Title: Evolutionary endocrinology and the problem of Darwin's tangled bank

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Abstract:

Like Darwin's tangled bank of biodiversity, the endocrine mechanisms that give rise to phenotypic diversity also exhibit nearly endless forms. This tangled bank of mechanistic diversity can prove problematic as we seek general principles on the role of endocrine mechanisms in phenotypic evolution. A key unresolved question is therefore: to what degree are specific endocrine mechanisms re-used to bring about replicated phenotypic evolution? Related areas of inquiry are booming in molecular ecology, but behavioral traits are underrepresented in this literature. Here, I leverage the rich comparative tradition in evolutionary endocrinology to evaluate whether and how certain mechanisms may be repeated hotspots of behavioral evolutionary change. At one extreme, mechanisms may be parallel, such that evolution repeatedly uses the same gene or pathway to arrive at multiple independent (or, convergent) origins of a particular behavioral trait. At the other extreme, the building blocks of behavior may be unique, such that outwardly similar phenotypes are generated via lineage-specific mechanisms. This review synthesizes existing case studies, phylogenetic analyses, and experimental evolutionary research on mechanistic parallelism in animal behavior. These examples show that the endocrine building blocks of behavior can have some elements of parallelism across replicated evolutionary events. However, support for parallelism is variable among studies, at least some of which relates to the level of complexity at which we consider sameness (i.e. pathway vs. gene level). Moving forward, we need continued experimentation and better testing of neutral models to understand whether, how – and critically, why – mechanism A is used in one lineage and mechanism B is used in another. We also need continued growth of large-scale comparative analyses, especially those that can evaluate which endocrine parameters are more or less likely to undergo parallel evolution alongside specific behavioral traits. These efforts will ultimately deepen understanding of how and why hormone-mediated behaviors are constructed the way that they are.

Keywords: parallel evolution; convergence; social behavior; steroid; nonapeptide; receptor; gene expression, experimental evolution

> *"It is interesting to contemplate a **tangled bank**, clothed with many plants of many kinds, with birds singing on the bushes, with various insects flitting about, and with worms crawling through the damp earth, and to reflect that these elaborately constructed forms, so different from each other, and dependent upon each other in so complex a manner, have all been produced by laws acting around us. … There is grandeur in this view of life… from so simple a beginning **endless forms** most beautiful and most wonderful have been, and are being, evolved."*
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> *– Charles Darwin, On the Origin of Species, p.490*

1. Introduction

Evolutionary biologists seek to understand the origins of diversity – the 'endless forms' of Darwin's 'tangled bank'. Proximate mechanisms have the potential to play an important role in this diversity because they give rise to variation that can be shaped by natural selection (Adkins-Regan, 2008; Hau, 2007; Hofmann et al., 2014; Ketterson et al., 2009) (Cox et al., 2016; Fuxjager and Schuppe, 2018; Lema, 2020; Ricklefs and Wikelski, 2002; Rosvall, 2013; Rosvall et al., 2016a; Taborsky et al., 2021). As we begin to consider diversity in complex multi-dimensional traits like behavior, it becomes clear that *many* different mechanisms lie between the genotype and phenotype, including gene activity, neuron firing, hormone secretion, and sensorimotor factors, to name just a few (Adkins-Regan, 2005; Mackay et al., 2009; Sapolsky, 2017; Schlinger et al., 2018). Many of these mechanisms interact with one another, they vary among cell types and tissues, and they vary with age, experience, and other environmental factors (Fischer et al., 2021a). Indeed, there may be mechanisms we have not yet discovered. These endless forms beneath the surface present a challenge as we seek to uncover general principles on the role of mechanisms in phenotypic evolution; beneath outwardly similar phenotypes, there can still exist a tangled bank of *mechanistic* diversity by which even a single phenotype has evolved.

Most of what we know about this issue comes from molecular ecology. This field includes an already large and growing body of literature exploring the degree to which the repeated, independent origin of a particular phenotype is derived via unique or similar mechanisms (reviewed in Arendt and Reznick, 2008; Blount et al., 2018; Bolnick et al., 2018; Rosenblum et al., 2014; Waters and McCulloch, 2021). At one extreme, mechanisms may be identical, such that evolution repeatedly uses the same internal mechanisms or toolkit – for instance, *parallel* changes in gene sequence or expression – to arrive at a particular trait (Rittschof et al., 2014; Toth et al., 2010). At the other extreme, the building blocks of a trait may be unique, such that outwardly similar phenotypes are generated via lineage-specific mechanisms across repeated evolutionary origins (Foote et al., 2015; Mikheyev and Linksvayer, 2015; Warner et al., 2019). These cases are referred to as *non-parallel*. If ancient, conserved mechanisms generate behavioral variation, then we may also see similarities stemming from deep homology and limited divergence (O'Connell and Hofmann, 2011; Waters and McCulloch, 2021). Resolution of these hypotheses lies in robust experimental and comparative analyses of whether and how mechanisms are re-used across evolutionary time.

There is hearty debate over terminology in this area of research (reviewed in Arendt and Reznick, 2008; Blount et al., 2018; Bolnick et al., 2018; Rosenblum et al., 2014; Waters and McCulloch, 2021). Some researchers use the terms 'parallel' and 'convergent' almost interchangeably. Many use ‘convergent’ when the lineages in question are *distantly* related and a particular trait arises independently, whereas ‘parallel’ is used when lineages more *recently* share a common ancestor, such as congeners or conspecifics. There is no clear demarcation between the two terms, yielding some recommendations to drop the term ‘parallel’ altogether (Arendt and Reznick, 2008); however, in the intervening 15 years, the number of publications using ‘parallel’ to describe cases in which the same gene(s) are re-used in trait evolution has only increased. Here, I follow the convention proposed by Rosenblum et al. (2014), who noted that convergent evolution may describe a phenotypic pattern, whereas parallel more aptly describes the mechanisms that underlie phenotypic convergence among lineages. These are the operational definitions use here.

In this synthesis, I highlight important knowns and unknowns related to mechanistic parallelism in behavioral evolution, with a focus on endocrine systems that may serve as important building blocks of behavioral variation. Evolutionary endocrinology has already greatly advanced our understanding of the evolution of hormonal systems (reviewed in Baker, 2019; Hau, 2007; Ketterson et al., 2009; Thornton, 2001; Wingfield, 2018; Zera et al., 2007, and many other reviews cited throughought the current manuscript). With exceptions noted below, it is generally unresolved whether certain components of endocrine systems are re-used to facilitate the repeated evolution of particular behavioral traits. My goal is determine whether the same (or different) mechanisms are employed repeatedly when a particular behavioral phenotype evolves in different taxonomic lineages. In doing so, I hope to reveal the tangled bank that lies beneath the skin, to better understand exactly how hormones give rise to behavioral variation in nature.

2. Building blocks in the endocrine-molecular architecture of behavior

Let’s first consider what neuroendocrine building blocks could be used by natural selection to bring about changes in behavior. Homologous brain structures and circuitry certainly play a role (Goodson, 2005), but this review concentrates on signals and the endocrine systems in which they are produced and received. In behavioral endocrinology, we tend to focus on a few specific endocrine systems that appear to influence both microevolutionary processes – those acting within a single population – and macroevolutionary processes – those that arise over vast periods of time, differentiating the behavior of species and other higher orders of taxonomic diversity.

For example, let’s start with testosterone. This sex steroid hormone often promotes aggressive, sexual, and other reproductive-related behaviors. Testosterone was featured in one of the first evolutionary frameworks in vertebrate endocrinology – the challenge hypothesis, which led to a set of predictions linking testosterone to variation in other traits (Wingfield, 2012; Wingfield et al., 2020; Wingfield et al., 1990), including inter-specific variation in aggression and mating competition, at least in some vertebrate groups. The challenge hypothesis continues to be tested, extended, and revised (Archer, 2006; Goymann et al., 2019; Moore et al., 2020; Rosvall et al., 2020), and we continue to gain insight on testosterone and its relationship with ecology, life history, and behavior (Garamszegi et al., 2005; Garamszegi et al., 2008; George and Rosvall, in press; Husak et al., 2021; Lipshutz and Rosvall, 2021).

Glucocorticoids, such as corticosterone or cortisol, also have strong connections to a variety of fitness related traits, including behavior (Martin, 2009; Sapolsky et al., 2000). Glucocorticoids facilitate the mobilization of resources under challenging conditions (Hau et al., 2010; Jessop et al., 2013; Schoenle et al., 2021; Vitousek et al., 2019), and they have some relationship to survival and reproduction (Bonier et al., 2009; Breuner and Berk, 2019; Cox et al., 2016; Ouyang et al., 2013; Schoenle et al., 2021), suggesting that these steroid hormones also may play some role in behavioral divergence.

Considering these many connections between hormones, behavior, and fitness-related traits, it is tempting to speculate that testosterone and corticosterone may form evolutionary 'hotspots' of behavioral change. In the context of molecular evolution, hotspots are defined as the 'repeated occurrence of de novo mutations at orthologous loci, causing similar phenotypic variation' (Martin and Orgogozo, 2013). In the context of behavioral endocrinology, we might view hotspots as endocrine components (or, building blocks) that repeatedly bring about the same behavioral changes across many lineages. How might this occur?

In some endocrine systems, the hormone itself is a building block and direct gene product, for instance oxytocin, encoded by the *OXT* gene, or vasopressin, encoded by the *AVP* gene. Within each of these systems, there are still multiple building blocks that may affect behavioral variation, including peptide release and multiple cross-reactive receptors (Donaldson and Young, 2009; Kelly and Goodson, 2014; Kelly and Ophir, 2015; Young, 1999).

Steroid hormones also appear to have some genetic basis (King et al., 2004; Mills et al., 2009; Pottinger and Carrick, 1999; Stedman et al., 2017), but they are not direct gene products. Instead, steroid production depends on an endocrine cascade across multiple tissues and enzymatic pathways. Within the hypothalamo-pituitary-adrenal (HPA) and hypothalamo-pituitary-gonadal (HPG) axes – we see an incredible array of potential building blocks (Adkins-Regan, 2005; Fuxjager and Schuppe, 2018). Including brain, pituitary, adrenal, and gonad, there are numerous interacting hormones and receptors affecting one another, any one of which could potentially be use in behavioral evolution. Enzymes regulating hormone synthesis can be behaviorally potent (e.g. aromatase; Forlano et al., 2006), and different metabolites can have unique downstream effects (Frankl-Vilches and Gahr, 2018). Hormones must be transported from their site of production to their site of action, a process facilitated by binding globulins (Deviche et al., 2001; Swett and Breuner, 2008). At the target cells, further conversion may occur towards more potent or less potent hormones (Schmidt et al., 2008). Also at the target tissue, transduction involves positive co-factors, negative co-factors, and tissue specific effects on gene expression (Peterson et al., 2013; Schuppe and Fuxjager, 2019; van Nas et al., 2009), including interactions with other important signaling systems (Heinlein and Chang, 2002). Receptor abundance and affinity also may vary, oftentimes independently of circulating levels of the hormone itself (Hunter et al., 2018; Lattin et al., 2015; Lipshutz et al., 2019; Rosvall et al., 2012), and different types of receptors may have different behavioral effects (Alward et al., 2020). Sequence variation in regulatory regions of the genome can affect when and how much each of these building blocks is expressed (Klinge, 2001), and sequence variation in coding regions can affect interactions among building blocks, such as binding affinity between hormone and receptor in behaviorally relevant tissues (Schuppe et al., 2020). Steroid hormones also may affect behavior via non-receptor mediated effects on secondary messenger systems (Remage-Healey et al., 2008; Stahn and Buttgereit, 2008) or via membrane-bound steroid hormone receptors (Balthazart, 2021). There are also interactions among the HPA and HPG axes, interactions with non-steroidal mechanisms of behavior, and interactions with other components of the body, such as the liver, gut, fat stores, and so on.

This list continues to grow as we learn more, leaving a truly massive tangled bank of endocrine-molecular building blocks that could change and affect behavioral evolution. Which, if any, of these building blocks are used repeatedly to elicit behavioral evolutionary change, and why?

3. Hypotheses on why re-use of particular mechanisms may occur

Given this almost absurdly large set of options, it may seem remarkable for evolution to use the same endocrine-molecular mechanisms to bring about behavioral change again and again, but there are also biological phenomenon that may favor re-use of the same mechanisms.

For instance, building blocks may vary in their mutability. Certain areas of the genome are more prone to mutation, with even some nucleotide substitutions being more probable than others (Storz, 2016). Given time and drift, these mutations may spread or go to fixation. Looking at androgen receptor as one example, regulatory domains appear more evolutionarily labile than functional domains (Schuppe et al., 2020). In androgen-dependent cancers, certain mutations are more likely than expected by chance (Gottlieb et al., 2012), and androgen receptor DNA binding sites also show elevated mutational rates (Morova et al., 2020), indicating some mutational biases in both coding and non-coding regions.

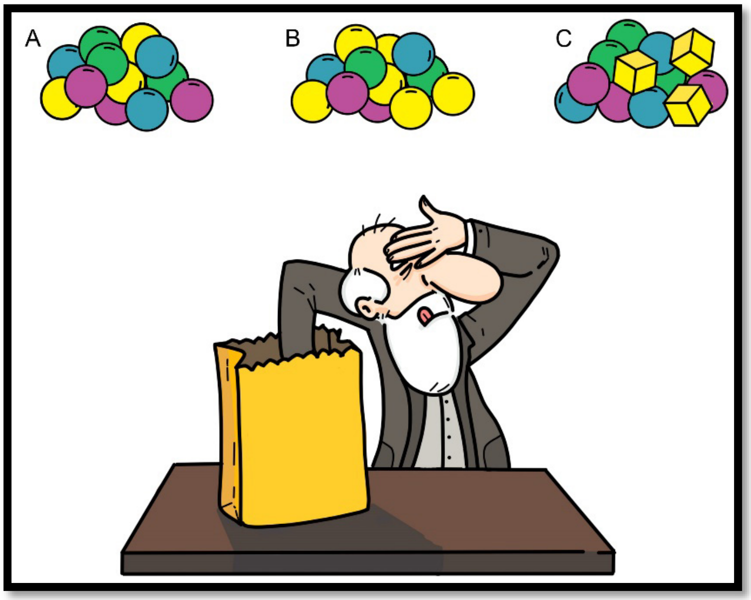
Even if the probability of a mutation in two building blocks is equally likely, changes in certain mechanisms may be better for some reason. This can occur if the mechanism *itself* is associated with some fitness benefit or cost. Well-known examples in molecular biology stem from epistasis (when the effect of one gene depends upon another gene) or pleiotropy (when one gene affects multiple phenotypes). Related ideas have been considered in evolutionary endocrinology for some time, *sensu* Hau (2007)'s evolutionary potential vs. constraint and Ketterson et al. (2009)'s integration vs. independence. Returning again to testosterone, its marked pleiotropic effects mean that selection may 'see' the behavioral mechanism (Dantzer and Swanson, 2017; Rosvall et al., 2020) via effects on genetic correlations (Cox, 2020; Cox et al., 2016) and many different fitness-related traits (Ketterson et al., 1992). Consistent with this view, some organisms seasonally switch away from behavioral mechanisms that depend on gonadal testosterone (Canoine et al., 2007; Demas et al., 2007; Quintana et al., 2021; Soma et al., 2015). This seasonal switch is thought to be adaptive, tied to a need for territorial aggression in the non-breeding season, alongside high costs and limited benefits of testosterone outside of the breeding season (Wingfield et al., 2001). There is also some evidence that females – who, like a wintering animal, have low testosterone but may nonetheless experience selection for higher aggression – also facilitate aggression via mechanisms that do not necessarily require high levels of testosterone in circulation (Bentz et al., 2021; Duque-Wilckens and Trainor, 2017; Munley et al., 2018; Rosvall, 2013; Rosvall et al., 2020).

These examples suggest that the adaptive value of the mechanism itself is important. These examples also show that different mechanisms can facilitate a particular behavior even within a single species, prompting the question as to whether similar ideas apply over macroevolutionary time.

With these concepts in mind, I envision at least three hypotheses in need of further testing. These hypotheses are illustrated via cartoon (**Figure 1**) in which evolution (depicted as Darwin) has different sets of building blocks to choose from in a bag, as he 'decides' how evolution should proceed (none of this is conscious, of course; this is a metaphor). Each building block is a unique endocrine parameter in the tangled bank of mechanisms described in **§2 –** a particular receptor, co-factor, enzyme, etc. Each building block has the potential to affect behavioral expression.

In one option (**A**), there may be many different building blocks, and their probability of use in each independent evolutionary origin of a trait may be random – yellow blocks are just as likely as any other color in the set of blocks in Darwin's bag. By example, we might see that paternal care is associated with a change in oxytocin receptor expression in one lineage, and a change in vasopressin peptide release in another. With this option, if parallelism does occur, it essentially stems from the fact that even rare, neutral events can occur repeatedly given enough time, and they may go to fixation in finite populations (i.e. neutral theory, Kimura, 1983). Next (**B**), some mechanisms may be more likely to occur – yellow is more frequent in the pool of building blocks, but it is no more fit than other building blocks. Adding to this toy example, oxytocin may be more commonly associated with paternal care if the *OTXR* gene is hyper-mutable, compared to other possible endocrine mechanisms that also affect paternal care. Finally (**C**), one mechanism may be better (or less likely to be deleterious); now, yellow is a superior building block. Here, parallelism may occur from the adaptive value of this particular mechanistic vs. others.

FIGURE 1. Colors denote different building blocks in the endocrine molecular architecture of behavior. Evolution, depicted by Darwin, 'chooses' building blocks from the bag of possible blocks. Options A, B, and C show different sets of building blocks within the bag of possibilities. Note how yellow blocks switch from equally probable in (A), to more probable but equally fit in (B), to equally probable but more fit in (C). *Illustration by Dr. Tamanash Bhattacharya*



There are many methods for evaluating parallelism, building from past work (reviewed in Arendt and Reznick, 2008; Blount et al., 2018; Bolnick et al., 2018; Rosenblum et al., 2014; Waters and McCulloch, 2021). We can quantify rates of molecular evolution at the gene level, say, in coding regions that affect enzyme efficiency or receptor binding, perhaps finding certain genes that consistently show signs of positive selection, typically measured as the rate of non-synonymous vs. synonymous nucleotide substitutions. We can also examine concordance (or, similarity) in gene expression, receptor abundance, or any other building block listed above, looking among lineages to see if the same components of endocrine systems are up- or down-regulated in relation to a particular behavior. We can scale these questions to higher levels of complexity, asking whether the same functions or pathways repeatedly co-occur with macro-evolutionary patterning in a particular behavioral trait. We can also compare across these mechanistic levels, asking whether parallelism is more likely to occur at the level of the amino acid, gene, or pathway (Rosenblum et al., 2014). In endocrine systems, we might further categorize building blocks into their functional role, such as receptor vs. enzyme, or HPA vs. HPG.

4. Evidence on mechanistic parallelism, non-parallelism, and the gray areas in between

This section reviews select examples in behavioral evolution, focusing on the degree of mechanistic parallelism underlying similar changes seen in different lineages. Examples are grouped by approach, first covering case studies using (a) a limited set of candidate mechanisms or (b) whole genome analyses; these first two sets do not use phylogenetic methods. Next, I discuss related research that more explicitly considers evolutionary history, including (c) phylogenetic comparative analyses and (d) experimental evolutionary approaches. Via this overview, my goal is not to understand the evolution of all endocrine systems per se, but rather, to understand whether, how, and eventually why, certain endocrine parameters are re-used in behavioral evolution across the tree of life.

a. Case studies

Paired comparisons of species that differ in their life history or ecology can tell us about how convergence or divergence proceeded in the past (Goymann and Schwabl, 2021). These case studies provide some evidence of mechanistic parallelism; however, they also demonstrate interspecific variation in endocrine systems, which we now know to be widespread.

It is clear, for example, that experimental treatment with a particular hormone can have different effects in different species (Bonier and Cox, 2020; Cox et al., 2009; Stiver and Alonzo, 2009). Behaviorally, androgens have opposing effects on communication signals in different weakly electric fish species (Smith, 2013), and supplemental testosterone inhibits parental care in some songbirds, but not in others (Lynn, 2008). With caveats for methodological variation such as dosage, these interspecific differences suggest that *some* components of the androgenic signaling system diverge over evolutionary time. Species do appear to share some commonalities in the gene networks that are activated by supplemental testosterone; these similarities are more striking at the pathway level, for instance with testosterone affecting pathways related to innate immunity, but different genes in different species (Newhouse and Vernasco, 2020; Peterson et al., 2013; van Nas et al., 2009).

Sex steroid hormone receptors appear to be commonly-used building blocks of sexual and social behaviors. Several studies link individual differences in aggression to variation in sex steroid sensitivity or metabolism in the brain (Goodson et al., 2012; Horton et al., 2014; Rosvall et al., 2012), suggesting there is standing variation upon which selection might act to bring about changes in behavior over evolutionary time. Across the songbird radiation, we know that the avian song system has diverged in its neural expression of androgen receptor and even more so estrogen receptor (Frankl-Vilches and Gahr, 2018), potentially accounting for some species-specific variation in behavior (Brenowitz and Beecher, 2005; Gahr, 2014). Androgen receptor mRNA abundance is also high in the longus colli ventralis (neck) muscle in two woodpecker species compared to a different species that does not use this neck muscle in sexually selected communication (Schuppe and Fuxjager, 2019). More analyses are needed to understand how sex steroid receptors are repeatedly used by evolution to enhance sexually selected traits (see also **§4c**). Examples from elaborate or acrobatic displays suggests that this may be true for androgen receptor (Fuxjager et al., 2022; Fuxjager et al., 2013; Schlinger et al., 2013), though evidence on the avian song system also reveals inter-specific variation in the molecular architecture connecting hormone receptors to behavior (Frankl-Vilches and Gahr, 2018).

Case studies on invasion dynamics and urban adaptation provide another set of replicated selective pressures, and several such examples point to the HPA axis as one source of repeatedly used building blocks for boldness, aggression, or other proactive behavioral traits.

Several studies report higher corticosterone secretion, at baseline or in response to a standardized stressor, in (i) invasive species (Brown et al., 2015; Liebl and Martin, 2012; Martin et al., 2017), (ii) populations that have moved into more urban areas (Atwell et al., 2012; Ouyang et al., 2019), and (iii) other naturally occurring range expansions (Duckworth and Badyaev, 2007; Dunlap and Wingfield, 1995; Walker et al., 2015). However, many other studies report the opposite or no difference (Fokidis et al., 2011; French et al., 2008), despite many examples of convergent behavioral traits. These mixed results were highlighted recently in a large comparative analysis on birds and reptiles, showing that, at least in the context of urban adaptation, changes in corticosterone are not predictable (Injaian et al., 2020). Of course, as populations diverge into species, we also may see differences in receptor expression (Liebl and Martin, 2013; Rosvall et al., 2016a), steroidogenic enzymes (Rosvall et al., 2016b), and negative feedback (Zimmer et al., 2020), indicating that changes among multiple building blocks can occur. However, we do not yet have a good understanding of whether one or another endocrine building block contributes consistently to behavioral variation each time populations diverge in response to colonization of new habitats.

Interspecific variation in social grouping and mating systems is also thoroughly researched for its neuroendocrine mechanisms, especially building blocks in the vasopressin/oxytocin family of genes (Kelly and Ophir, 2015; Potticary and Duckworth, 2021; Soares et al., 2019). For example, looking at areas of the social behavior network in the brains of Estrilid finches, Goodson and colleagues showed that highly gregarious species have more vasotocin immunoreactive neurons compared to territorial species, and these neurons are activated by affiliative stimuli (Goodson and Wang, 2006). Comparable findings were also found among other groups of birds, rodents, and frogs (Anacker and Beery, 2013; Fischer et al., 2019; Goodson et al., 2012; Turner et al., 2010; Young et al., 1998). Yet in the details, these examples reveal multiple paths to a particular behavioral phenotype (e.g. change receptor expression in one group, change peptide signaling in another).

b. Omics case studies

Massively parallel sequencing and gene expression analyses are useful for integrating mechanistic approaches into a hypothesis-driven framework (Travisano and Shaw, 2013; Zuk and Balenger, 2014). Qualitatively, many early transcriptomic analyses of non-model systems pointed to aspects of neuroendocrine systems as repeatedly used building blocks of behavioral variation (reviewed in Harris and Hofmann, 2014; Rittschof and Robinson, 2016).

Quantitatively, this bears out in pairs of comparisons that ask whether the genomic signatures of a behavioral trait in one species are concordant in another species. For example, in colonies of honeybees, some individuals show muted behavioral responses to otherwise provocative stimuli; the brains of these socially unresponsive bees share transcriptomic similarities to humans diagnosed with autism-spectrum disorder (ASD), a set of phenotypes also characterized by social avoidance (Shpigler et al., 2017). This suggests that ASD-like behaviors either share a deeply conserved genomic signature, or they arose independently via parallel genomic mechanisms.

Many studies have measured gene activity to understand how social behavior affects the brain. Drnevich et al. (2012) combined several of these in songbirds and found marked heterogeneity in the neurogenomic response to each of six different experiments in which birds heard or interacted with a conspecific. With caveats for the brain areas and developmental stages across the six experiments, these findings suggest that *different* mechanisms respond to similar behavioral stimuli in different species. Using a broader taxonomic scale and RNAseq, two later analyses measured gene expression in bees, fish, and mice (Rittschof et al., 2014; Saul et al., 2019), and reported widespread *similarities* in the brain's response to social challenge across these distantly related taxa. Notably, these similarities operate at the pathway level; the specific genes that respond to social challenges and the time course of those responses are not fully parallel. In addition, these analyses do not formally use a phylogenetic framework, leaving uncertainty as to whether similarities stem from convergence or shared use of ancestral patterns.

c. Phylogenetic comparative data

Over the past 15 years, explicitly phylogenetic comparisons have vastly expanded data on mechanistic parallelism in phenotypic evolution. In animals, well known morphological examples include repeated evolutionary changes in body size, which are associated with different genetic loci (Wilches et al., 2021) or convergent evolutionary changes in pigmentation that are associated with parallel changes in melanocortin signaling (Hubbard et al., 2010). Physiological traits also show some mechanistic parallelism, including salinity tolerance (Reid et al., 2016) and altitude tolerance (Lu et al., 2020; Witt and Huerta-Sanchez, 2019), although the degree of conservation varies among taxonomic groups and the level at which sameness is considered, with several examples of lineage-specific changes at the gene level.

Quantitative phylogenetic approaches have been historically neglected in animal behavior (Price et al., 2011), but this is changing rapidly (e.g. Antonson et al., 2020; Cowen et al., 2020; Mason et al., 2017; Miles et al., 2020; Odom et al., 2014). Similarly, behavioral endocrinology was slower to adopt formal phylogenetic analyses (but see Garamszegi et al., 2005; Garamszegi et al., 2008) though this too has shifted, particularly in the last few years following the publication of HormoneBase (Vitousek et al., 2018). Despite these gains, we are still in the early days of robust phylogenetic analyses that co-consider the evolution of endocrine systems alongside behavior.

Most phylogenetic research on the molecular mechanisms that facilitate behavioral evolution has focused on a few discrete or extreme behavior differences, such as loss of flight (Pan et al., 2019) or transitions to subterranean living (Davies et al., 2018). Among the most interesting examples is a comparative analysis of marine adaptation in three mammalian lineages (walrus, manatee, cetacean), vs. 17 non-marine lineages (Foote et al., 2015). Results highlight 44 parallel non-synonymous amino acid substitutions in the three marine lineages, and nearly all stemmed from the *exact same* nucleotide change. Roughly a third of these substitutions also showed evidence of positive selection (i.e. non-synonymous substitutions) in at least one marine lineage. However, this incredible case of phenotypic and molecular convergence is not the full story – the authors identified *even higher* rates of molecular convergence among the non-marine sister lineages (cow, dog, elephant). Thus, parallel molecular evolution may be common as a neutral background process. A subsequent paper also showed that adding more species to this analysis erodes support for parallelism, eliminating ~80% of the previously identified parallel substitutions (Thomas et al., 2017). These complexities highlight a number of methodological considerations, such as the null hypotheses against which we test mechanistic parallelism, a topic I will unpack further below (see **§5a**).

Eusociality has also arisen independently at least 10 times in bees, wasps, ants, and termites. Some studies find eusociality-associated similarities in sequence evolution (Woodard et al., 2011) and caste-related brain gene expression (Toth et al., 2010), providing evidence of mechanistic parallelism. However, most evidence suggests that the molecular toolkit for eusociality is ‘loose’ (Berens et al., 2015), with lineage-specific solutions at the gene level coupled and greater similarities at higher levels of organization (Rehan and Toth, 2015; Woodard et al., 2011). These conclusions apply within the Hymenoptera (Kapheim et al., 2015) and Isoptera (termites) (Harrison et al., 2018). Both groups show signatures of positive selection on genes related to chemoreception, but they differ in the specific class of receptors that are affected. Although some of these analyses point to endocrine-related building blocks of eusociality, their utility for questions in evolutionary endocrinology is more related to the sheer complexity of these systems. Like endocrine systems, they involve many interacting and redundant parts, each of which has pleiotropic and epistatic effects that may shape exactly how each building block is used over evolutionary time.

Focused broadly on animal communication, the data thus far point to high degrees of mechanistic parallelism across different lineages. Hawaiian swordtail crickets have diverged in their mating song, and Blankers et al. (2019) found striking parallel molecular evolution involving the same regions of the genome across three separate pairs of *Laupala* species that differ in song pulse rate. Electric communication has evolved in three separate lineages of fishes, which show convergent amino acid substitutions (Wang and Yang, 2021) and similar transcriptomic profiles at the pathway level, with fewer similarities at the gene level (Liu et al., 2019). Vocal learning has also arisen independently in several mammalian and avian lineages (Petkov and Jarvis, 2012). When four such lineages were compared (humans, songbirds, parrots, and hummingbirds), their brains showed significant similarities in transcriptional profiles (Pfenning et al., 2014). Here, the details include building blocks related to amine signaling, including *MAOB*, *DRD1*, and *HTR1B*.

Analyses on other aspects of social behavior, including mating and parenting, provide mixed support for mechanistic parallelism. On the one hand, asexual reproduction shows marked similarity in its transcriptomic profiles across five independent origins in stick insects (*Timema* spp) (Parker et al., 2019). On the other hand, a comparative analysis of four pairs of cichlid species found no consistent relationship between cooperative breeding and mRNA expression for five key genes in the oxytocin-vasopressin family (isotocin, vasotocin, and receptors) (O'Connor et al., 2015). In other words, different species arrive at cooperative breeding via different endocrine-molecular mechanisms, albeit all of those related to nonapeptide signaling. Somewhere in the middle between non-parallelism and parallelism is a comparative analysis on the evolution of monogamy across multiple, deeply diverged vertebrate lineages (Young et al., 2019). Among ~2000 orthologous gene groups expressed in all 5 species pairs, about 6% shared consistent expression patterns in the brains of monogamous vs. non-monogamous species. This is statistically more than expected by chance, thought it bears repeating that tens of thousands of genes were not expressed in parallel among monogamous lineages. In addition, the common building blocks of social behavior – such as biogenic amines, sex steroids, glucocorticoids, and related receptors – were *not* present in this shared signature of monogamy, and targeted analyses of these neuroendocrine parameters show limited concordance in their expression among the monogamous vs. non-monogamous species pairs.

Other phylogenetic analyses focused on androgen receptor do find support for the hypothesis that neuroendocrine parameters may be hotspots of behavioral evolution. For instance, androgen receptor is more highly expressed in the forelimb muscles of frogs that have independently derived foot flagging as a sexually selected display behavior (Anderson et al., 2021). Androgen receptor expression in forelimb muscles also correlates with display frequency in anoles (Johnson et al., 2018), and this gene is upregulated in association with the wing-snap display in manakins (Pease et al., 2022).

These diverse phylogenetic analyses provide some evidence that genomic parallelism underlies behavioral convergence, though parallelism is more marked at higher levels of complexity. Critically, there are gray areas in our consideration of parallel mechanistic evolution. If only a tiny portion of the genome shows parallel evolution, is that parallelism? Functional experimentation with candidate building blocks will be key to understanding whether even small degrees of parallelism matter, behaviorally. More importantly, classical differential expression analyses may not appropriately estimate null models of background drift, and phylogenetically informed analyses are a key advance (see Pease et al., 2022). Moving forward, I urge future research to investigate cases of non-parallelism and why they occur, in addition to small but statistically significant cases of parallelism.

d. Experimental evolution

Artificial selection provides an especially powerful tool for identifying the scope of changes that could occur over evolutionary time (reviewed in Garland et al., 2016). These experiments typically mirror the process of divergence because they begin with the same starting population. Despite this non-independence in ancestral states, these experiments have been useful in drawing inferences on the mechanisms that facilitate the evolution of complex traits like behavior. For instance, in great tits (*Parus major*), females from lines selected for a bolder (fast) coping style exhibited more robust elevation of estradiol in response to a GnRH challenge (Caro et al., 2019), suggesting that one or more components of the HPG axis have diverged alongside selection for boldness. These bold vs. shy lines do not differ in glucocorticoid responses to a novel object (Baugh et al., 2017), in contrast to artificial selection on bank voles (*Myodes glareolus*), which leads to muted elevation of corticosterone in a line selected for predatory boldness (Lipowska et al., 2020). Artificial selection on aggression in mice involves changes in corticotropin-releasing hormone-binding protein (*CRHBP*) and in adrenergic receptors (D'Anna et al., 2008; Gammie et al., 2006; Scotti et al., 2011). Similarly in cattle, selective breeding for aggression is associated with changes in the promotor region of monoamine oxidase A (*MAOA*) (Eusebi et al., 2020) as well as differences in brain gene expression for *DRD2*, *IGF2*, *BDNF*, and *CRHBP* (Eusebi et al., 2021). Eusebi and colleagues also combine their analysis with a set of ~1700 genes previously linked with aggression in other species (Zhang-James et al., 2019). Across humans, rodents, foxes, cattle, and dogs, mechanistic parallelism was low: zero genes were associated with aggression in all contrasts, and fewer than 10% of genes appear in just two contrasts, most notably *AVPR1A* and *MAOA*. Via these examples, we see some commonalities in the endocrine building blocks that change alongside artificial selection on specific behavioral traits, but we also see differences and we do not yet know why.

Replicated selection experiments allow us to take these inferences a step further, using an ‘evolve and re-sequence’ (or, evolve and re-assay) approach, which is key to quantitative evaluation of mechanistic parallelism. Garland’s replicated selection lines for high levels of wheel-running in mice reveal some consistent endocrine differences, including plasma levels of adiponectin and corticosterone (Malisch et al., 2007; Vaanholt et al., 2007). In fruit flies, Dierick and Greenspan (2006) found several hundred differentially expressed genes between any paired comparison of lines selected for high aggression vs. control lines, but only 80 of these genes consistently differed in the same direction in replicate high vs. control lines. Also in *Drosophila*, Edwards et al. (2006) reported ~1500 differentially expressed genes that consistently differed in relation to selection on aggressiveness in flies. Notably, there were differences in the specific genes and the scope of parallelism in these two *Drosophila* experiments, suggesting some degree of non-parallelism, at least when the ancestral states are non-identical (i.e. different stock populations). Malki et al. (2014), working with three independently derived and artificially selected populations of mice found 70 genes were consistently differentially expressed in the brains of all three contrasts of high vs. low aggression mice; however each replicate also had its own unique set of aggression-related genes. Only 7 of these genes (~10%) were also differentially expressed in the brains of fish exposed to aggressive experiences (Malki et al., 2016), suggesting that there may be some building blocks of behavior that are repeatedly used, but genetic background, standing variation, and stochastic processes play a role in the degree of mechanistic parallelism (Robin et al., 2007).

Replicated domestication can also shed light on the mechanisms underlying behavioral convergence because domesticated animals often display diminished anxiety or aggression. Work in foxes and dogs indicates that serotonin receptors may be differentiated during domestication, though each independent case reports expression or sequence divergence in a *different* serotonin receptor (Akey et al., 2010; Bence et al., 2017; Kukekova et al., 2011; Li et al., 2013; Wang et al., 2018). Hou et al. (2020) extends similar comparisons to the domesticated vs. wild chicken, cat, cattle, horse, and rabbit, finding only 26 genes that were concordantly expressed among any two pairs of contrasts. At higher levels of complexity, though, mechanistic similarities emerge. For instance, 7 pathways involving neurotransmission were enriched in domesticated species, but each lineage varied in exactly how that was achieved (i.e. endocannabinoids vs. catecholamines). Again, mechanistic parallelism appears stronger at the pathway or functional levels than at the gene level.

5. Next steps in applying this framework to evolutionary endocrinology

There is intuitive appeal to the hypothesis that mechanisms of behavior are tailored to specific life history and ecological contexts, just as there is appeal to the hypothesis that nature repeatedly uses the same, or parallel, mechanisms in replicated cases of phenotypic evolution. The historically separate study of function and mechanism in animal behavior has limited progress on these hypotheses, though there is fast progress on this integration in behavioral endocrinology, as summarized above. As the discipline moves towards larger and more quantitative analyses in the age of big data, we are well poised to unveil further insights on the degree of sameness in behavioral evolution. In this final section, I propose greater attention to asking: If there is mechanistic parallelism, then why? And if not, why not?

a. Robust testing of adaptive hypotheses

Modern behavioral ecology was born from early insights that behavior can evolve – that life history and ecological factors shape behavioral diversity among species (Birkhead and Monaghan, 2010; Cullen, 1957; Tinbergen, 1963), a perspective that is also woven through the incredible successes of early evolutionary endocrinology (Wingfield, 2018; Zera et al., 2007). Now is an exciting time to learn more about how this same 'ecology of selection' shapes what lies beneath the skin. Summarized above, it is clear that the building blocks of behavior can have some elements of parallelism across replicated evolutionary events. However, support for parallelism is variable among studies, at least some of which relates to the level of complexity at which we consider sameness (i.e. pathway vs. gene level). Now is a good time to extend these areas of inquiry, asking: why exactly do we see mechanism A in one lineage and mechanism B in another?

Key to these efforts will be devising adaptive hypotheses and testing them against alternatives. Why do we see the same mechanisms used repeatedly for some behaviors or for some species, but not for other behaviors or other species? Is there something adaptive about one particular mechanism in a particular context? In an intriguing example related to hypoxia, Natarajan et al. (2018) synthesized different intermediary forms of hemoglobin, asking how each affected oxygen binding affinity. One variant was superior, suggesting parallel molecular evolution would be adaptive; however, that variant does *not* occur in wild geese adapted to high-altitudes (Natarajan et al., 2015). This example is analogous to using pharmacological experimentation to understand functional effects of one or another endocrine building block, and asking whether those experimental manipulations mirror what occurs during natural phenotypic differentiation in the wild. For instance, Ketterson and Nolan's now classic work on testosterone in juncos showed us why testosterone levels are not higher in nature (Gerlach and Ketterson, 2013; Reed et al., 2006). Comparable experiments manipulating a range of endocrine-molecular building blocks will allow us to better see when and why experimental and natural variation align. In essence, this will formally test hypotheses A, B, and C from **Figure 1**, ultimately shedding light on if, then, and why selection ‘cares’ exactly how particular traits are built.

We already have some evidence that the distribution of hormone receptors is less evolutionarily labile than ligands (O’Connell and Hofmann, 2012). Likewise, there is a rich history on the molecular evolution of steroid receptors and steroidogenic enzymes (Baker, 2019; Thornton, 2001), but these analyses have not yet been broadly layered atop behavioral variation. Are metabolic enzymes more or less likely to predict behavioral variation than receptors or co-factors? To what degree are the components of sex steroid signaling systems more or less likely to undergo parallel evolution, compared to say, nonapeptide systems? Answering these questions will help us to resolve which building blocks (if any) are overrepresented during phenotypic evolution and why (see **Figure 1**). I imagine, as others have noted before (see **§3)**, that some mechanisms may better generate adaptive pleiotropy or better minimize maladaptive pleiotropy. This is an empirical question we can address.

b. Clarifying neutral expectations

It is also critical that we better account for the background occurrence of neutral molecular evolution, even without layering on hypotheses of adaptation. This will require revised statistical tools, large phylogenetic comparisons of multiple endocrine parameters, and appropriate null models, a potentially tall order in a comparative, largely field-based discipline. Carefully devised species comparisons can help us to achieve this goal, clarifying null expectations on mechanistic parallelism (see discussion of Foote et al., 2015, above). Evolutionary distance also may shape these null hypotheses. Time and drift may lead to the accumulation of lineage-specific mechanisms, or alternatively, they also may allow lineages to acquire the same mechanistic solution, the latter of which finds some support in both guppies (Fischer et al., 2021b; Ghalambor et al., 2015) and stickleback (Kingman et al., 2021). In addition, the number of building blocks we analyze is related to the probability of finding parallelism (Conte et al., 2012). Therefore, evaluation of specific, functional candidates may be an important step towards understanding the scope of parallelism (Marcovitz et al., 2019). Though there are certainly undiscovered mechanisms that will eventually warrant inclusion, endocrinology is well positioned to remain focused on a core set of biologically relevant candidates, informed by past experimental knowledge on the building blocks that alter behavioral expression.

Sources of variation also shape inferences on mechanistic parallelism in a number of ways. Mutation across the genome is non-random (Storz, 2016), and we need more analyses on how this plays out across the diverse neuroendocrine building blocks of behavior if we are to robustly test deviations from this null. Standing variation is a related factor (Barrett and Schluter, 2008) because is not always clear whether: (i) there was pre-existing variation in the common ancestor that was later (independently) shaped by drift or selection; (ii) new mutations arose (independently) and were later shaped by natural selection; or (iii) lineages hybridized leading to parallelism via introgression (Lee and Coop, 2019; Waters and McCulloch, 2021). Evolutionary endocrinology can address these questions in new and interesting ways, integrating micro-evolutionary questions on individual differences with comparative analyses that can evaluate how functional axes of behavior change over evolutionary time. Natural variation (within and among species) is ideally suited to explore these problems because it can tell us how evolution has proceeded when faced with an incredible array of options like what we see in neuroendocrine systems.

This tangled bank adds one final complication: the pace of evolution is generally thought to be inversely related to complexity (Mauro and Ghalambor, 2020). In endocrine systems, though, there is support that hormones can both constrain or facilitate adaptive evolution (Hau, 2007;

Ketterson et al., 2009). For instance, a change in testosterone secretion may hasten evolution if testosterone's pleiotropic effects change many different traits at once; however, if pleiotropy is costly, then the very same change may impede the pace of evolution, more so than say, a targeted change in androgen receptor in one tissue. Complexity also introduces the potential for redundancy and epistasis, which can reduce the potential for convergence (Zou and Zhang, 2017). By example, a mutation that affects the final step of steroidogenesis may have limited phenotypic consequences unless it occurs alongside (or subsequent to) a change in earlier, rate-limited steps in steroidogenesis. Classic neuroendocrine experimentation may again help to resolve these unknowns as we see what combinations of mechanisms do or do not change behavior in repeatable ways across species. In doing so, we can better understand whether endocrine systems play any sort of unique role in behavioral evolution.

6. Conclusion

Evolutionary endocrinology is well poised to unveil the degree to which parallel mechanisms generate variation in behavior across scales. The field has already plunged deeply into the functional effects of hormones and how these processes vary within and among species. Continued synthesis with molecular ecology and use of nature's diversity as a natural laboratory will be central as we move towards a functional understanding of behavioral mechanisms and their role in evolutionary processes. This is how we will disentangle the tangled bank.

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