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Evolution of the androgen receptor: Perspectives from human health to dancing birds

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ABSTRACT

Androgenic hormones orchestrate the development and activation of diverse reproductive phenotypes across vertebrates. Although extensive work investigates how selection for these traits modifies individual elements of this signaling system (e.g., hormone or androgen receptor [AR] levels), we know less about natural variation in the AR sequence across vertebrates. Our knowledge of AR sequence mutations is largely limited to work in human patients or cell-lines, providing a framework to contextualize single mutations at the expense of evolutionary timescale. Here we unite both perspectives in a review that explores the functional significance of AR on a domain-by-domain basis, using existing knowledge to highlight how and why each region might evolve. We then examine AR sequence variation on different timescales by examining sequence variation in clades originating in the Cambrian (vertebrates; > 500 mya) and Cretaceous (birds; > 65 mya). In each case, we characterize how the receptor has changed over time and discuss which regions are most likely to evolve in response to selection. Overall, domains that are required for androgenic signaling to function (e.g., DNA- and ligand-binding) tend to be conserved. Meanwhile, areas that interface with co-regulatory molecules can exhibit notable variation even between closely related species. We propose that accumulating mutations in regulatory regions is one way that AR structure might act as a substrate for selection to guide the evolution of reproductive traits. By synthesizing literature across disciplines and highlighting the evolutionary potential of specific AR regions, we hope to inspire new avenues of integrative research into endocrine system evolution.

1. Introduction

Endocrine systems are fundamental regulators of animal life, mediating physiological and behavioral processes necessary for an individual to survive and reproduce. Because hormone action guides so many fitness-linked traits (Adkins-Regan, 2005), endocrine systems are thought to be shaped by selection for components that positively influence viability and/or reproductive fitness. However, endocrine function is dictated not only by the hormone itself, but also its interactions with a diverse cast of other 'molecular machines'. To gain a more complete understanding of endocrine evolution, it is therefore important to understand how function arises as an emergent property of the interactions between all agents in a signaling cascade. A logical step toward filling this gap is to examine how different proteins that facilitate endocrine action evolve on a molecular basis.

Sex steroids have emerged as a popular system for understanding endocrine adaptation, perhaps because they underlie changes in physiology and/or behavior that are readily observable. Circulating sex steroids are primarily released from the gonads (Miller, 1988), although other tissues may also secrete them in small quantities (Miller, 2002). Functionally, sex steroids guide the development of reproductive phenotypes as an animal reaches sexual maturity, facilitating the expression of secondary sexual characteristics and behaviors that are necessary to find a mate and reproduce (McCarthy et al., 2009). Later in life, the same hormones regulate how these traits are expressed, both on predictable/ timescales (e.g., seasonal cycles) and in response to unpredictable stimuli (e.g., shift in social context) (McCarthy et al., 2009). Because sex steroids are closely linked with the expression of key traits necessary for reproduction, they present many opportunities for selection to influence how the signaling system evolves.

To date, most studies considering the evolution of sex steroid systems focus on tissue-specific expression patterns of genes whose proteins product mediate different elements of steroid signaling. For example, increased expression of steroid receptors in a specific region of the brain can help mediate the production of behavior associated with the area (McGinnis et al., 1996; Sato et al., 2004; Juntti et al., 2010;

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Rosvall et al., 2012; Alward et al., 2013). Alternatively, locally expressing more steroidogenic enzymes can also amplify steroid function by increasing the amount of available hormone or converting it into more potent forms (Soma et al., 2008; Saldanha et al., 2011; Anuka et al., 2013). Of course, every element in the androgenic system can also evolve when genetic mutations change the amino acid sequence that defines a protein's structure and function. Such effects, in theory, have the capacity to 'turn up' or 'turn down' the regulatory capacity of the steroid system as whole.

At the same time, a mutation on the gene that encodes a protein necessary for steroid function might invoke similar changes in a signaling system's function. For example, substituting an amino acid at a particular residue could plausibly increase/decrease its binding affinity and yield similar effects as changing expression levels. However, the close proximity of steroid signaling to reproductive fitness also means that similar mutations risk disrupting portions of the signaling cascade entirely. The latter outcome certainly appears to be of evolutionary importance, as many studies emphasize the tendency for proteins involved in steroid signal transduction to be highly conserved (Henttu et al., 1997; Robert, 2003; Yamamoto et al., 2011; Kattoula and Baker, 2014; Edman et al., 2015). Characterizing which proteins (or which domains on a protein) are more or less prone to change over time is therefore necessary to identify the molecular substrates that facilitate endocrine adaptation.

Here we assess the protein evolution of one key element in the steroid signaling network: androgen receptor (AR). AR is an intracellular receptor that binds androgenic steroids, such as testosterone (T) or dihydrotestosterone (DHT). It then acts transcription factor, dimerizing with another androgen-AR complex and translocating into the cell's nucleus. Then, it binds to cofactors that modify how AR affects gene expression. Therefore, androgen-dependent traits are therefore expression through these changes to the target cell's transcriptional milieu. To this end, the fact that steroid action plays such a fundamental role in biological systems may explain why certain components, like the basic structure of AR itself, are highly conserved: dramatic changes to these proteins are highly likely to cascade into deleterious or fatal physiological effects (La Spada et al., 1991; Ahmed et al., 2000; Marcelli et al., 2000). At the same time, it is this same characteristic of steroid signaling that makes the system so compelling from an evolutionary standpoint, as non-deleterious modifications can theoretically modify how AR interacts with other players in the signaling cascade. Such instances may be more likely to balloon into functional changes that support phenotypic evolution.

With this in mind, we examine variation in AR structure in two clades that have diversified on different timescales: vertebrates (origin during the Cambrian explosion > 500 mya; Shu et al., 1999) and birds (first emerged in the late Mesozoic > 70 mya; Pacheco et al., 2011; Jarvis et al., 2014; Prum et al., 2015). Our goals are two-fold. First, we probe AR protein sequences to characterize the receptor's evolutionary history and identify which regions are most likely to evolve in response to selection for reproductive traits. Second, we draw on studies from various fields (including the medical sciences) to contextualize natural AR variation and highlight important topics for future study. Functional studies of AR variants are almost entirely restricted to humans and model organisms, and we must recognize the speculative aspects of this endeavor. Our aim is to prompt a broader discussion around steroid evolution, by connecting research with an organismal, evolutionary, and/or ecological focus to ideas in endocrinology that are seldom encountered outside of fields such as molecular biology and biochemistry. By connecting insights across these disciplines, we hope to highlight new routes of inquiry for understanding the evolution of sex steroid systems.

2. Structure-function of androgen receptor (AR)

AR is the functional lynchpin of androgenic signaling, as it is responsible for binding hormones (in tetrapods and lobe-finned fishes, T or DHT; in other fishes, T or 11-ketotestosterone [11-KT]) and then modifying gene expression. Like many other nuclear receptors, AR is characterized by four main domains: *(i)* the N-terminal domain (NTD), which plays an impressive variety of regulatory roles; *(ii)* a DNA-binding domain (DBD), responsible for binding to specific DNA sequences called androgen response elements (AREs); (*iii*) the hinge domain, which links the NTD and DBD; and (*iv*) a ligand-binding domain (C-terminal domain; LBD), which binds both hormone and co-factors. Because each region is associated with a different function in androgenic signaling, some regions may be more variable than others over evolutionary time. For instance, mutations near the binding residues for DNA or the androgenic ligand (the DBD and LBD, respectively) may introduce a greater probability of system failure. On the other hand, all four domains feature residues that allow for binding with a diverse cast of proteins to modify how AR regulates gene expression (Shah and Bradbury, 2015). This fact may hold the key for how AR evolves to facilitate adaptation, as modifying regulatory regions makes it possible for AR to have different effects on gene expression without destabilizing the entire signaling system. Below we provide a brief overview of these four regions, setting the stage for further consideration about how they might evolve in response to selection to shape androgenic signaling.

2.1. The N-terminus domain

Most of the AR is occupied by the NTD (also referred to as Activation Function 1 [AF-1]; Bevan et al., 1999). The NTD plays a key role in modulating androgenic signaling once T or DHT binds. For example, multiple regions across the NTD interact with various proteins, including other portions of AR itself, to ensure that the receptor maintains its structure during ligand-binding and translocation to the cell nucleus (Claessens et al., 2008). Like several other nuclear hormone receptors, AR is stabilized by heat-shock proteins until a ligand is bound (Smith and Toft, 2008). This is largely carried out by the NTD, which features multiple motifs to recruit factors that interact with heat-shock proteins. For example, there is a motif that also binds a ligase integral to recycling the heat-shock protein complex, thus destabilizing and inactivating AR (He et al., 2004). In this way, the NTD both preserves AR and facilitates its degradation. As is the case with much of our understanding of steroid receptors, we know this from work studying deleterious mutations in the human AR. Specifically, mutations that impede the turnover of heat-shock proteins result in greater AR activity in both humans patients and *in vitro* models of prostate cancer (Han et al., 2001; Callewaert et al., 2006).

The NTD also features motifs that allow it to interact with the LBD of this protein. After AR binds a ligand, it must dimerize with another bound AR before translocating to the cell nucleus. When AR dimerizes, interactions between the NTD and the hydrophobic surface of the LBD help to stabilize the dimer's structure and retain the ligand (He et al. 2000, 2001, 2002). Human mutations that strengthen this interaction lead to enhanced transcriptional activity and aggressive forms of prostate cancer (Wilson, 2009).

Another important aspect of the NTD is its capacity to recruit and bind auxiliary proteins called cofactors. Cofactors determine how AR will change gene expression, where co-activators increase transcription (e.g., SRC-1 Perissi et al., 2010) and co-repressors down-regulate expression (e.g., NCOR1 Perissi et al., 2010). Cofactors primarily bind to residues on the NTD called transcription activation units (tau-1 and tau-5), which are also required for AR to begin modifying transcription (Claessens et al., 2008). Within these regions are residues that bind both SRC-1 (Bevan et al., 1999; He et al., 2000; Christiaens et al., 2002) and NCOR1 (Chmelar et al., 2007), which can occur even before AR is bound by a ligand (Dehm and Tindall, 2007). Because interacting with cofactors determines whether gene expression is increased or decreased, changes that influence the NTD's affinity for SRC-1 or NCOR1 should result in elevated or depressed androgen action overall.

Finally, extensive research on the NTD has identified repeated sequences of glutamine (Q) and glycine (G), which vary in length among individual humans (Brinkmann, 2001). These length differences have consequences ranging from severe to benign. For example, long poly-Q regions can be found in persons with spinal bulbar muscular atrophy (SBMA; Kennedy's disease) (La Spada et al., 1991; Kobayashi et al., 1998; Cortes et al., 2014). Progressively longer poly-Q regions make AR more likely to be cleaved by caspase-3, which truncates AR and renders it non-functional (Kobayashi et al., 1998). This leads to progressive motor neuron loss over time, which eventually results in loss of basic motor functions. Conversely, too much shortening of the poly-Q region can lead to aggressive forms of prostate cancer (Giovannucci et al., 1997; Hakimi et al., 1997). At the same time, more subtle variation in poly-Q and poly-G length are associated with an individual's propensity for aggressive or impulsive behavior (Rajender et al., 2008; Westberg et al., 2009; Aluja et al., 2011). This polymorphism is found in rodents as well as humans, and is thus thought to have its origins in ancestral mammals before being exaggerated in primates (Choong et al., 1998). Although we know relatively little about the role of poly-Q and -G region length in other organisms, this example illustrates how one region of the NTD may have the capacity to underlie adaptive evolution, particularly of social behavior—but only for mutations that do not impose an unsustainable cost on viability.

2.2. DNA-binding domain

Androgenic signaling works by changing patterns of gene expression, which means that AR must bind to DNA. This is, of course, the primary function of the DBD. The DBD also contains a sequence that anchors the AR dimer to the promoter regions of androgen-responsive genes by interacting with androgen response elements (Jenster et al., 1991; Shaffer et al., 2004). These short DNA sequences are typically located in promoter or enhancer regions of androgen-responsive genes (Shaffer et al., 2004; Wilson et al., 2016). Disrupting the DNA-binding process or AR's capacity to dimerize (which is a necessary prerequisite for DNA binding) will thus inhibit androgen signaling (Wong et al., 1993). For example, substituting a cysteine for tyrosine at position 619 in the human DBD results in complete loss of transcriptional activity (James et al., 1999). Given the important functional role of this region, it is not surprising that there is strong conservation of this region across vertebrates.

At the same time, not all mutations in the DBD functionally abolish androgen signaling. For instance, this region contains two zinc finger regions, which may be mutable without deleterious consequences. Swapping an alanine for a threonine at position 575, for example, increases AR activity by causing promiscuous binding to other hormone response elements (Monge et al., 2006). Other mutations within the zinc finger regions generate comparable changes to DNA binding affinity (Hu et al., 2010). Therefore, despite the overall conservation of the DBD, mutations might still arise to alter the expression of androgen dependent traits. Nevertheless, such mutations likely have broad physiological costs, and thus would only evolve under a narrow set of circumstances.

2.3. Hinge domain

Adjacent to the DBD is the hinge domain, which is similarly small. Based on early work across different types of nuclear receptors (Mangelsdorf and Evans, 1995), researchers originally posited that this region served as a flexible link between domains. It was thought that the flexible nature of the hinge might permit changes in the folding of this protein necessary to form this promiscuous dimer (Mangelsdorf and Evans, 1995). Subsequent work has since also demonstrated that the hinge domain also plays other roles that are indispensable for normal AR function (Zhou et al., 1995; Gioeli et al., 2006). For instance, in conjunction with the DBD, the first 12 amino acids of this domain play a significant role in ARE binding (Denayer et al., 2010; Helsen et al., 2012b).

Additionally, the hinge contains residues important for translocation to the cell nucleus (Zhou et al., 1994), a process that requires a number of biochemical interactions to take place, including phosphorylation (S650), acetylation (K630,632,633), ubiquitylation (K630,632,633), and methylation (K630 and 632) (reviewed in Clinckemalie et al., 2012). Studies have shown that altering these events can influence AR's ability to translocate into the cell and initiate its effects, as is the case when the phosphorylation of serine 650 is disrupted (Gioeli et al., 2006; Zuccarello et al., 2008). Many growth factors (e.g., insulin-like growth factor 1) can even form complexes with the kinases that phosphorylate this residue, leading to a non-canonical and androgen-independent activation of AR (Connolly and Rose, 1990; MacGrogan et al., 1992). This suggests that S650 is an important site for the regulation of AR function. Yet relative to the other domains, few naturally occurring mutations in humans have been identified in the AR hinge region (Gottlieb et al., 2012). Rather than suggesting that the hinge is not evolvable, it is more likely that restricted variation is the expected outcome when examining a species that has only existed for a few hundred-thousand years.

2.4. The ligand binding domain

Finally, the LBD is the region of the AR protein that contains the hormone-binding pocket. In almost all vertebrates, the main androgenic ligands that bind to this region are T and DHT (Kemppainen et al., 1999). One exception are non-Sarcopterygian fishes, which have a stronger affinity to 11-KT (Hossain et al., 2008). Structurally, the LBD is composed of 12 alpha helices, many of which have highly specific functions (Helsen et al., 2012a; Eisermann et al., 2013). For example, the third and fourth helices contribute to the ligand binding pocket and participate in the recruitment of co-factors (Claessens et al., 2008). Another important region of the LBD is the AF-2 motif, which is involved in recruiting SRC1 to enhance the transcriptional output of AR (Claessens et al., 2008). This region also plays an important region in mediating the interaction between NTD and LBD (*see above*).

3. Evolution of the androgen receptor (AR)

The capacity for AR sequence variation to generate phenotypic differences is well-known, arising both from engineered mutations and natural variation (Feng et al., 2010; Juntti et al., 2010; Rosvall et al., 2012; Fuxjager et al., 2015; Mangiamele et al., 2016; Yong et al., 2017; Ramzan et al., 2019; Schuppe and Fuxjager, 2019). Whether or not this leads to broader variation in AR across evolutionary time remains largely unknown, and we attempted to address this question through a series of exploratory analyses (See *Supplemental Methods*). We first characterized how AR sequences vary across 8 species from major vertebrate groups to gain a preliminary view of how different regions were modified over millions of years. After comparing sequence variation on a domain-by-domain basis, we identified specific mutations known in humans to better contextualize the mutations we uncovered. We then took a more recent view of AR variation using a second analysis in birds. A rich literature on the evolution of avian reproductive morphology, physiology, and behavior allowed us to contextualize our findings in the phenotypic realms most likely to be shaped by AR variation. Altogether, we aim to uncover new avenues of research for scientists interested in steroid endocrinology, vertebrate evolution, and reproductive traits alike.

3.1. Variation among major vertebrate groups

All four AR domains are present in the 8 vertebrate species we examined (Fig. 1A), but there is considerable variation in sequence length. Given that AR's size can significantly alter its transactivation

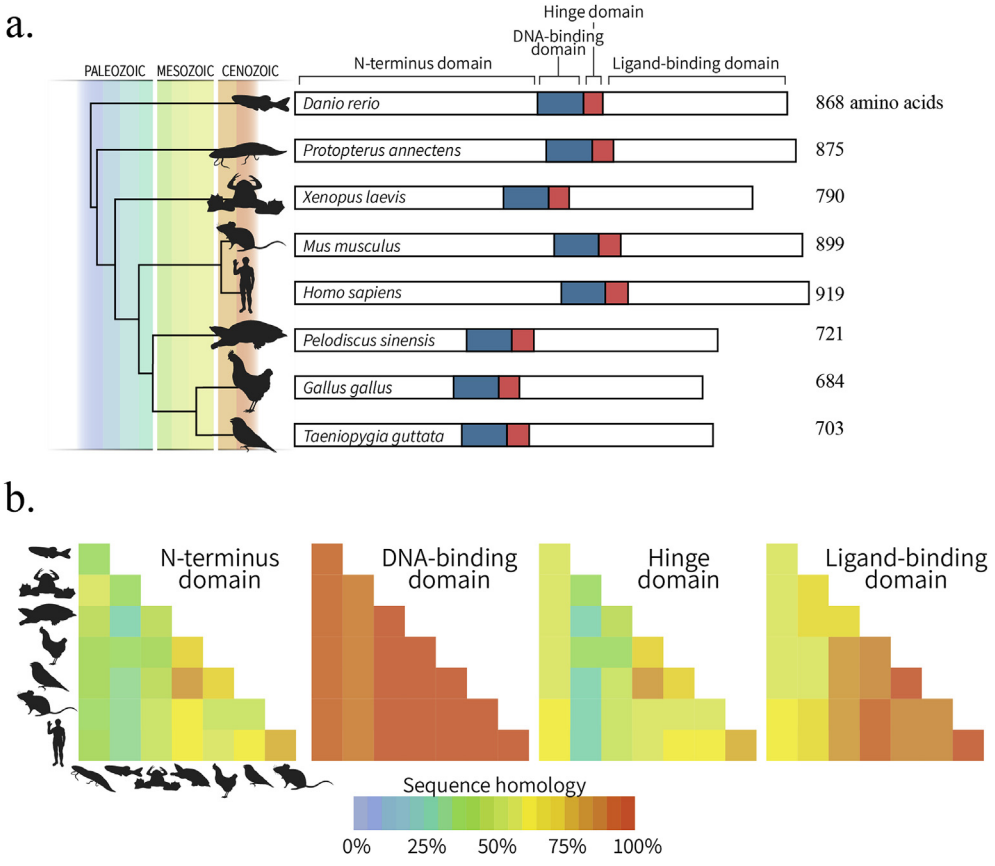

Fig. 1. (a) Protein sequence overview for androgen receptor (AR) in key organisms across the vertebrate tree of life. Each rectangle is scaled by amino acid sequence length and differentiates between the four AR domains. These schematic illustrations of the protein demonstrate that there are numerous evolutionary truncations (the most pronounced in the bird lineage) of the AR protein. However, there was also a significant expansion of the AR protein in mammals. In both instances the change in length is mostly a result of modifying the N-terminal domain (NTD). Part of this NTD expansion in mammals is larger tau-1 and -5 motifs, which are responsible for binding co-regulatory proteins (Claessens et al., 2008). In humans, at least, this is also due to polymorphic repeats that are absent in all other vertebrate lineages (Eisermann et al., 2013). (b) Pairwise comparisons of sequence homology characterize the degree to which each domain-specific sequence is similar among species, where 0% homology reflects no similarity and 100% reflects identical sequences. Each cell in the matrix reflects a comparison between the row and column species, with warmer values indicating greater similarity and cooler values indicating greater disparity. This analysis indicates that there is striking divergence, even among more closely related species, in both the NTD and hinge domain. Yet, the DNA binding and ligand-binding domains (LBD) exhibit greater sequence conservation.

ability (Wadosky and Koochekpour 2017)—and thus ostensibly how AR shapes a variety of reproductive and/or behavioral traits— it is theoretically plausible that receptor size may be a target of selection. However, this possibility must be weighed against the fact that differences in sequence length may simply be a byproduct of domain-specific modifications that also influence length. Different patterns from domain to domain offer hints as to AR's role in shaping animal evolution.

3.1.1. N-terminus domain

Consistent with previous reports (Thornton and Kelley, 1998; Ogino et al., 2009), we demonstrate that the NTD varies across vertebrates (21–81% conserved). Although the length of the most ancestral NTD sequence in this analysis (lungfish; 875 a.a.; Fig. 1A) is similar to the mammalian sequences (899–919 a.a.; Fig. 1A), they share very little sequence homology (32% conserved; Fig. 1B). Of course, this may not be surprising considering that fish and mammals last shared a common ancestor some 450 million years ago (Hughes et al., 2018). Yet even within modern animal groups, the NTD exhibits some degree of sequence dissimilarity. For example, mice and humans likely shared a common ancestor > 80 mya (Springer et al., 2003; Lartillot and Delsuc, 2012), and retain 88% sequence homology in the NTD (Fig. 1b). Birds also had their origins in the Mesozoic era, at least 70 mya (Pacheco et al., 2011; Jarvis et al., 2014; Prum et al., 2015), and exhibit similar levels of sequence similarity (83%; Fig. 1B). Therefore, the NTD has the capacity to accrue major variation within animal groups, although this process unfolds over tens of millions of years.

We next looked at how mutations are concentrated on regions of this domain, particularly where substitutions might influence its regulatory behavior. The functional core of the NTD is the AF-1 motif, which we found to be 10–87% conserved among the vertebrates. This region exhibits striking variation across many vertebrate groups (Fig. 1B). In particular, we find that many of the amino acids present in the human AF-1 are absent in non-mammalian species. One example is the Tab2 binding sequencing ($^{183}LX_7LL^{192}$), which helps recruit the co-repressor NCOR1 (Zhu et al., 2006). All vertebrates exhibit a leucine at the beginning of this motif, however, several vertebrates groups lack the additional leucines thought to be necessary for Tab2 binding. Although fishes lack both leucines at the end of this motif, frogs are only missing the second leucine. Because turtles exhibit the characteristic Lx_7LL sequence, this suggests that this region more recently became a site to depress androgen action by permitting the formation of the NCOR1 complex (Zhu et al., 2006; Claessens et al., 2008).

Other residues with similarly critical functions appear to be relatively conserved. For example, one particularly important motif (AKELCKAVSVSMGL) for binding proteins that interact with heat-shock proteins (e.g., Hsp70-interacting protein E3 ligase CHIP) is rather homologous among vertebrates, with 9 of 15 amino acids shared. Moreover, point mutations on this motif typically result in less dramatic changes to AR function (Han et al., 2001; He et al., 2004). Considering that CHIP essentially triggers the breakdown heat-shock protein clients including AR (He et al., 2004), mutations that alter ability for CHIP to interface with AR might serve to decrease or increase AR levels. On the one hand, an increased capacity to bind CHIP could lead to an enhanced rate of AR degradation (He et al., 2004; Paul and Ghosh, 2014). At a functional level, decreasing AR's activity in this way could buffer an organism against the costly effects of androgens. However, if mutations hinder the binding of this ligase (thereby interrupting AR degradation), the likely result would be a marked increase in the steady state levels of AR. This, in theory, would increase androgen action and potentially facilitate the expression of androgen-dependent traits (He et al., 2004; Paul and Ghosh, 2014).

Another important role of the NTD is to bind cofactors (Dehm and Tindall, 2007; Wilson, 2009). Even at a critical cofactor recruitment site (tau-5; WxxLF motif), we detected two substantial shifts. First, all

species except zebrafish begin this motif with a tryptophan. Another clearly identifiable shift in this motif was the ancestral proline in both fishes that was mutated numerous times across vertebrates. Whereas turtles and birds have a phenylalanine at this position, all other species have a leucine. The functional significance (if any) of this difference remains unknown; however, mutating the same leucine in human cell culture alters how the NTD binds SRC-1 (Bevan et al., 1999). If a similar effect occurs in other animal groups, then this mutation may correspond to a decrease in androgenic potency by way of altered co-activator binding. In turn, this could imply that certain amino acids within this motif are site where selection can act to fine-tune androgen-mediated transcriptional capabilities via interactions with other molecular machinery.

Finally, motifs within the NTD also mediate the interaction with the LBD. Most research suggests this important inter-domain interaction occurs at the FxxLF (He et al. 2000, 2002). We find that motif was FENVYY (lungfish), a complete shift from the teleost sequence (YQSVF). This is interesting because this sequence is distinct from the other species, suggesting the possibility that forming the essential N/C termini interaction could be relatively weaker. Unless the lungfish AR operates by a completely novel mechanism, other regions must be able to support this interaction, as it is required to initiate AR's transcriptional capabilities (Claessens et al., 2008). Among the tetrapods we studied, all species share the same pattern (FxxLF), with one exception: in birds, the leucine is now a phenylalanine (FxxFF). Functionally, mutations to these residues reduce the ability of this motif to the bind the LBD and alter the kinetics of ligand binding (He et al., 2004b). However, it is quite possible that the shift in birds introduces no functional differences, as these amino acids have similar properties including charge and hydrophobicity.

3.1.2. DNA-binding domain

To exert its potent downstream effects, AR must bind and dimerize at specific regions of genomic DNA. Since AREs are thought to follow a conserved pattern (Wilson et al., 2016), it is not surprising that the DBD is similarly conserved among vertebrates: mutations on the ARE and DBD could lead to a complete breakdown of the signaling cascade if they impede AR binding the DNA (Denayer et al., 2010; Helsen et al., 2012a). Indeed, there is 100% homology in the DBD among amniotes, although this sequence is different from both fish species. The DBD has apparently remained more-or-less unchanged for almost 500 million years—a testament to how functional constraints continuously restrict variation. How might these changes in fish alter androgen action? Of the seven changes that we found in either the lungfish or zebrafish, three are in zinc finger region. In humans, mutations in this region of the DBD cause AR to promiscuously bind to other hormone response elements, while simultaneously increasing overall transcriptional activity (Monge et al., 2006; Hu et al., 2010). The remaining changes are in amino acids that are directly involved in ARE binding, but not translocation (Claessens et al., 2008; Denayer et al., 2010). One possibility is that the sequence divergence in lungfishes and other teleosts may have allowed the ancestral AR to accommodate binding to other ARE sequences (Helsen et al., 2012a). It is also possible that the DBD of fishes has a greater propensity to promiscuously bind to other hormone response elements.

3.1.3. Hinge domain

The hinge domain, which links the DBD and LBD, is highly variable (32–82% similar). However, the residues important for ARE binding and nuclear translocation are highly conserved. Again, the only major sequence dissimilarity occurs in non-Sarcopterygian fishes—which is perhaps unsurprising, because the fish AR primarily binds 11-KT instead of T or DHT. Since these residues are presumably essential to AR function, they have likely been conserved over evolutionary history. This may further suggest that fishes are able to bind additional AREs or might promiscuously bind to other hormone response elements. We also find that lungfishes lack S650, an important phosphorylation site (Wong et al., 2004). Mutating this serine to an alanine results in significantly reduced AR activity by decreasing nuclear-cytoplasmic shuttling of AR (Zhou et al., 1995; Gioeli et al., 2006). Perhaps the most intriguing finding is that most amino acids without a known function are highly variable (Clinckemalie et al., 2012). Since different residues within the hinge region are important phosphorylation, acetylation, and ubiquitination targets, mutations in this region may further modulate androgen action and abundance. Accordingly, variation in this region could be a way to further regulate the expression of adaptive reproductive traits.

3.1.4. Variation in the LBD

Because of the conserved molecular structure among vertebrates (except fishes), one might expect that the residues of the LBD associated with ligand binding to remain similarly conserved. However, other residues in the LBD associated with ligand affinity may be modified. Such an arrangement may allow for androgenic signaling function to be conserved, while still providing the potential for adaptive modification its effects. Indeed, we find that the LBD exhibits strong conservation between turtles, birds, and mammals. Yet, we also expect that evolutionary shifts in the affinity of different androgenic metabolites should mirror notable changes in the amino acid sequence. Consistent with this idea, this domain is less conserved between teleost and Sarcopterygian fishes (~70%). One possible explanation is that these sequence differences confer marked differences in the affinities for different androgenic molecules. Certainly, in many vertebrates DHT is the main androgenic hormone, but this is not the case in fish species. In lobe-finned fishes (e.g. lungfish) T appears to be the primary androgen, whereas the primary functional androgen in teleost fishes is 11-KT (Hossain et al., 2008).

How might these changes in amino acid sequence influence aspects of the structure that allow significant evolutionary shifts in the binding of androgenic ligands? To help explore this question, we generated predicted protein models for each species based on the crystal structure of this domain in humans (Fig. 2A). First, when we compare the LBD structure of humans to birds (zebra finches and chickens), we find minor structural variation (Fig. 2B). In some cases, amino acid changes confer no change in overall structure, such as an AA to SN mutation at 698 and A to S at 735. Whereas other mutations (e.g., N to S at 692; Fig. 2B) generate minor deviations in the overall structure of AR. Given that previous literature that demonstrates the binding affinities of different androgens are comparable to other mammals (Katoh et al., 2006), we believe that the functional effect of these differences is likely minor. In this way, the structural differences in the LBD may not significantly alter ligand binding or AR activation. However, when we compared LBD between humans and fishes, we see greater variation (Fig. 2C). In particular, it appears that the structure substantially differs in helix 11 (Fig. 2C), especially with respect to the end of this helix and the portion of this domain that transitions into the 12th helix. Prior work shows that mutations in this area of the human LBD can lead to an expansion of the hormone binding pocket potentially altering androgen affinity (Bohl et al., 2007). These differences might explain why the affinity for certain androgens differs in zebrafish (Hossain et al., 2008). In particular, this notable structural change in the LBD may be what allows the teleost AR to preferentially bind 11-KT, a ligand that has relatively weak binding affinity for mammalian AR (Katoh et al., 2006; Hossain et al., 2008). Yet, studies that investigate the functional ramification of each of these structural changes are lacking, and thus we are unable to definitively infer the effect of these differences between fishes and other vertebrates.

3.1.5. Conclusions about regions variability in AR

Overall, we find that most of the AR protein is highly conserved across vertebrates. Nonetheless, some domains do exhibit notable sequence variation across the vertebrate groups studied. This variation is

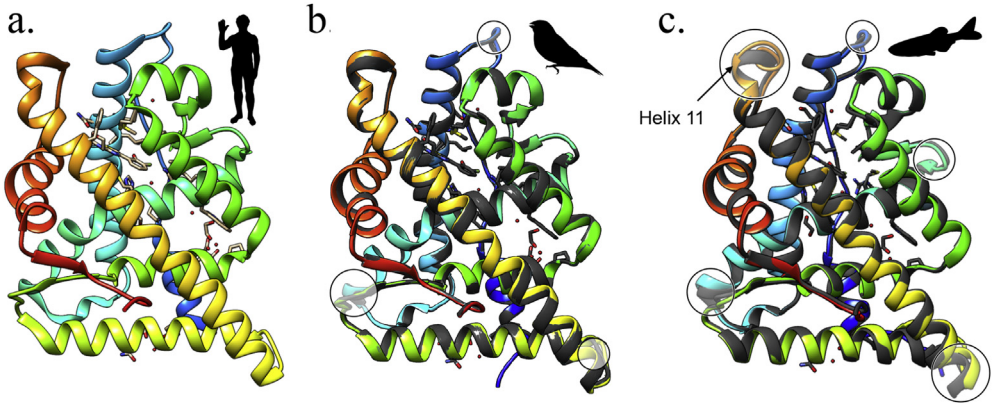

Fig. 2. Predicted crystal structures of the AR ligand-binding domain (LBD) in model organisms: (a) human, (b) zebra finch, and (c) zebrafish. In (b) and (c), the human structure is superimposed in grayscale to highlight structural differences that have emerged over millions of years. The circled areas highlight instances in which mutations that have led to significant structural changes between the human and zebrafish or zebra finch LBD. Humans and birds exhibit strong sequence homology, and thus share a striking structural resemblance. However, fishes and humans exhibit striking sequence divergence that has led to several changes in the overall structure of the LBD. One of the most notable changes in zebrafish are the mutations in 11th helix that confer substantial differences structure of the LBD. Helices were annotated based on pervious literature and alignments in Chimera (Sakkiah et al., 2018).

perhaps most striking in motifs within the NTD that are essential for binding co-regulatory proteins that ultimately modify the effects of androgens on the genome (He et al., 2004; Perissi et al., 2010). One variable NTD motif is AF-1, which is responsible for binding co-factors (including SRC-1) and molecules (e.g. CHIP ligase) that ultimately lead to the destruction of this receptor. One intriguing possibility is that selection for androgen-dependent traits may favor mutations in regions that are responsible for binding regulator molecules. In this way, the key functions of AR (e.g., ligand and DNA binding) can remain unchanged but the activity or longevity of the receptor is augmented. This could theoretically enhance expression of reproductive traits without modifying other elements of the androgen signaling system, including the expression of the receptor or co-repressors.

3.2. Evolution of the avian androgen receptor

We next explore how ideas derived above through our deep-time analysis of AR evolution apply to a single vertebrate group: birds (Class: Aves). As a radiation that evolved in the past 60–80 Ma, the clade provides a temporal intermediate between the extremes of vertebrate evolution (> 500 Ma) and human-focused studies (< 400 ka). Birds are also well studied in the context of sexual selection, and androgen action is important for activating the avian reproductive phenotype. We now have access to a large number of well-sequenced avian genomes, which provide an unprecedented opportunity to evaluate gene evolution amongst these taxa (Zhang et al., 2014), allowing us to examineprotein sequence variation in 44 species. We did not include the NTD in this analysis because few species have it fully sequenced. However, we fully recognize that variation in the NTD is likely important in the context of adaptive evolution of avian AR.

3.2.1. Points of variation across avian species

Similar to our other analyses (see above), the DBD is highly conserved among birds (92–100% conserved). There appear to be four species (barn owl [*Tyto alba*], Anna's hummingbird [*Calyte anna*], southern ostrich [*Struthio camelus australis*], wild turkey [*Meleagris gallopavo*]) with sequence differences in this domain. Of the seven mutations that we identified, six were in the DBD zinc fingers. As mentioned above, mutations in this region may have the capacity to enhance androgen action, but often simultaneously promote promiscuous binding to other hormone response elements (Monge et al., 2006; Hu et al., 2010). With this in mind, we suspect that any functional differences arising from these mutations would likely affect which genes are transcribed and/or the rate of transcription. Notably, the four species with a different DBD sequence are not closely related and almost certainly reflect independent mutations. Future work should begin to assess whether these differences result in a functional shift, be it adaptive, neutral, or negative.

We next compared the hinge domain across species. On average, we found that a low level of variation (82–97% conserved) with numerous conserved residues. For example, residues responsible for translocation were identical in all taxa except for Japanese quail (*Coturnix japonica*) (Fig. 3). This is consistent with findings that show these mutations are usually detrimental to AR function (Clinckemalie et al., 2012), and thus

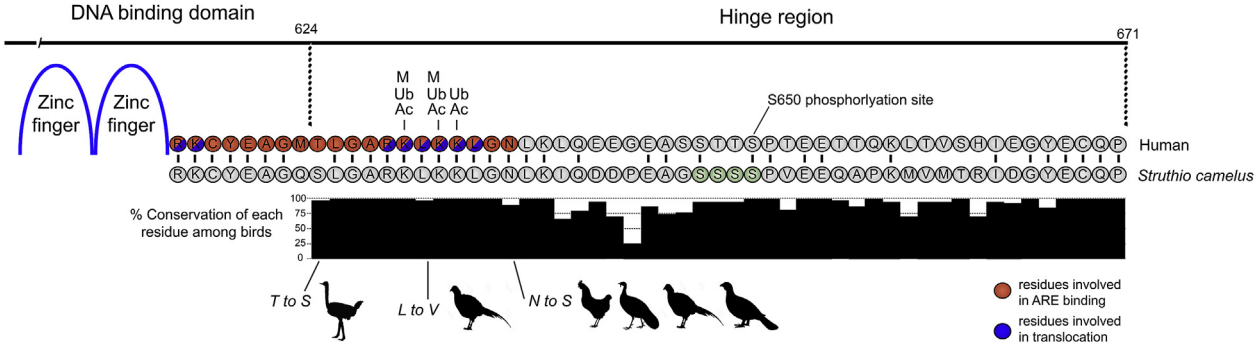

Fig. 3. Schematic illustration of the amino acids that make up the hinge domain and the relative conservation of each residue among birds. Residues highlighted in red play a critical role in allowing the androgen receptor (AR) to bind to androgen response elements (ARE), and those in blue are essential for translocation. Many of these same residues also play an important role in post-translational modification of AR (acetylation [Ac], ubiquitination [Ub], and methylation [M]) that functionally suppress androgen action (Clinckemalie et al., 2012). Surprisingly, we identified three mutations in these regions that are critical for normal AR function. Two mutations occurred in residues that are only involved in ARE binding, including a T to S shift at 625 (*Struthio camelus*) and a N to S shift at 636 (*Gallus gallus*, *Coturnix japonica*, *Meleagris gallopavo*, and *Numida meleagris*). Finally, we identified an L to V mutation at 630 in *C. japonica*. Although there is significant variation in the other residues that make this domain, many have no clear function.

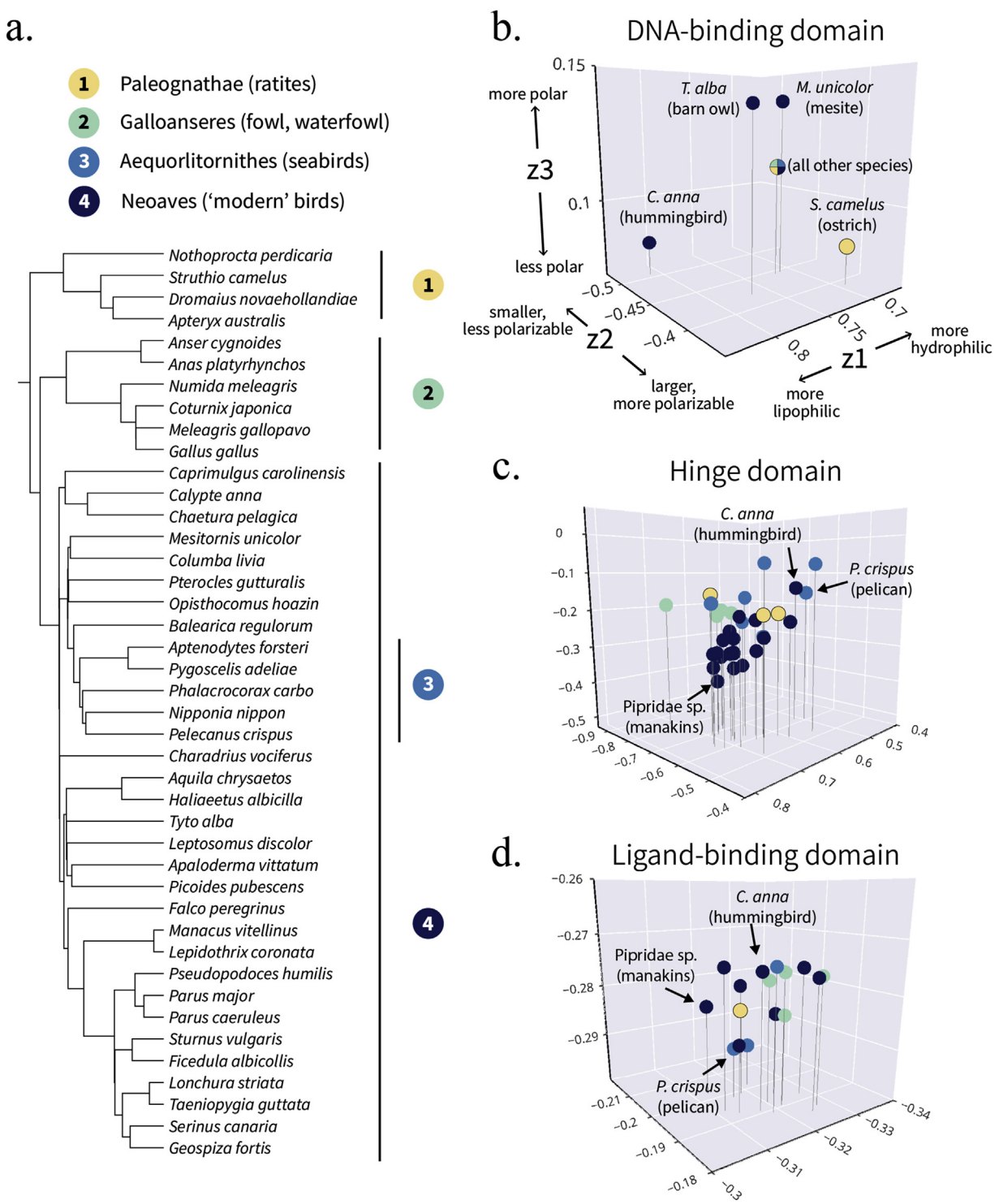

Fig. 4. (a) Phylogeny of bird species for which androgen receptor sequences are publicly accessible; topology determined by maximum clade consensus among 1,000 sample trees from http://birdtree.org. (b) 3D scatterplot of protein z-scores on the DNA-binding domain of the avian AR; z1 reflects differences in nonpolar surface area and solubility that broadly reflect the degree to which a protein will dissolve in nonpolar solvents (i.e., lipophilic) vs. polar solvents (i.e., hydrophilic); on the y-axis, z2 scores increase for proteins with greater molecular weight and/or polarizable (higher probability of changing polarity when interacting with another molecule); on the z-axis, higher values reflect increasing polarity (greater separation of positive and negative charges). (c) Protein z-scores on the hinge domain. (d) Protein z-scores on the ligand-binding domain.

likely to be selected against. How exactly this mutation—a leucine substitution to valine at 630—influences androgenic signaling in quail is currently not known; but, given that both of these amino acids have hydrophobic side chains, the structure and ability to bind to AREs might remain unhindered. Thus, it is likely that the mutation is functionally neutral.

We also identified variation in residues responsible for ARE binding within the hinge domain (Fig. 3). Such changes may alter the affinity and ability of AR to bind to genomic response elements, potentially changing the degree to which AR can influence gene expression. This is

because mutations to this region that do not completely eliminate AR's DNA-binding ability can instead lead to off-target binding of other DNA regions. An increased number of genomic binding sites result in a more robust transcriptional response (Monge et al., 2006; Hu et al., 2010).

Equally important, we also uncovered substantial species-level variation along portions of the hinge domain that may be targets of post-translational regulation, including both ubiquitination and phosphorylation (Clinckemalie et al., 2012, Fig. 3). In particular, birds exhibit several substitutions for serine, adding potential phosphorylation sites within this domain (Clinckemalie et al., 2012). Phosphorylation of serine in the hinge domain precedes nuclear export (Zhou et al., 1995). If these serines can be phosphorylated, it might increase the probability that a given AR complex will leave the nucleus. Compounded over time, an increased rate of export should therefore correspond to a decrease in the average time that AR spends anchored to DNA and modifying gene transcription. If true, this would indicate that mutations that produce (or remove) post-translational modification sites may provide an additional route to regulate the potency of androgens. The many other amino acid changes in this region—as well as the interactions among amino acids—might similarly alter fundamental properties of the hinge domain, and thus confer a slight functional change to the receptor as a whole.

The LBD of avian AR is also highly conserved (96–100% conserved). However, mutations that alter key LBD functions may still alter AR potency without necessarily being lethal (McPhaul et al., 1992; He et al., 2000; Matias et al., 2000). Although the functional effects of the species variation in the LBD remain unclear, it is well established that even a single point mutation can significantly alter N/C termini interactions, ligand binding, or cofactor binding (Wilson, 2009). Moreover, these small sequence changes may confer meaningful structural changes that ultimately modify how hormones interact with the binding surface (*see below*). It is also possible that these changes subtly alter inter-domain interactions or regulation by co-regulatory proteins.

3.2.2. Sequence variation alters the physio-chemical properties of AR

Extensive work characterizing the biochemical properties of different amino acids—both individually and as part of a protein—has led to the development of metrics to broadly classify physical and chemical properties of a protein. Among these metrics are z-scores, which characterize principal components of biochemical action. Three z-scores (z1, z2, and z3) are easily tractable to salient aspects of protein biochemistry, such as hydrophobicity, molecular weight, polarity, and polarizability (Collantes and Dunn Iii, 1995). Thus, z-scores offer a way to guide inference about relative functional differences rooted in protein structure, especially when it is not possible to directly test such function in the laboratory. Of course, the degree to which variation in any one z-score might influence AR performance is unknown. Below, we measure and compare z-scores in the AR domains of birds. Rather than attempting to make broad conclusions about species variation in z-scores, we instead draw out a subset of species that might highlight how and why AR has evolved. These ideas should be treated as hypotheses that lay the foundation for future research, rather than robust conclusions.

3.2.2.1. Manakins. One of the first groups of species to show significant variation in z-scores belongs to a small family of oscine Passerine called manakins (family: Pipridae). The two species included in our analysis (*Manacus vitellinus* and *Lepidothrix coronata*) both have identical AR sequences. Coincidentally, there is a rich literature that characterizes the role played by androgens to shape the remarkable courtship dances of these birds. Male manakins of most species must dance to find a mate, and some species have evolved remarkable performance traits as a consequence—including one of the fastest vertebrate limb muscles on record in *M. vitellinus* (Fuxjager et al., 2016). In this species, females prefer to mate with fast-displaying males (Barske et al., 2011) and muscle contraction-relaxation cycling speeds specifically impose a limit on display evolution (Miles et al., 2018). Importantly, androgenic signaling mediates neuromuscular adaptations for male display, with selection favoring increased expression of AR in key wing muscles (Feng et al., 2010; Fuxjager et al., 2015) to help prime these tissues for rapid movements (Fuxjager et al. 2013, 2017).

Given that AR supports sexually selected displays in these birds, it is not surprising that we find pronounced changes in the z-scores of their hinge domain (Fig. 4B) and LBD (Fig. 4C). First, both species exhibit the least polar hinge domains in the entire analysis. The functional ramifications of these modifications are unclear, but it is possible that accumulating amino acids with more polar side chains could more strongly tether co-regulatory molecules, amplifying androgen action (Krishnan et al., 2002; Cantin et al., 2007). Second, the manakins exhibit one of the least polarizable LBD (Fig. 4D). Again, how this changes the structure and function of AR is unknown. However, we do know that making a protein more polarizable can alter its ability to interact with other molecules (Quillin et al., 2000). Given this, it is possible that these changes might alter a variety of salient functions of AR including ligand binding and recruiting co-factors to the AF-2 region.

Broadly speaking, it is intriguing to observe taxonomic variation in receptor z-score that is associated with a molecular system that evolved in response to extreme sexual selection. The implication is that the biochemical properties of AR have functional effects that can be traced to reproductive fitness, setting the stage for additional studies that explore how and why this happens.

3.2.2.2. Hummingbirds. We also find substantial variation in AR z-scores of the Anna's hummingbird (*Calypte anna*). This species has a more polarizable DBD, as well as a hinge domain that is far more hydrophilic and less polar than other species (Fig. 4B). However, its LBD is much more on par with the various taxa in our analysis. If the manakins are any guide (see above), such deviation in the biochemical properties of AR may be a signature of strong adaptive evolution of the androgenic system. This idea dovetails nicely with what we know about hummingbirds, which have a unique locomotory program that forms the basis of their courtship behavior (Clark, 2009). Thus, if selection for exaggerated flight (diving) behavior drives the evolution of supportive muscular or neuromuscular adaptations, then the androgenic system may be the hormonal substrate through which this process unfolds. This is because androgens often regulate genes responsible for enhancing skeletal muscle performance (Yeagle et al., 1983; Sassoon et al., 1987; Holmes et al., 2007; Fuxjager et al., 2012), which can otherwise constrain how elaborate a courtship trait can diverge (Miles et al., 2018). Of course, it is unclear how such effects might manifest through modifications to the polarity of key AR domains, but this is nonetheless an intriguing issue to unravel.

3.2.2.3. Pelecaniformes. Finally, we note a group of seabirds that consistently clusters away from other species based on notable differences in physio-chemical properties of their AR, with more dramatic variation found in the Dalmatian pelican (*Pelecanus crispus*) and the great cormorant (*Phalacrocorax carbo*; Figure B–C). Substitutions in the hinge region of these species make it more hydrophobic, less polarizable, and less polar (Fig. 4B). Many seabirds are socially monogamous and are unlikely to undergo the same selection regimes for display performance seen in the polygynous manakins. Instead, we interpret this finding as an indicator that other aspects of an animal's life history may also correspond with modified androgenic signaling. Most seabirds (including the species above) rear only one or two offspring each year, and both parents provision the young for months until they reach independence. If the pelecaniform AR is distinctive due to adaptation, this might reflect modifications to support parental behavior. One alternative hypothesis is that AR variation instead arises due to its influence on physiology and behavior of offspring in the nest. Pelicans in particular exhibit brood reduction; pairs will lay multiple eggs but only raise one chick, which

either starves or is killed by its sibling (Johnston, 2018). Bird parents select which chick to feed based on begging intensity, which can apparently be amplified by androgen-dependent hypertrophy of a muscle necessary for hatching and begging (Lipar and Ketterson, 2000). Various birds also deposit more maternal androgens in the first egg laid (Navara et al., 2006; Müller et al., 2007; Poisbleau et al., 2012; Merkling et al., 2014), which speeds development of the first chick and gives it a competitive edge over its siblings (Poisbleau et al., 2012). To our knowledge, this phenomenon remains unstudied, but the fact that only one pelican chick will survive to adulthood is certainly a context where strong selection pressure would exist on any traits that change an individual's chance of outcompeting its siblings for food. A discovery that AR differences present in pelicans and their relatives is more likely to increase transcription of genes that express begging-related traits would certainly be consistent with the literature to date.

4. Future directions

With all this in mind, it is important to consider how other molecular and genetic modifications to AR may lead to variation in androgenic signaling. Extensive clinical and lab studies have illustrated that *i)* spice variants, *ii)* methylation in non-coding regions of AR, and *iii)* genetic rearrangements near the AR locus can significantly modify the AR structure and function (Jarrard et al., 1998; Jagla et al., 2007; Viswanathan et al., 2018). As such, these phenomena represent additional pathways by which evolution can shape processes of androgen signaling to influence phenotypic change. Yet, there is almost no research that investigates these regulatory avenues outside of the context of human health. In this final section, we briefly explore this literature, and how it may contribute to phenotypic adaptation and diversification.

4.1. Splice variants

Although AR sequence variation is one way to modify androgenic signaling, there is also mounting evidence that AR splice variants could have similar effects (Jagla et al., 2007). Unlike permanent sequence changes (e.g. non-synonymous mutations), splice variants result from enzymatic modification or proteolytic cleavage of AR. AR splice variants exhibit losses of key regulatory regions, including portions of the NTD or LBD (Jarrard et al., 1998; Wadosky and Koochekpour, 2017; Viswanathan et al., 2018). Producing alternatively spliced AR does not require any change to the DNA sequence, so a cell can express the prototypical full-length AR as well as a number of alternative spliced ARs. In theory, this opens new avenues by which androgenic hormones might impact cell physiology outside of the canonical androgenic pathway.

The potential importance of these alternative splice variants in phenotypic evolution is underscored by two ideas. First, these variants often regulate distinct target genes compared to the full-length AR (Guo et al., 2009; Sun et al., 2010; Guo and Qiu, 2011). This suggests that generating splice variants may alter pathways that would otherwise rarely (or never) be regulated by the prototypical AR, leading to novel phenotypes. In addition, many AR variants are able to actuate transcriptional changes in a ligand-independent manner. Although these variants have only been investigated in the context of cancer biology, numerous AR variants expressed in diverse cell types exhibit ligand-independent activity (Jagla et al., 2007; Dehm et al., 2008; Haile and Sadar, 2011). Thus, it is possible variant expression may facilitate tissue-specific maintenance of reproductive traits when levels of androgenic hormones are otherwise basal.

4.2. Methylation of the AR gene

Another emerging area of research focuses on how epigenetic modifications, such as methylation, contribute to changes in androgenic signaling (Jarrard et al., 1998; Sasaki et al., 2000; Keil et al., 2014). In general, such changes occur when DNA methyltransferases add methyl groups to specific nucleotides (CpG islands) located within untranslated regions of a gene (Jarrard et al., 1998). Adding methyl groups to the AR promoter, for example, leads to a pronounced down-regulation of AR (Jarrard et al., 1998). Methylation is reversible and can be induced in a tissue specific manner, and thus constitutes an intriguing mechanism to explain the evolution of tissue and/or seasonal differences in AR expression.

Recent work has provided promising evidence that methylation can lead to marked differences in reproductive traits. Perhaps the best example linking methylation of AR to differences in behavior comes from work conducted in the white-rumped munia (*Lonchura striata*), an oscine songbird, and its domesticated relative the Bengalese finch (*Lonchura striata domestica*). Compared to the white-rumped munia, the Bengalese finch produces songs with less variability and complexity (Wada et al., 2013). Species and individual differences in vocal ability are linked to variation in AR expression in brain regions that controls vocal skill (Wada et al., 2013); that is, the species with less complex song exhibits reduced AR expression in Area X (homolog to the human basal ganglia). More intriguing was that species and individual differences in singing behavior were also linked to the methylation of several CpG islands in the AR promoter (Wada et al., 2013). These findings therefore suggest that the epigenetic program of the AR gene can mediate biologically meaningful differences in a trait that is under strong sexual selection. Thus, exploring patterns of methylation in untranslated regions of AR in other species should help further our understanding of how variation in AR arises at the species and individual levels.

4.3. Changes to the AR locus

A final intriguing avenue for future inquiry centers around how modifications to the AR locus may generate functionally relevant changes in androgen signaling. Recent work suggests that intra-chromosomal rearrangements at or near the AR locus is associated with the severity of prostate cancer (Viswanathan et al., 2018). The duplications of genes upstream of AR can similarly increase the expression of markers associated with cancer (Viswanathan et al., 2018), further suggesting that modifying the AR locus can dysregulate pathways modulated by the receptor. Accordingly, future research is needed to investigate how rearrangements near the AR locus contribute individual or species differences in adaptive traits.

5. Conclusions

Here, we explored the evolution of the AR both among vertebrates, with a subsequent deeper dive into functional variation of this receptor a single vertebrate class (birds). Our aim was to merge our understanding of sequence variation in this receptor with studies of human health and molecular endocrinology, which assess the effects of point mutations in the AR gene on its ultimate functionality. We broke our analyses down into the different domains that characterize AR, finding that overall the protein is highly conserved. Unsurprisingly, regions of this protein with greatest sequence conservation play an integral role in translocation of this receptor to the cell nucleus, interfacing with AREs, and N/C termini interactions – all of which are necessary for activation of AR (Claessens et al., 2008). At the same time, we uncover sequence variation that in theory can confer adaptive changes to how AR works, enhancing or reducing its potency. Intriguingly, some of the most common mutations that we identified occur in key regulatory motifs. This is important as most work suggests that mutations or truncations in these regions are extremely detrimental. We instead posit that the accumulation of mutations in these regions may a strong candidate for supporting adaptation, provided the mutations do not result in the signaling system's breakdown. Maintaining certain regions along the

protein that can support more mutations may allow these regions to be shaped by selection, either to augment or suppress androgen action. In this sense, modification of steroid-dependent traits can certainly occur, at least on a theoretical basis, without actually modulating the signaling machinery in a given tissue. Thus, future work is needed to clarify the functional importance of many of the mutations that we identified.

Within birds, our analyses uncover relatively less variation in the AR protein across species (which is to be expected based on large differences in evolutionary time). The only major domain that shows obvious species differences is the hinge domain, potentially pointing to changes in post-translational modification (acetylation, ubiquination, etc), ARE binding, or translocation. In theory, mutations in this region can substantially modulate the potency of androgen action by altering the ability of AR to bind to DNA or slowing down the degradation of this protein. Regardless, these mutations likely confer meaningful effects to AR function, and may therefore regulate adaptive physiological and behavioral processes that are modified by androgen action. Other regions, including the DBD and LBD, exhibit minor sequence variation across bird species. Nevertheless, the minor differences found in these domains may not be benign. By exploring the physical and chemical properties of the major protein domains, we find species that exhibit notable deviations in these properties have also evolved extreme androgen-dependent phenotypes. One example are the manakins, a group of species known for performing intricate courtship flight displays that are androgen-dependent. These findings lend credence to the idea that selection may alter the AR protein to modify the expression of complex sexual traits. More studies are needed to thoroughly evaluate this hypothesis, but our hope is that these data highlight important topics to guid future work in comparative endocrinology.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2019.110577.

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