Supergenes on Steroids

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Abstract

At the birth of supergenes, the genomic landscape is dramatically re-organized leading to pronounced differences in phenotypes and increased intrasexual diversity. Two of the best-studied supergenes in vertebrates are arguably the inversion polymorphisms on chromosomes 2 and 11 in the white-throated sparrow (*Zonotrichia albicollis*) and the ruff (*Calidris pugnax*), respectively. In both species, regions of suppressed recombination determine plumage coloration and social behavioral phenotypes. Despite the apparent lack of gene overlap between these two supergenes, in both cases the alternative phenotypes seem to be driven largely by alterations in steroid hormone pathways. Here, we explore the interplay between genomic architecture and steroid-related genes. Due to the highly pleiotropic effects of such genes and their universal involvement in social behavior and genomic architecture, forces favouring their linkage are likely to have substantial effects on the evolution of behavioral phenotypes, individual fitness, and life history strategies. We propose that the differentiation of steroid-related genes, inside both supergenes, lies at the core of phenotypic differentiation in both of these interesting species.

Keywords: Aggression, alternative reproductive tactic; androgen; estrogen; inversion polymorphism; life history strategy

1. **Steroid hormones and the evolution of vertebrate social behavior**

1.1 Steroid hormone pathways: Ubiquitous and pleiotropic

Any consideration of the mechanisms underlying social behavior, in any vertebrate, is likely to prominently feature the steroid hormones testosterone and estradiol.[*] As an animal enters a state of reproductive readiness, either at puberty or at the onset of a breeding season, these hormones are secreted by the gonads and promote the production of gametes. The hormones enter the bloodstream and thus are available to any tissue that needs to provide a supporting role to reproductive effort. Sensitivity to these hormones, for example via expression of steroid receptors, allows such tissues to coordinate their activity with gametogenesis [2, 3]. For example, well-timed and well-positioned steroid receptor expression in the brain can ensure that mate seeking, receptivity, and territorial defense occur at the appropriate time, when gametes are available to be released. Steroid hormones can therefore play a coordinating role in reproduction, promoting gametogenesis while at the same time promoting many of the behaviors and other signals that ensure that a mate is available and that gametes will meet each other [3, 4].

Because the suites of behaviors that promote reproduction are heterogeneous across species, the effects of steroid hormones on behaviors must, by necessity, be similarly diverse. These effects are subject to diverse selection pressures, which produce a variety of solutions to the problem of coordinating reproductive behavior with gamete production. Because they are steroids, not proteins, steroid hormones themselves are not encoded in the genome; however a multitude of proteins, encoded by genes, participate in the relevant pathways. These proteins can take the form of steroid receptors, as noted above, or synthetic or metabolic enzymes that can increase or decrease availability of a particular steroid or convert that steroid into a more or less active form. Variation in the sequence of any of these genes can affect not only the specificity and functionality of an enzyme or a receptor, but how much of it is expressed and in which tissues.

Through selection on these genes, evolution creates immense diversity in the mechanisms by which steroids control reproductive behavior. Thus, although proceptivity, receptivity, and territoriality are fairly universally influenced by steroid hormones across vertebrates, the details regarding how and when they do so vary widely from species to species. It is this rich diversity that invites investigation into the ultimate and proximate sources of variation in how steroid hormones coordinate reproduction, and in particular how genetic variation in the genes within steroid pathways contributes to behavioral diversity. Below, we explore how genomic architecture interacts with the biology of steroid hormones to promote diversity of reproductive strategies in two very special species of birds.

1.2 Steroid hormones and territoriality

[*] Estradiol and testosterone have traditionally been called “sex steroids” or “gonadal steroids” to distinguish them from adrenal hormones such as cortisol, which are associated more with stress than with social behaviors. Both of these labels are now outdated, however. “Sex steroids” is a misnomer because it is not the case that either hormone is more important in one sex than another, or that their functions are limited to sexual reproduction. “Gonadal steroids” is similarly misleading because these hormones can be synthesized *de novo* by non-gonadal tissues, namely the brain [1]. In this review, we will use “steroid hormones” with the caveat that we mean androgens and estrogens, not necessarily glucocorticoids.

One of the behaviors associated with reproduction, yet not directly related to the production of gametes or to copulatory behavior, is the defense of breeding territories. In birds, territoriality assumes many forms. Members of some species defend large territories of several hectares while others defend only a few square meters. In migratory populations of New World sparrows, males arrive before the females, giving themselves time to compete for and establish territories containing the best possible resources. When the females arrive, they choose mates according to the quality of those resources. Once a female has chosen a mate, she helps defend the territory. In contrast, in lekking species such as grouse, manakins, and some shorebirds, males defend a small area devoid of resources, using their territories only for the purpose of displaying to females [5]. After mating, the female leaves the territory to rear the young herself. In both types of territoriality, defending territories coincides largely with the breeding season and is clearly linked to mate attraction and reproductive effort.

In territorial songbirds, the seasonal peak of territorial aggression generally coincides with peak levels of plasma testosterone. In song sparrows (*Melospiza melodia*), plasma testosterone reaches its seasonal high when males are initially establishing territories and slowly declines until the females are incubating fertile eggs, when territory disputes are rare [6]. In lekking species such as black grouse (*Tetrao tetrix*), sharp-tailed grouse (*Pedioecetes phasianellus*), and greater prairie chicken (*Tympanuchus cupido*), plasma testosterone is high in males exhibiting overt aggression during the establishment of territories within leks. Treating these males with exogenous testosterone does not, however, alter the level of ritualized aggression or the size or location of a male's lek once it is established [7-9]. Thus, testosterone seems to be important only during periods of social instability, when interactions with rivals may be unpredictable. Notably, in many territorial birds, treatment with testosterone can result in increased territorial defense [6, 10-13], suggesting that the suite of behaviors is under the control of steroid hormones.

Although steroid hormones exhibit causal effects on territorial behavior, it is also clear that this hormone-behavior relationship is bidirectional [14]. Male song sparrows treated with testosterone responded to a simulated territorial intrusion (STI) with more aggression than did control males and were able to defend much larger territories [13]. Testosterone levels also rose, however, in the untreated neighboring males, suggesting that simply being a neighbor of a more aggressive male can increase testosterone. These results show not only that testosterone increases aggression, but also that engaging in aggressive interactions increases testosterone. The effect on testosterone is rapid; increases are detectable within minutes of a territorial challenge [15, 16] and can remain elevated for days. This rise in testosterone has been hypothesized to heighten vigilance in anticipation of a sustained challenge [17]; however, the function of challenge-induced testosterone secretion in birds remains unclear [18]. In mice, challenge-induced testosterone pulses have been shown to alter social decision-making, social vigilance, and the probability of winning future encounters [19]. These transient increases are rewarding and reinforcing, suggesting that a challenge-induced increase in testosterone may increase the probability of subsequent aggression because responding to the challenge was reinforcing.

1.3 Steroid hormones, life history strategies, and alternative phenotypes

Territorial defense, particularly the defense of breeding territories, is part of a collection of related behaviors that characterize a "life history strategy" prioritizing short-term gains over longer-term investments [20, 21]. Short-term mating relationships, high intrasexual aggression, and low parental behavior can characterize this strategy, particularly in males (McGlothlin et al., 2007). In birds, the trade-off between fighting off rivals and parental care has been hypothesized

to be mediated by testosterone [22, 23]. In free-living male dark-eyed juncos (*Junco hyemalis*), treatment with testosterone increased song rate [24] as well as the size of the home range [25], suggesting increased territoriality and mate-finding. At the same time, the testosterone treatment decreased provisioning trips to feed offspring [24]. Although these findings do not generalize to all species [26], they illustrate an important concept. If two suites of behaviors, such as territoriality and parental care, require incompatible endocrine states, such as high and low testosterone, then these behaviors would be unlikely to occur simultaneously in the same individual—that is, assuming that the behaviors are not easily uncoupled from their respective hormonal mechanisms [18, 22, 27, 28].

When two different life history strategies rely on incompatible underlying mechanisms, also called antagonistic pleiotropy, selection favours individuals that express either one strategy or the other [29, 30]. Differentiation with respect to life history trade-offs is often sex-typic; in mammals, high levels of parental behavior are regarded as more typical of females, whereas territorial aggression is more typical of males (although this is not always the case; see [11]). In mammals, the genes ultimately responsible for sexual differentiation are located on the Y sex chromosome, on which recombination is profoundly suppressed and genes responsible for male-typical development are inherited together with other genes that are more advantageous in males than in females. Thus, the XY system of sex chromosomes in mammals represents an extreme case of genotypic divergence underlying phenotypic divergence in life history strategy, with steroid hormones at the core of this differentiation.

Distinct sexes are just one example of alternative phenotypes driven by antagonistic pleiotropy. Phenotypic differentiation can become dissociated from sex, occasionally leading to the emergence of within-sex “morphs” with strikingly different reproductive strategies [31]. The differences in reproductive behavior between the alternative phenotypes are frequently mediated by differences in steroid hormones. For example, in species with both territorial and non-territorial males, the territorial males typically have higher levels of the most potent androgen (i.e., testosterone in mammals/birds, ketotestosterone in fish), whereas ‘sneaker’ males have lower levels of this hormone but higher levels of other androgens [31]. The hormonal differences are paralleled by differences in gonadal investment: sneaker males have larger gonads relative to their body size than territorial males as they invest more into sperm competition than resource control [32]. Thus, the evolution of these alternative strategies is likely linked to differentiation of genes that govern steroid pathways. Below, we explore the genomic mechanisms leading to the most well-understood examples of genetically-based alternative phenotypes and how, in some cases, those mechanisms have affected behavior by altering steroid-related genes.

2. Supergenes

2.1 Adaptive consequences of chromosomal inversions

Alternative phenotypes that are fixed for the life of an individual typically have a genetic basis. For example, some of the best-known examples of behavioral polymorphism are linked to chromosomal rearrangements such as inversions [33]. Dobzhansky [34] hypothesized that inversions confer a selective advantage when they link alleles that function well together. That is, individuals with a particular allele of one gene might benefit from also having particular allele of another. Imagine a hypothetical example: a hormone with two isoforms A and B, each of which interacts optimally with the corresponding receptor isoforms A and B, respectively. Having the A allele of both hormone and receptor would be more advantageous than a mismatched set. Binding these co-adapted alleles together ensures that they stay together, benefiting both the

individual and the allele. The necessary tight link can be achieved by suppressed recombination between different haplotypes of an inversion region (Fig. 1A). The inversion results in disruption of crossing over as recombinant chromosomes of inverted and non-inverted haplotype are frequently inviable. Thus, the combinations of alleles from different genes inside the inversion are locked in together and co-inherited as a 'supergene', which can lead to the emergence of new phenotypes [reviewed by 35, 36]).

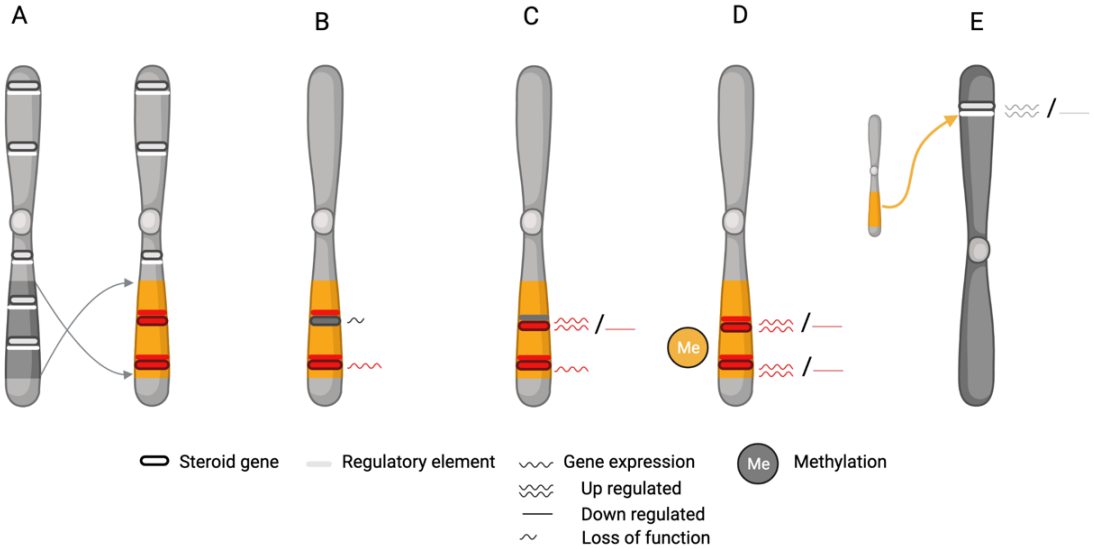

Figure 1. Evolutionary consequences of supergene variation on steroid-related genes. (A) A chromosomal inversion captures co-adapted alleles (red) of steroid-related genes in a new supergene variant (orange). The genes in this inversion haplotype will then evolve together because of the inhibition of recombination. As steroid-related genes are typically highly pleiotropic, changes to their regulation may have particularly strong effects on phenotypic variation. The resulting new phenotype has initially higher than average fitness and will rise in frequency within a population. (B) Degeneration of coding sequences in steroid-related genes leads to loss-of-function mutations. Occasionally, such mutations can be adaptive. (C) Mutations in regulatory elements cause changes in expression at specific steroid-related genes. (D) Changes in patterns of methylation may lead to widespread changes in the expression of steroid-related genes within the supergene variant. (E) Mutations within the supergene variant may affect not only genes within the supergene but also the regulation of other steroid-related genes located outside of the inversion.

2.2 Supergenes and antagonistic pleiotropy

Supergenes themselves evolve over time as they advance through their life cycle [37, 38]; their DNA sequences evolve faster than recombining chromosomal regions. The lack of recombination leads to the accumulation of deleterious mutations that are often recessive. These mutations include transposable elements, frameshift or nonsense mutations. When the inversion haplotype is rare, there are few homokaryotypes to expose these mutations to purifying selection. As a result, the inversion haplotype will accumulate further mutational load resulting in lower fitness [39-41]. Ultimately, the resulting fitness loss stemming from this degeneration will limit the spread of an inversion haplotype [37]. Instead, inversion haplotypes will either disappear or remain as a balanced polymorphism within a population.

The degeneration of inversion haplotypes alters the costs and benefits for their bearers. In addition, the costs and benefits of supergene variants may also vary across sexes or life stages [41-43]. In these cases, the inversion haplotype may impact fitness components such as survival and fertility differentially through antagonistic pleiotropy. As noted above in Section 1.3, genes with antagonistic pleiotropic effects have alleles that are beneficial for one fitness component but are detrimental for another. The concept was originally developed to explain

senescence observed late in life, for example a gene may impact fitness positively early in life but negatively later in life [44]. The idea is also relevant to many genetic traits underlying life history trade-offs. One prominent example of antagonistic pleiotropy is sexually antagonistic effects. Males and females largely share their genome but may have different optima for phenotypic traits such as reproductive strategies. Thus, some alleles may be beneficial for one sex but detrimental for the other, creating sexual conflict over their expression. By accumulating antagonistic genes on sex chromosomes, the sexual conflict can be reduced as the expression of antagonistic alleles becomes sex-biased [45].

Similar to the sexes, alternative reproductive phenotypes also differ in the phenotypic optima. Consequently, supergene variants that segregate with such phenotypes have been hypothesized to reduce genomic conflicts [46] and result in antagonistic pleiotropy. Antagonistic pleiotropy has been demonstrated to operate on inversion polymorphisms in yeast. Experimentally induced inversion haplotypes were favoured during periods of asexual reproduction, whereas the ancestral strains lacking the chromosomal rearrangements had higher fitness under sexual reproduction. In yellow monkey flowers and seaweed flies, inversion polymorphisms are maintained by differences in viability and fecundity between haplotypes [42, 47]. Intriguingly, genes in steroid pathways themselves have many well-described antagonistic pleiotropic effects [22, 48]. This means that inversions that capture one or several steroid-related genes may provide prime examples for the evolution of distinct alternative phenotypes.

Below, we summarize the research on two colorful avian examples of pronounced intraspecific phenotypic diversity: the white-throated sparrow (*Zonotrichia albicollis*) and the ruff (*Calidris pugnax)*. In both species, supergenes provide a potential genomic substrate for variation in steroid regulation and reproductive behavior.

3. The white-throated sparrow

3.1 The bird with four sexes

White-throated sparrows are territorial, socially monogamous songbirds found throughout much of North America [49]. Within any population, about half of the birds have black and white stripes on the crown and a white throat, whereas rest have tan and brown stripes and a streaked throat (Fig. 2A). These plumage morphs, called ‘‘white-striped’’ (WS) and ‘‘tan- striped’’ (TS), are fixed for life; individuals of this species cannot switch to the other color pattern. Almost all breeding pairs consist of one TS and one WS bird, earning this species the nickname “the bird with four sexes” [50]. Together with the plumage morphs, this disassortative mating system makes them unique among songbirds.

The morphs of this species are particularly interesting to behavioral biologists because they differ with respect to territorial and parenting behaviors. WS birds of both sexes engage in more territorial singing and other aggression than do the TS birds (Fig. 2B; [49, 51]). The morphs differ also in the rate at which they provision nestlings; TS birds make more trips to the nest to feed their young than do WS birds [51-53]. Thus, the strategies employed by the two morphs remind us of the aggressive versus parental phenotypes predicted by life history trade-offs [21]. The fact that these sex-typic, steroid-dependent behaviors have become dissociated from sex and sex chromosomes makes this species an exciting model for understanding the hormonal and evolutionary mechanisms underlying life history strategies.

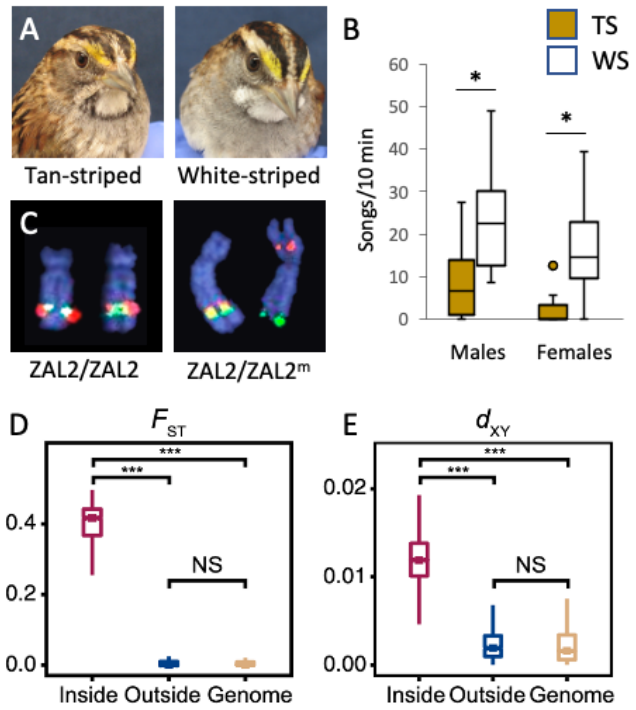

Figure 2. Polymorphism in white-throated sparrows. (A) Males and females occur in two plumage morphs, tan-striped (TS) and white-striped (WS). (B) WS birds of both sexes respond to simulated territorial intrusions with more vocal aggression (song rate is plotted here) than TS birds. (C) Fluorescent *in situ* hybridization shows two zebra finch BAC clones (red and green) both mapping to the long arm of ZAL2, but because of a series of inversions, they map to opposite arms of ZAL2^m. (D) Fixation index (F_{ST}) indicates a high degree of population differentiation (suppression of recombination) inside the rearrangement. (E) Pairwise nucleotide divergence (d_{xy}) indicates significant genetic differentiation within the rearrangement compared with the rest of the genome. (A) and (C) are from [59]; (B) is from [91]; (D) and (E) are from [57].

3.2 The ZAL2/ZAL2^m chromosomal system

The plumage polymorphism in white-throated sparrows has a genetic basis originally discovered by Thorneycroft [54, 55]. TS birds have two copies of a submetacentric version of chromosome 2, whereas WS birds have at least one copy of a rearranged metacentric homolog (Fig. 2C). Thus, the WS phenotype is inherited as a dominant trait linked to the metacentric version of the chromosome. Decades after this original discovery, Thomas et al. [56] showed that the metacentric arrangement, now known as ZAL2^m, contains at least two inversions relative to the submetacentric version, ZAL2. With more than a thousand genes, the rearranged region represents one of the largest known inversion polymorphisms [56, 57]).

Almost all white-throated sparrows with a copy of ZAL2^m are heterozygous; that is, WS birds have one copy of ZAL2^m and one copy of ZAL2. Homozygotes for the ZAL2^m chromosome can arise only from WS-WS matings, which are extremely rare because of the disassortative mating system. Given the known prevalence of WS-WS pairings, and genotyping data from thousands of wild specimens, ZAL2^m/ZAL2^m homozygotes occur at or perhaps even below the expected frequency (1/500 birds; [58, 59]). The low frequency of ZAL2^m/ZAL2^m homozygotes renders the ZAL2^m chromosome unable to recombine with itself, due to its near-constant state of heterozygosity. As a result, mutations are accumulating on ZAL2^m independently of ZAL2, and the two haplotypes have diverged (Fig. 2D) by 1-2% [57, 60].

Despite the suppression of recombination, ZAL2^m does not show strong signatures of degeneration. Recent analyses have revealed only minor increases in nonsynonymous polymorphisms [58] and low levels of markers of degeneration, such as repetitive sequences and pseudogenization [57, 61]. Although the level of degeneration of ZAL2^m is minor, the chromosome is differentiating (Fig. 2E). This differentiation is affecting behavior, as evidenced by the well-documented morph differences in singing, other forms of territorial aggression, and parental provisioning [49, 51]. The only behaviorally characterized ZAL2^m homozygote was

extremely aggressive and sang at an unusually high rate [59], suggesting that alleles on ZAL2^m may affect aggressive behavior in a dosage-dependent manner.

4. The ruff

4. 1 Fighting Independents, flashy Satellites and sneaky Faeders

Ruffs are renowned for their spectacular lekking behaviour, which is an expression of intense male-male competition. The previous scientific name, *Philomachus pugnax* ('combative battle lover'), reflects more adequately the nature of the male contests and the aggressive courtship involved. On leks, individual Residents vigorously defend small courts against other competitors. Aggression helps individual Residents to climb to the top of the dominance hierarchy [62] with copulation success positively related to dominance and endurance [62-65].

Not all males compete through aggression, however. Residents are competitive members of the Independent morph, a morph that accounts for only about 85% of the ruff population. Independent males are large and feature dark plumage and colored feathery ornaments such as peculiar-looking ruffs and head tufts (Fig. 3A). The males of two other morphs, Satellites and Faeders, engage in alternative reproductive tactics (ARTs). Faeders and Satellites exploit the elaborate courtship of Residents for opportunistic matings. Satellites are slightly smaller than Independents and have paler plumage ornaments [66]. Satellite males use a semi-cooperative strategy, in which they choose a Resident partner on the lek for co-display on the Resident's court. The co-displaying unit is very successful in obtaining copulations [66, 67]. Satellite males do not engage in antagonistic interactions with Residents; instead, they secure matings by efficiently exploiting Residents distracted by conflicts with neighbours [67]. Faeder males are smaller and rarer than males of the other morphs and resemble females in appearance and behavior. They can thus sneak copulations without elaborate courtship.

4.2 An autosomal inversion underlies discrete phenotypes

Remarkably, the three ruff morphs are fully genetically determined and encoded by variants of an inversion supergene located on chromosome 11 [68, 69]. Satellites and Faeders carry distinct inversion haplotypes, whereas Independents are homozygous for the ancestral arrangement (Fig. 3A). The initial inversion event gave rise to the Faeder morph about 4 million years ago [69]. A few million generations after the initial inversion event, a rare recombination event involving a double cross-over between an ancestral and inversion haplotype created the Satellite haplotype and morph. Such recombination events required the formation of an inversion loop that led to a double crossover, a rare phenomenon since the chromatin pairing of the inverted and non-inverted segment is severely hampered [70, 71]. Some regions of this haplotype are broadly similar to the Faeder haplotype, whereas others are more similar to the ancestral non-inverted sequence characteristic of the Independents. Thus, Satellites are true hybrids between Independent and Faeder morphs, albeit both inversion haplotypes share the same breakpoints [68, 69]. One of these breakpoints disrupted the CENPN gene, whose product is essential for centromere assembly during mitosis [72]. As a result of the gene disruption, the inversion haplotypes are homozygous lethal meaning that all Faeders and Satellites are heterokaryotes who require an ancestral allele for survival [68].

Because the Satellite and Faeder haplotypes cannot recombine with each other, they have differentiated and show signs of advancing degeneration. The differentiation is apparent through strong nucleotide divergence and high FST values between haplotypes [68, 69]. A large number of deletions, insertions and duplications of segments and missense mutations within the

inversion region on both the Satellite and Faeder haplotypes point towards gradual erosion of the Faeder and Satellite variants [68, 69]. Intriguingly, this degeneration may actually have had positive fitness implications for the mating success of those males. The functional erosion of some gene sequences seemingly led to an adaptive loss of aggressive and courtship behaviour although it has negatively impacted the reproductive fitness of Faeder females [43].

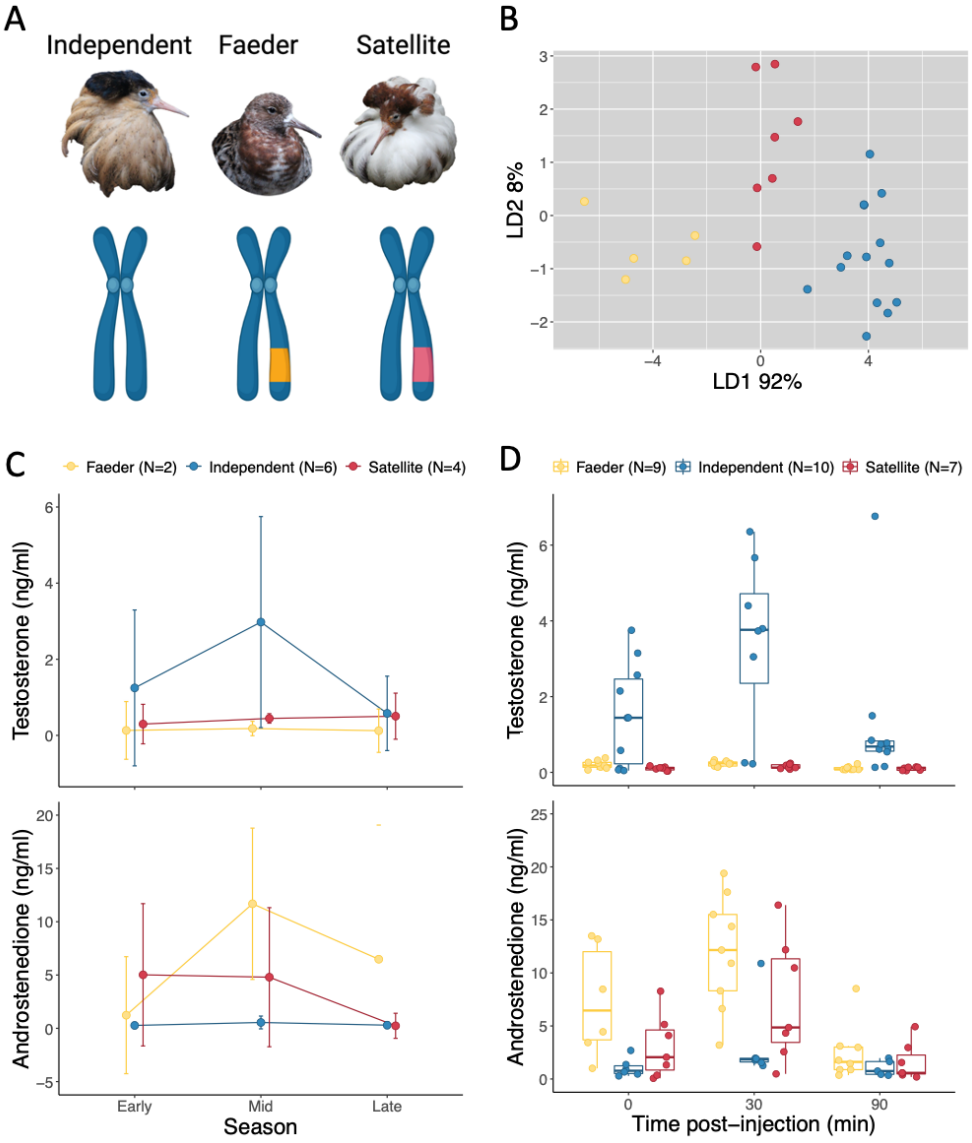

Figure 3. Supergene variants and steroid-related variation between morphs in ruffs. (A) A chromosomal inversion region determines three reproductive morphs that are most pronounced in males [68, 69]. Independents are homozygous for the ancestral arrangement. Faeders are heterozygous for a slowly degenerating inversion haplotype, whereas Satellites are heterozygous for a recombined inversion haplotype. (B) The three morphs can be distinguished on the basis of testicular expression profiles of 14 steroid-related genes, analyzed using a least discriminant (LD) analysis [73]. (C) Morph differences in circulating levels of testosterone (top) and androstenedione (bottom) in lekking males during the breeding season [68]. For better comparison, only data from males for which endocrinal data at all three time points were available were plotted. (D) A GnRH challenge elicits an increase in circulating testosterone levels in Independent males but not in males of the inversion morphs (top). Instead, GnRH injection leads to an increase in androstenedione in Faeders and Satellites (bottom) [85]. Photos of ruffs reprinted from [73].

5. Steroid-related genes as candidates underlying morph differences in behavior

5.1 White-throated sparrows and ruffs: different yet similar

Ruffs and white-throated sparrows offer a fascinating opportunity for understanding the mechanisms by which genetic variation contributes to behavioral variation. In both species an inversion region provides the only genetic differences between plumage morphs. The supergenes themselves share no evolutionary history, differ in type and size, and have captured different sets of genes (Table 1). Nonetheless, they show striking similarities at least on the surface. First, the autosomal inheritance means that both males and females carry inversion haplotypes. Second, in both species, homozygous inversion carriers are rare (white-throated sparrows) or absent (ruffs) meaning that for the inversion haplotypes, recombination is severely hindered. Third, for genes with clear differences in sequence, allelic imbalance in gene expression seems to be a common feature of genes inside these inversions [57, 73, 74]. Here, we have taken a close look at the genes inside each of these inversion polymorphisms to determine what other commonalities may exist at the genetic level. To test whether steroid-related genes are overrepresented in either supergene, we performed a gene ontology enrichment analysis using AmiGO version 2 [75]. For the baseline, we identified the steroid-related genes in chicken *Gallus gallus,* i.e., all genes identified with the keyword “steroid”, which provided a list of 418 (2.3%) of the 17851 annotated chicken genes. Out of the genes with known function in the two supergenes, two (2.1%) in the ruff supergene were steroid-related whereas for the supergene in the white-throated sparrow ZAL2 we found 22 (2.4%) steroid-related genes (Table 1). Thus, there is no support for an overrepresentation of the steroid-related genes in either of the supergenes (Fisher Test: P=0.98).

Table 1. Comparisons of genomic and evolutionary characteristics between two avian inversion supergenes.

	White-throated sparrow	**Ruff**
Location*	Chromosome 2	Chromosome 11
Size	100 Mbp	4.5 Mbp
Estimated age	2.2 MYA	3.9 MYA
Rearrangement type	Pericentric	Paracentric
Number of inversions	≥ 2	1
Number of distinct morphs/haplotypes	2	3
Number of annotated genes **~with known function**	1292 900	125 96
Steroid-related genes	22 TGFB2, ESRRG, PROX1, UFL1, APOB, CGA, BMP5, STRN, BMP2, LATS1, ESR1, LBR, C1D, HNRNPU, SMYD3, RWDD1, ARV1, LHCGR, SRD5A2, LBH, TCF21, FSHR	2 SDR42E1, HSD17B2
Fate of homokaryotes	Viable	Unviable
Suspected mechanism of balancing selection	Disassortative mating, antagonistic pleiotropy	Negative frequency dependent selection, antagonistic pleiotropy

*We follow the chromosome numbering of Thorneycroft [54] for the white-throated sparrow and Küpper et al. [68] for the ruff.

The lack of overrepresentation of steroid related genes within both avian supergenes does not mean that their importance within the inversion region can be discounted. On the contrary, because the behaviors that differ between the morphs in both ruffs and white-throated sparrows are known to be steroid-dependent, the steroid-related genes inside the rearrangement are strong candidates for mediating the behavioral phenotype. Alternatively, the supergenes may impact the regulation of steroid-related genes outside of the inversion. These scenarios are not mutually exclusive and previous studies have found support for both of them (Fig. 1). Below, we summarize insights from recent research on the importance of steroid-related genes in differences between morphs in ruffs and white-throated sparrows.

5.2 Steroid-related genes and behavioral polymorphism in white-throated sparrows

In white-throated sparrows, birds of the aggressive WS morph have higher breeding levels of plasma testosterone and estradiol than those of the less aggressive TS morph (Fig. 4A) [51, 76, 77]. In addition, plasma levels of these hormones positively predict aggressive responses to territorial intrusion [51]. On the surface, these findings may suggest a relatively simple explanation for morph differences in behavior. But when plasma levels of testosterone or estradiol were experimentally equalized in laboratory-housed birds (Fig. 4A), the morph differences in singing and other aggressive behaviors persisted (Fig. 4B) [78, 79]. Therefore, morph differences in these behaviors are not caused solely by morph differences in plasma levels of these steroid hormones. Instead, the behavioral polymorphism could be mediated by morph differences in steroid metabolism or sensitivity, particularly in brain regions relevant to social behavior. Two genes inside the ZAL2^{m} rearrangement are strong candidates for this task: *SRD5A2* and *ESR1*, which encode 5-alpha reductase and estrogen receptor alpha, respectively. Both genes are differentiating inside the inversion but neither has accumulated changes to the coding regions that would result in loss of function (Fig. 1B). Instead, variation in regulatory regions is expected to alter expression in a morph-dependent manner (Fig. 1C). In turn, these morph differences in expression could result in differential steroid action locally in the brain.

Five-alpha reductase irreversibly converts testosterone into the more active androgen dihydrotestosterone, making it unavailable for conversion to estradiol. Thus, differential expression of *SRD5A2* could cause differences in local levels of dihydrotestosterone or estradiol in the brain, affecting behavior in a morph-specific way. Grogan et al. [80] measured the expression of *SRD5A2* mRNA in a variety of behaviorally relevant brain regions, and found no compelling evidence that *SRD5A2* is differentially expressed. Also not differentially expressed were the genes for aromatase or androgen receptor, which are not located inside the rearrangement.

In contrast to *SRD5A2*, the gene *ESR1* is differentially expressed between the morphs in many areas of the brain [81]. This finding suggests that the morphs may differ in their sensitivity to estradiol. The magnitude of this difference in sensitivity depends on the brain region. In the nucleus taeniae of the amygdala (TnA), also known as the ventromedial arcopallium [82], WS birds have several fold higher expression than do TS birds (Fig. 4C) [80, 81]. This expression is tightly correlated with the rate of territorial singing [81]. The variation in expression likely results from differential regulation of expression of the two alleles. Such variation could be caused by divergence of *ESR1* cis-regulatory regions; indeed, differentiation of the 2kbp upstream of the *ESR1* start site has affected the binding sites of nearly 300 transcription factors [83] (Fig. 1C). In addition, this differentiation has affected the sites at which DNA is methylated, resulting in differential methylation of the two *ESR1* alleles (Fig. 1D) [83]. When expression of the ZAL2 and ZAL2^{m} alleles of *ESR1* were measured separately in TnA, it became clear that it is the ZAL2^{m}

allele of *ESR1*, not the ZAL2 allele, that predicts singing behavior with astonishing precision. The more the supergene allele is expressed, relative to the standard allele, the more aggressive the bird [83] (Fig. 4D). These results suggested the possibility that the morph difference in aggression in this species could be explained by the expression of this one steroid receptor in this one brain region.

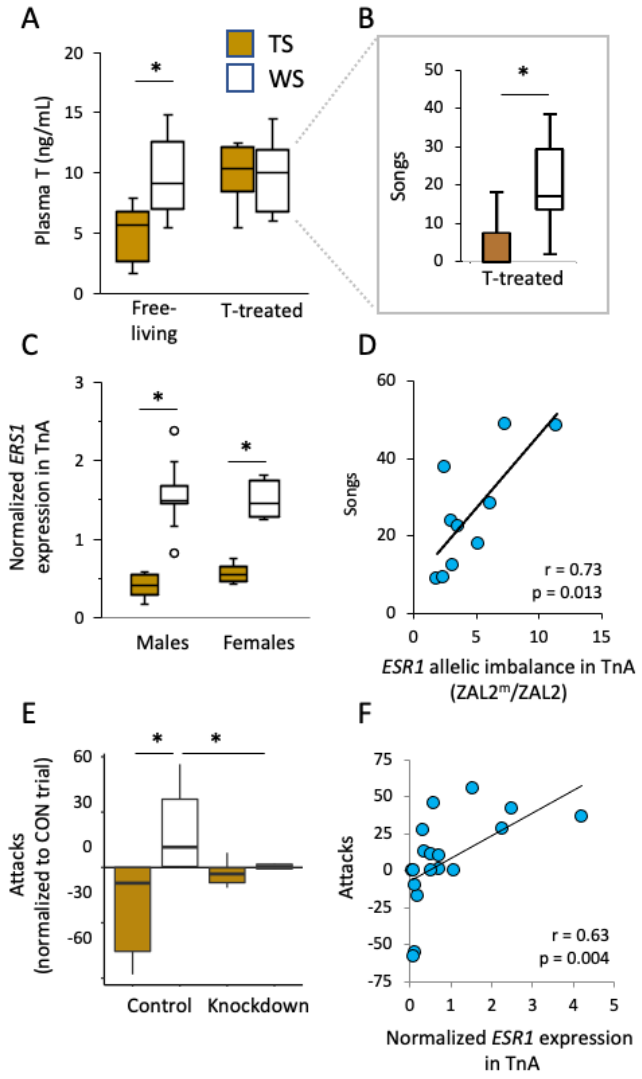

Figure 4. Testosterone and ESR1 in white-throated sparrows. (A) Free living birds of the WS morph have higher plasma T than birds of the TS morph (male data shown). In lab-housed birds with experimentally equalized plasma T (left portion of A), WS birds nonetheless sing more than TS birds (B). (C) The expression of *ESR1* is higher in nucleus taeniae of the amygdala (TnA; also known as the ventromedial arcopallium) in WS than TS birds. (D) Singing in response to simulated territorial intrusion is correlated with the extent to which the ZAL2^m allele of *ESR1* is overexpressed, relative to the ZAL2 allele, in TnA. (E) In lab-housed birds, estradiol-induced aggression is higher in WS than TS birds; that difference is eliminated in birds receiving *ESR1* knockdown in TnA. (F) The expression of *ESR1* is correlated with aggression in lab-housed birds. Data in (A) are from [51]and [78]; (B) is from [78]; (C) is redrawn from [81]; (D-F) are redrawn from [83].

To test whether expression of *ESR1* might be causal for the behavioral polymorphism, Merritt et al. [83] performed an experimental manipulation of *ESR1* expression in laboratory-housed birds. They asked whether knocking down *ESR1* expression in TnA could alter the degree to which exogenous estradiol increased aggression toward a subordinate conspecific. For birds receiving control treatment (no knockdown), estradiol facilitated aggression in the WS but not the TS birds (Fig. 4E). Thus, the WS birds were more sensitive than TS to the behavioral effects of estradiol (see also [79]). This morph difference disappeared, however, in the birds receiving *ESR1* knockdown. When *ESR1* expression was inhibited in TnA, both morphs behaved like TS birds (Fig. 4E), showing that the differential expression of ESR1 explains the morph difference in aggression. The behaviors that differed, including attacks directed toward the subordinate, were directly proportional to the expression of *ESR1* in TnA in animals without manipulated ESR1 expression (Fig. 4F). This series of studies represented the first causal evidence that a

particular gene inside a supergene, in this case *ESR1*, contributes to the differentiation of a behavioral phenotype associated with that supergene.

The downstream consequences of variation in *ESR1* expression are unlikely to be limited to rapid effects of estradiol on aggression. Because estrogen receptor alpha is a transcription factor, it regulates the expression of other genes across the genome (Fig. 1E). Of all the genes expressed in TnA, for example, the ones correlated with both plumage morph and territorial singing form a module enriched for genes in the estrogen receptor signaling pathway [84]. That is, the expression of *ESR1* predicts not only behavior but also the expression of a large number of other estrogen-responsive genes, suggesting a mechanism for widespread pleiotropy and the complex, steroid-dependent behavioral phenotypes that characterize the morphs in this species.

5.3 Steroid-related genes and behavior in ruffs

Ruffs show typical endocrinal variation of ARTs. Instead of maintaining high levels of testosterone, the two non-aggressive morphs, Satellites and Faeders, have high levels of androstenedione. Independents show the opposite relationship between these two androgens (Fig. 3C)[68, 85]. In addition, Satellites and Faeders invest in testes instead of territories; they have larger gonads than Independents relative to their body size [68, 85].

Within the supergene, two steroid-related genes *HSD17B2* (hydroxysteroid 17-beta dehydrogenase 2) and *SDR42E1* (short chain dehydrogenase/reductase family 42E, member 1) are strong candidates for the morph differences in reproductive behavior in ruffs. The enzymatic product of *HSD17B2* converts testosterone to androstenedione, making it a prime candidate to explain observed differences in plasma levels of these two androgens between morphs. The *SDR42E1* protein appears to be involved in regulating both androstenedione and progesterone levels [73]. *HSD17B2* and *SDR42E1* are less than 30 kbp apart and the Faeder and Satellite haplotypes share at least three major deletions near the two genes [68, 69], suggesting changes to *cis* regulatory elements, which may impact their expression (Fig. 1B).

Two recent studies have investigated the expression of steroid-related genes located inside and outside the inversion [73, 85]. Loveland et al. [73] reported pronounced differences in gene expression between morphs that were generally tissue-dependent. When assessing the entirety of variation in gene expression for all 14 steroid-related genes in testes sampled in spring, the three morphs could be clearly discriminated from each other (Fig. 3B). *HSD17B2* was one of the genes with strongest influence on morph separation. However, overall expression of neither *HSD17B2* and *SDR42E1* was notably different from the expression of twelve steroid-related genes outside of the inversion and the contribution of individual genes to these observed differences was additive. This finding suggests that the chromosomal rearrangement is affecting the expression of steroid-related genes not only within the supergene but also outside it, via other genes or regulatory elements located within the supergene (Fig. 1E).

The second study used an experimental challenge of exogenous gonadotropin releasing hormone (GnRH) to examine morph differences in reproductive physiology and gene expression in males [85]. Gonadotropins are important messenger molecules of the hypothalamus-pituitary-gonad (HPG) axis, which regulates reproductive physiology in vertebrates and plays a key role in the control of sex steroid synthesis and spermatogenesis [86]. GnRH-challenged Faeder and Satellite males were able to synthesize androstenedione but were unable to convert the newly produced androstenedione to testosterone (Fig. 3D). An analysis of nine key genes involved in regulating the HPG axis in pituitary and gonads showed similar expression between morphs, with the notable exception of the *STAR* gene, which was generally upregulated in Faeder and

Satellite gonads. *STAR*, which is located outside of the supergene, encodes the steroidogenic acute regulatory protein, which provides cholesterol for steroid synthesis. Therefore, the paucity of testosterone in Faeders and Satellites could reflect an impairment of the final catalytic step in the conversion of androstenedione to testosterone. As androstenedione is converted into testosterone within the Leydig cells [87] the morph differences in the capacity to produce testosterone seem to lie in the gonads rather than further up in the HPG axis. In addition, the differences in expression among steroid-related genes located outside of the inverted region point to a potential role of *trans* regulatory effects by elements of the inversion region.

6. Conclusions

Supergenes that have arisen in areas of chromosomal rearrangements frequently underlie pronounced within-species variation that culminates in distinct morphs. In birds, supergenes are prominently related to divergence of reproductive traits and strategies [68, 88-91]. Ruffs and white-throated sparrows provide classic examples of extraordinary within-species diversity in reproductive behaviour associated with inversion polymorphisms. The supergenes of these species are dissimilar to each other in many ways: they share no genes, they are associated with different behaviors, they have experienced different evolutionary trajectories, and the mechanisms that maintain diversity are unique to each species. In both species, however, the behaviors that characterize the reproductive strategies are steroid-dependent. As we have shown, each supergene has captured steroid-related genes that are likely to play major roles in defining the features of intraspecific variation in these species. Because variation in steroids often underlies life history variation, differentiation of steroid-related genes is likely to underlie differentiation of life history strategies in other species as well. Future studies should investigate the relatively understudied consequences of this type of genomic variation on development [92, 93] and fitness, including the consequences for survival and reproductive success [43, 94, 95]. Such work will help to elucidate the mechanisms that maintain adaptive genetic and phenotypic variation.

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