The candidate gene *HSD17B2* that is located in the inversion region, showed differential expression in embryonic brains. These differences in steroid metabolism may play an important role in mediating morph-specific developmental trajectories. Overall, our study suggests that supergenes may shape phenotypes of alternative reproductive tactics through similar mechanisms as sex chromosomes shape sexual differentiation.

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Data Accessibility Statement

All raw data and all R scripts necessary to reproduce the reported results are available in Edmond the Open Research Data Repository of the Max Planck Society, https://doi.org/10.17617/3.ZUZMB3/v1 (Giraldo-Deck, Loveland, Goymann, Lank, & Küpper, 2022).

Benefit-Sharing Statement

Not applicable. All data were taken from a captive population. This population was established 1985, thus before the Nagoya Protocol. All stakeholders involved in the study are included as authors.

Author Contributions

LMGD, JLL, DBL, and CK designed and conceived the experiments, LMGD and JLL collected the samples, WG supervised hormone measurements, JLL supervised gene expression measurements, LMGD analyzed the data and LMGD wrote the manuscript with input from CK. All authors gave their final approval for publication.

Tables and Figures

Table 1. Variance analyses of plasma testosterone, androstenedione and progesterone concentrations for each morph. Means and 95% CrIs of the random variance and the variances explained by differences among individuals or among sampling years. For testosterone, the probabilities of Independents having a higher random variance than Satellites or Faeders were higher than 0.99. Conversely, for androstenedione, the probabilities of Independents having a higher random variance than Satellites or Faeders were lower than 0.01. For progesterone, the probabilities of Independents having a higher random variance than Satellites or Faeders were lower than 0.40 and 0.16 respectively. The variances explained by ID or sampling year did not differ significantly among morphs in any of the hormones.

Hormone	Morph	Variance	Variance	Variance
		Random	**ID**	**Year**
Testosterone	Independent	0.745 (0.613; 0.938)	0.153 (0.102; 0.218)	0.335 (0.054; 1.794)
	Satellite	0.283 (0.224; 0.374)	0.028 (0.017; 0.044)	0.177 (0.028; 1.002)
	Faeder	0.179 (0.130; 0.274)	0.070 (0.035; 0.126)	0.109 (0.014; 0.533)
Androstenedione	Independent	0.159 (0.129; 0.204)	0.008 (0.005; 0.012)	0.040 (0.006; 0.226)
	Satellite	0.584 (0.451; 0.803)	0.036 (0.020; 0.057)	0.108 (0.014; 0.608)
	Faeder	0.601 (0.423; 0.977)	0.014 (0.004; 0.028)	0.067 (0.005; 0.384)
Progesterone	Independent	0.184 (0.149; 0.238)	0.029 (0.019; 0.043)	0.047 (0.007; 0.243)
	Satellite	0.192 (0.149; 0.264)	0.012 (0.007; 0.019)	0.058 (0.008; 0.337)
	Faeder	0.226 (0.161; 0.358)	0.046 (0.023; 0.120)	0.026 (0.002; 0.144)

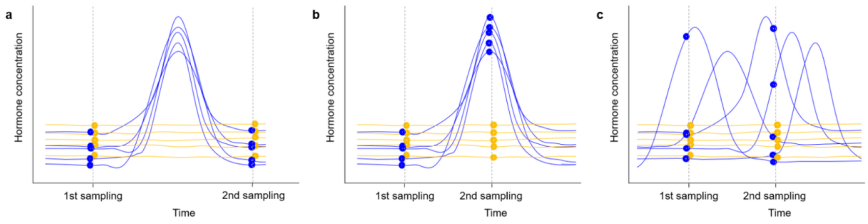

Fig. 1. How sampling time and individual variation may shape observed variation in hormonal titers between groups. Curves represent within individual changes in circulating hormone concentrations. Circles represent concentration at sampling time. Colours (blue and orange) represent different groups, e.g. sexes or morphs and for simplicity, we show only two sampling events. **a** Variation in hormone levels occurs in-between sampling events, meaning that existing differences between groups will pass unnoticed. **b** When sampling coincides with group-specific peaks, mean concentrations will differ between groups. **c** Between individual variation in timing of the peaks will increase observed variation in variance and skewness between groups.

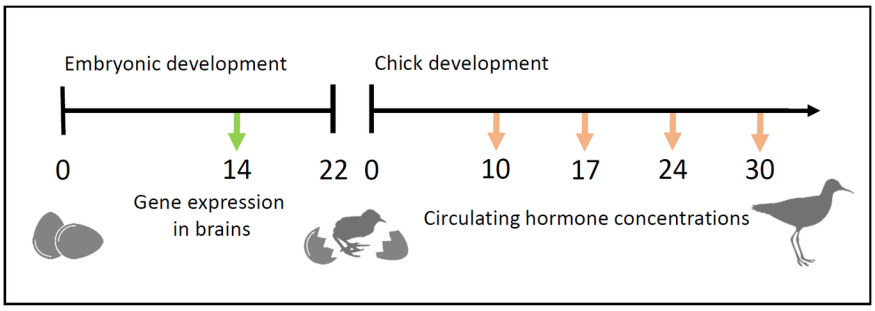

Fig. 2. Timeline of data collection . We evaluated gene expression of seven candidate genes in brains of 14-day-old embryos (green arrow). Circulating steroid concentrations were evaluated at days 10, 17, 24 and 30 after hatching (orange arrows).

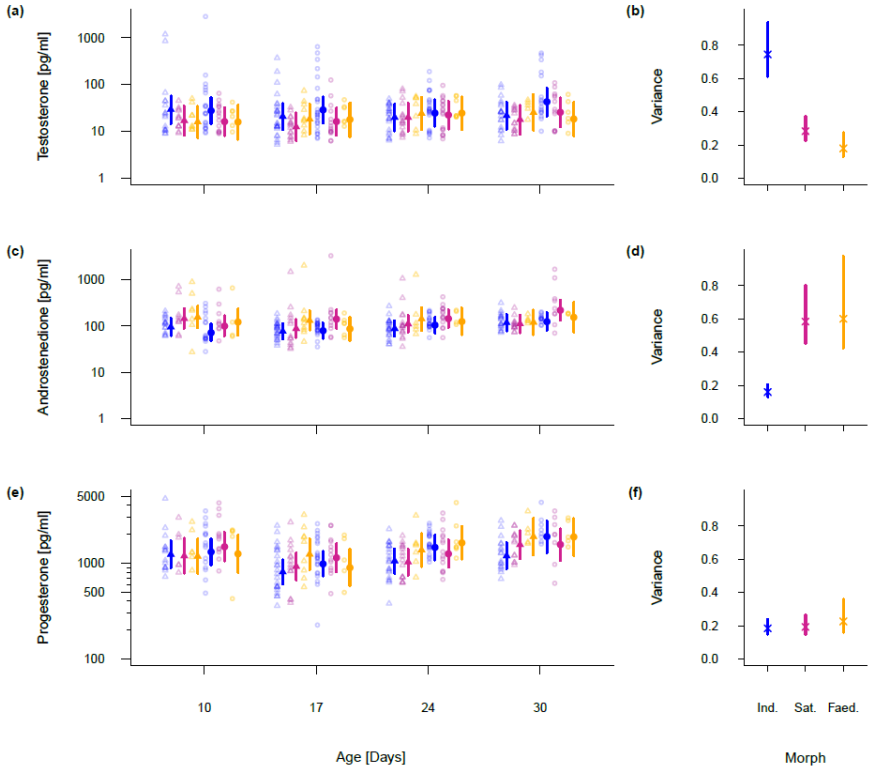

Fig. 3. Plasma steroid concentrations in ruff juveniles. **a** Mean testosterone concentrations (means: filled symbols, lines: 95%CrI) were similar between sexes (males: triangles, females: circles), morphs (Independents: blue, Satellites: purple, Faeders: orange) and age groups, **b** but variance was greater in Independents compared to Satellites or Faeders. **c** Mean androstenedione concentrations were similar between sexes, morphs and age groups, **d** but variance was higher in derived morphs compared to Independents**c** Mean progesterone concentrations were similar between sexes, morphs and age groups and **d** variances were also similar between morphs. All models controlled statistically for individual ID and sampling year. Full model details are given in Table 1 and Supplementary Table S2.

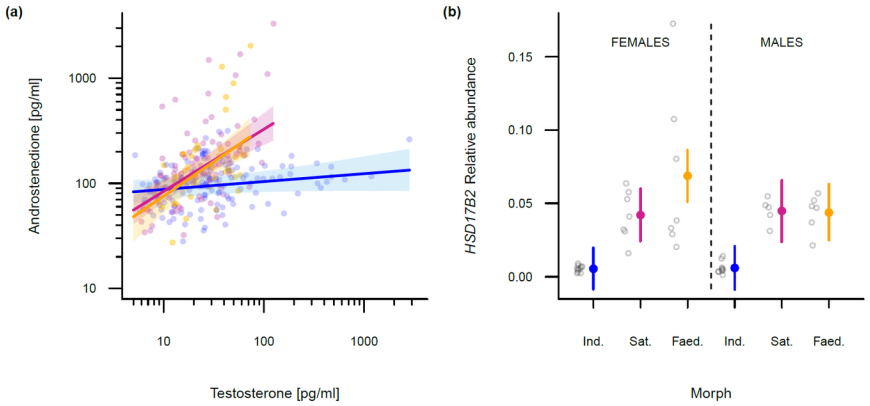

Fig. 4. Variation in androgen regulation between morphs. **(a)**Relationship between circulating testosterone and androstenedione concentrations in juveniles. In Independents (blue), testosterone and androstenedione concentrations were not correlated, whereas in Satellites (purple) and Faeders (orange) androstenedione increased with increasing testosterone concentrations. **(b)** Relative gene expression of neural *HSD17B2* (means and 95%CrI) in male and female ruff embryos separated by morphs. In both sexes, the two derived morphs (Satellites, purple; Faeders, orange) have higher expression of*HSD17B2* compared to Independents (blue). Full model details are given in Supplementary Table S4.