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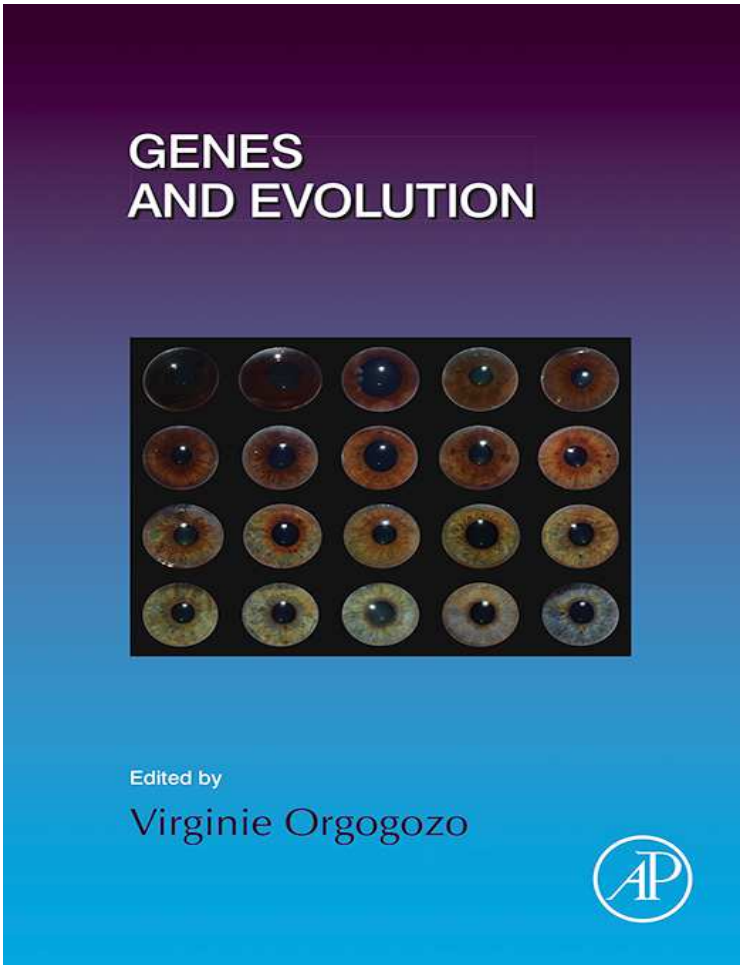

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CHAPTER FIVE

Behavioral Genetic Toolkits: Toward the Evolutionary Origins of Complex Phenotypes

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Contents

Abstract

The discovery of toolkit genes, which are highly conserved genes that consistently regulate the development of similar morphological phenotypes across diverse species, is one of the most well-known observations in the field of evolutionary developmental biology. Surprisingly, this phenomenon is also relevant for a wide array of behavioral phenotypes, despite the fact that these phenotypes are highly complex and regulated

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by many genes operating in diverse tissues. In this chapter, we review the use of the toolkit concept in the context of behavior, noting the challenges of comparing behaviors and genes across diverse species, but emphasizing the successes in identifying genetic toolkits for behavior; these successes are largely attributable to the creative research approaches fueled by advances in behavioral genomics. We have two general goals: (1) to acknowledge the groundbreaking progress in this field, which offers new approaches to the difficult but exciting challenge of understanding the evolutionary genetic basis of behaviors, some of the most complex phenotypes known, and (2) to provide a theoretical framework that encompasses the scope of behavioral genetic toolkit studies in order to clearly articulate the research questions relevant to the toolkit concept. We emphasize areas for growth and highlight the emerging approaches that are being used to drive the field forward. Behavioral genetic toolkit research has elevated the use of integrative and comparative approaches in the study of behavior, with potentially broad implications for evolutionary biologists and behavioral ecologists alike.

1. INTRODUCTION

One of the most well-known discoveries from the field of evolutionary developmental biology (Evo-Devo) is that many of the major genes involved in developmental patterning are conserved across all bilaterally symmetric animal phyla. These "toolkit genes" consist of a relatively small set of master regulatory genes, including transcription factors and signal transduction molecules (Carroll, 2008; Wilkins, 2014). The most well-known examples of toolkit genes include the *Pax* family of transcription factors in the context of eye developmental patterning, and the *Hox* genes, which are involved more generally in patterning the body axes (Gellon & McGinnis, 1998; Newman, 2006). That certain genes are highly conserved in terms of sequence and function across the majority of animal phyla, despite the immense diversity in form represented at the phenotypic level, remains a surprising observation that raises fundamental questions about evolution at the molecular level and the origins of phenotypic diversity (reviewed in Wilkins, 2014).

Over the last decade or so, interest in the toolkit gene phenomenon has spread to the study of animal behavior (Ben-Shahar, Robichon, Sokolowski, & Robinson, 2002; Fitzpatrick et al., 2005; Toth & Robinson, 2007). Though behaviors are complex and regulated by many genes, behavioral ecologists have long observed similarities in behavioral phenotypes across diverse species, presumably the result of shared ecological

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conditions and selection pressures (eg, Brockmann, 1993). With a similar comparative scope, researchers have addressed whether shared behavioral phenotypes, like morphology, have a shared genetic basis (eg, Berens, Hunt, & Toth, 2015; Campbell, Reep, Stoll, Ophir, & Phelps, 2009; Ferreira et al., 2013; Kapheim et al., 2015; Rittschof et al., 2014; Scharff & Petri, 2011; Sumner, 2014; Toth & Robinson, 2007, 2009; Toth et al., 2014). Of particular interest is whether certain conserved genes are reused over evolutionary time to give rise to convergent behavioral phenotypes. Given the complexity of behavioral phenotypes, and the variation in brain structure, function, and behavior represented across animal species, such a phenomenon would have significant implications for the study of behavioral expression, adaptation, and evolution.

A few key discoveries in behavioral genetics (Fitzpatrick et al., 2005) provided the first evidence that there may indeed be genetic toolkits for behavior. One famous example involves the genetic regulation of foraging behavior. In *Drosophila melanogaster*, genetic variants at the *foraging* locus exhibit differences in larval foraging behavior (Sokolowski, 2001), and studies in honey bees (Ben-Shahar et al., 2002), along with subsequent studies in ants, *Caenorhabditis elegans*, and other arthropod species, associated orthologs of *foraging* with diverse forms of foraging behavior (reviewed in Fitzpatrick et al., 2005), evidence that this gene is a behavioral toolkit gene. In vertebrates including humans, *FoxP2* and its orthologs have been repeatedly associated with speech, song, and other types of vocalizations (Scharff & Petri, 2011), a second example of a behavioral toolkit gene. These findings and the potential significance of the widespread occurrence of behavioral genetic toolkits motivated a wealth of additional studies over the last decade.

These studies have taken a range of experimental approaches across diverse behavioral contexts to reveal ample evidence for genetic toolkits for behavior. The evidence for behavioral genetic toolkits takes many forms as a result of the types of analytical approaches used to assess toolkit presence, the multigene nature of behavioral regulation, the myriad organizational levels between genes and behavior, and the intricacies of gene–gene relationships across species. This area of research has led to many exciting discoveries and has raised many important questions about the nature of the evolution of complex phenotypes.

Our overarching goals for this chapter are to review the use of the behavioral genetic toolkit concept and highlight some emerging research directions that are driving the field forward. To achieve these goals, we begin by describing the unique challenges involved in applying a concept rooted

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in morphology to the study of behavior. These challenges include defining cross-species similarities in behavior, and localizing behavior, an emergent phenotypic property, to a tissue in which gene expression occurs. We then review the scope of behavioral genetic toolkit research, much of which is rooted in the emerging discipline of behavioral genomics. One implication of genome-wide assessment of behavioral genetic toolkits (as opposed to traditional single-gene approaches) is that it emphasizes the role of multiple genes or gene networks in modulating phenotypes. Thus we review the significance of single vs multigene comparative approaches in the context of behavioral genetic toolkit research, the various definitions of orthology and gene networks, and factors that influence network lability.

Though much of the work surrounding behavioral genetic toolkits has been groundbreaking in and of itself, further conceptual advances depend on distilling the themes from previous studies to determine fruitful areas of future research. In an attempt to do this, we end this chapter with a review of the scope of theoretical research objectives and questions that are either rooted in or influenced by the genetic toolkit concept. Clarification of these sometimes-divergent theoretical perspectives will help provide a framework to develop and test hypotheses relevant to the toolkit at the molecular and organismal scales; we highlight some ways in which studies are already beginning to move in this direction.

2. EXTENDING A MORPHOLOGICAL CONCEPT TO BEHAVIOR

Studies have assessed behavioral genetic toolkits for a range of behavioral phenotypes, from relatively simple behaviors that occur over a short time frame, eg, aggression or dominance displays (Kravitz & Huber, 2003; Rittschof et al., 2014; Toth et al., 2014), to complex phenotypes like eusociality, which encompass a range of behaviors and show variation over evolutionary time at the species level (Harpur et al., 2014; Kapheim et al., 2015; Simola et al., 2013). Unusual or evolutionarily significant behavioral phenotypes that have repeatedly evolved across diverse species have also been targets of toolkit type studies, eg, echolocation as a prey capture strategy (Parker et al., 2013) and behavioral phenotypes associated with domestication (Trut, Oskina, & Kharlamova, 2009). Work on behavioral genetic toolkits has successfully mirrored the scope and diversity of behaviors of interest to evolutionary biology and behavioral ecology researchers (reviewed later). However, there are special challenges associated with

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comparing complex behaviors across diverse species, and rooting a behavioral phenotype in a single tissue for molecular analysis.

2.1 Defining Cross-Species Similarity in Behavior

A perennial challenge to behavioral genetic toolkit studies, and comparative studies in general, is assessing the evolutionary history of a phenotype. For complex phenotypes like behaviors, this challenge exists at two nested levels: (1) determining whether two behaviors can truly be considered "similar" across species, and if so (2) determining whether two similar behaviors are homologous, meaning they have a shared ancestry, or homoplasious, meaning they evolved independently (Bertossa, 2011; Barker et al., 2014; Rendall & Di Fiore, 2007). Because the genetic toolkit concept historically has been applied to both homologous and homoplasious phenotypes (depending on the question of interest), behavioral genetic toolkit studies need not focus on just one type of evolutionary pattern. However, defining clear criteria for behavioral similarity is still an important challenge to be addressed, as it applies regardless of evolutionary history.

Over short evolutionary distances, behavioral similarity may seem obvious because closely related species share similarity at other organizational levels, such as the neural or endocrine systems (O'Connell, 2013; O'Connell & Hofmann, 2011a). In contrast, over large evolutionary distances, it can be difficult to determine which behaviors are comparable across species. Behavioral ecology research, however, has set a precedent for such broad phylogenetic comparisons, suggesting it is possible to infer common extrinsic factors shaping such phenotypes (eg, Maher & Lott, 2000); behavioral genetic toolkit research suggests that similar inferences are possible for intrinsic factors.

From a "bottom-up" perspective, it is also possible to use genomic data itself to provide support for behavioral similarity. For instance, genomic patterns are used as independent markers of variation in behavioral phenotypes within species (Rittschof & Robinson, 2014; Whitfield, Cziko, & Robinson, 2003). In addition, although homology at the genetic level is not always an indicator of homology at the behavioral level (Bertossa, 2011), genomic data could be used to provide support for behavioral similarity across species. Appropriately defining shared behaviors likely requires a combination of both top-down and bottom-up approaches, since comparisons at the behavioral and molecular levels each have distinctive benefits and limitations. Much research to date has yielded important insights at the molecular level

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by using relatively liberal criteria for behavioral similarity across species. Developing criteria for behavioral comparison is an important issue in behavioral genetic toolkit research and deserves further consideration.

2.2 Grounding Behavior in a Tissue: The Brain

Behaviors are highly dynamic phenotypes that represent the effects of genes acting in multiple tissues and systems in the organism, sometimes throughout life. As a result, linking gene activity in a single tissue to a behavioral output is intuitively more difficult than for a morphological feature, which is by definition a single structure with relatively constrained plasticity (Bertossa, 2011). However, demonstrating functional associations or causal relationships between a gene and a behavior requires evaluating how genes influence the structure or function of a particular tissue, and the obvious choice is usually the brain. The brain is a dynamic and heterogeneous tissue, divided into many functionally distinct regions and subregions. Moreover, a high proportion of genes in the genome are expressed in the brain (Lein et al., 2007). These two features can make it difficult to tie particular genes to a significant portion of behavioral variation. Here we review the various contexts in which gene function in the brain is linked to behavior.

2.2.1 Brain Development and Function Both Influence Behavior

Behavioral phenotypes remain environmentally responsive long after the period of brain tissue development has ended, and even rapid shifts in behavior that are mediated by transient brain electrical signals result in changes in gene expression in neurons (Clayton, 2000). Thus behavioral responses to brief stimuli may be influenced by variation in genome sequence and the activity of genes (Clayton, 2000, 2013; Dong et al., 2009; Fernald & Maruska, 2012; Zayed & Robinson, 2012). Moreover, longer term learning and memory processes, and variation in behavior at the timescale of life history traits, such as the seasonal scale, have all been linked to changes in brain gene expression (reviewed in Harris & Hofmann, 2014; Wong & Hofmann, 2010). Thus candidate toolkit genes for behavior include not only those involved in brain developmental patterning but also any genes that regulate the structural or functional plasticity of the brain (Ament, Corona, Pollock, & Robinson, 2008; Grozinger, Fan, Hoover, & Winston, 2007; Kapheim et al., 2015; Kocher, Richard, Tarpy, & Grozinger, 2008; Manfredini, Brown, Vergoz, & Oldroyd, 2015; O'Connell, 2013; Patalano et al., 2015; Rittschof et al., 2014; Rogers, Gagnon, & Bernatchez, 2002; Toth, Bilof, Henshaw, Hunt, & Robinson, 2008; Whiteley et al., 2008;

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Woodard, Bloch, Band, & Robinson, 2014). It is important to note that the different functions of even a single gene throughout different life stages and across different tissues are often unknown. The genes associated with neural plasticity later in life, at short or long timescales, may be the same or different from the genes involved in developmental patterning of the brain (Kiecker & Lumsden, 2005; Rittschof et al., 2014). Determining the features of genes associated with behavioral plasticity at different timescales is an exciting area of future work.

2.2.2 Brain Heterogeneity and Its Implications for Behavioral Genetic Toolkit Inferences

In addition to structural and functional dynamics during development and throughout life, the brain is highly spatially heterogeneous, with variable cell types and circuitry. Understanding how information in the brain is integrated to give rise to behavioral phenotypes under natural conditions remains a major and unresolved goal in neuroscience. Moreover, knowledge of the relationships between behavior and region- or circuit-specific neural and gene activity varies greatly across species. As a result, studies differ strongly in their approach to accommodating heterogeneity in the brain in the course of identifying toolkit genes. Many behavioral genomic studies collect data from the whole brain (Aubin-Horth, Landry, Letcher, & Hofmann, 2005; Renn, Aubin-Horth, & Hofmann, 2008; Toth et al., 2014), and some toolkit studies have even had success comparing whole brain transcriptomic signals from one species with transcriptomic patterns observed within specific brain regions in other species (Rittschof et al., 2014).

Brain heterogeneity and circuitry can be parsed in many ways, eg, in terms of regions activated metabolically (Raichle & Mintun, 2006) or genetically (Guzowski et al., 2005) in correlation with expression of a behavioral phenotype, or in terms of the locations of activity of specific neurotransmitters or neuromodulators known to have behavioral associations (Kiya & Kubo, 2010; Sari, 2004). Particularly among closely related species, there is often conservation of regions functionally linked to a behavioral phenotype. For example, in vertebrates, there is evidence that brain regions associated with social behavior and decision making are highly conserved in terms of neurochemistry (O'Connell & Hofmann, 2012) as well as other measures including topography and gene expression during development (O'Connell & Hofmann, 2011b). As a result, gene activity in these regions at different life stages can be broadly compared to identify evidence of behavioral genetic toolkits. Across distantly related species, it may be

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that behavior is more similar than brain structure and function, although studies increasingly support the idea of functionally analogous brain regions across vertebrate and invertebrate brains, suggesting a basis for region-specific comparisons across broad phylogenetic distances (Strausfeld & Hildebrand, 1999). Moreover, certain neurotransmitter and neuromodulator systems are widely shared and associated with similar behavioral phenotypes across species (eg, dopamine and reward (Barron, Sovik, & Cornish, 2010), or serotonin and aggression (Dierick & Greenspan, 2007; Gillette, 2006; Keele, 2005; Kravitz & Huber, 2003; Takahashi, Quadros, de Almeida, & Miczek, 2011)), suggesting that for certain behaviors, spatial neurotransmitter expression information can be used to target regions of behavioral relevance across diverse species for further genetic toolkit analyses. It is important to note that analyses of gene activity, even those focused on a particular brain region, typically assess multiple cells and cell types in a single sample, which masks cell or cell type variation in expression patterns (Lein et al., 2007). This level of heterogeneity could affect inferences about the functional significance of gene expression patterns at the level of the single gene or gene network (discussed later). Finally, it is important to consider that gene activity in response to a stimulus has a temporal component, which, though operating on a longer scale and with a different functional outcome, corresponds to the path of the electrical signal as it propagates through a circuit (Clayton, 2000; Guzowski et al., 2005). The spatiotemporal properties associated with gene expression dynamics, particularly if they are variable across species, could influence behavioral genetic toolkit inferences, and investigating these dynamics is an important area of future work.

2.2.3 The Importance of Peripheral Tissues

The brain is a central point of information perception and integration, and it coordinates behavioral response. But despite the importance of the brain, it is not the only organ system associated with variation in cognitive function and behavior; the brain communicates with peripheral tissues, and the actions of multiple tissues influence behavior and organismal homeostasis (DeWall, Deckman, Gailliot, & Bushman, 2011; Schilder & Marden, 2006; Stranahan et al., 2009; Yue & Lam, 2012). Actions in peripheral tissues are particularly relevant to consider for shifts in behavior associated with physiological adaptation, eg, temperature or hypoxia tolerance (Marden, 2013), as these physiological changes may be the target of selection, with secondary behavioral effects. For example, a number of studies have noted relationships between metabolic rate, a physiological property influenced in

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part by mitochondrial function, and behavioral phenotypes; the ecological factors that cause these effects, and thus whether these act directly or indirectly to influence behavior, are not always clear (Biro & Stamps, 2010; Mathot & Dingemanse, 2015). Moreover, from an evolutionary perspective, genes may be expressed in multiple tissues either simultaneously or at different time points throughout life (Johnson, Atallah, & Plachetzki, 2013), and thus changes in protein-coding regions or regulatory regions surrounding a gene could have pleiotropic effects (Fitzpatrick, 2004; Mank, Hultin-Rosenberg, Zwahlen, & Ellegren, 2008). Assessing gene activity in nonbrain tissues may improve inferences about behavioral adaptation, and in some cases may provide a more accurate means to assess behavioral genetic toolkits.

There is a long research history surrounding the role of peripheral tissues in regulating behavior, especially in invertebrates. For example, foundational studies on moths showed that copulation and egg laying do not require a head, let alone a brain (Kellogg, 1966). Behavioral genetic toolkit studies in invertebrates have evaluated gene function in the brain as well as peripheral tissues including the fat body (the insect equivalent of the liver; Ament et al., 2008) and the ovaries (Kocher et al., 2008). The involvement of systems outside of the brain in regulating behavior extends to the vertebrates as well. For example, hormone signaling in the hypothalamic-pituitary axis affects the brain as well as peripheral tissues, producing an organism-wide stress response (Peters et al., 2004; Wommack & Delville, 2007). In addition, morphological or physiological constraints may influence the development or expression of a behavior and vice versa, as has been shown for mouth morphology and scale-eating behavior in a cichlid fish (Lee, Kusche, & Meyer, 2012), and gill salinity tolerance and habitat range in killifish (Whitehead, Roach, Zhang, & Galvez, 2012). Incorporating information from peripheral tissue is also used to infer processes in the brain and to link gene expression patterns with behavioral variation in circumstances where brain measurements are impossible. For example, sampling transcriptomic dynamics in white blood cells has been used to infer neural functional correlates of stress and mental health disorders in humans (Cole, 2009, 2010; Miller et al., 2009, 2008). It is possible to make relevant inferences about behavioral genetic toolkits across a range of tissue types; the appropriate approach depends on the salient research question. In particular, if there is interest in understanding specific physiological adaptations that are linked to behavioral change and are potentially the primary target of selection, it may be fruitful to assess gene function in peripheral tissue in addition to the brain.

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3. THE SCOPE OF BEHAVIORAL GENETIC TOOLKIT RESEARCH

Behavioral genetic toolkit studies have examined a wide range of behaviors. In the absence of well-understood relationships between genotype and behavioral phenotype, creative analytical approaches are critical to assessing behavioral genetic toolkits. The concept of behavioral genetic toolkits should be considered here as a broad term which includes not only the genes that harbor the mutation(s) causing evolutionary changes in behavior but also the genes whose changes in expression pattern are associated with the evolution of a new behavior, even though the underlying mutation(s) may lie in an upstream regulator gene and not in these genes themselves. In exploring the scope of behavioral genetic toolkit research, we grouped studies based on the approach used to determine behavioral genetic mechanisms and to compare these mechanisms across species. These approaches include functional genomics and DNA sequence-level comparisons, in addition to perspectives from Neuro-Evo-Devo, which is perhaps the most similar to traditional genetic toolkit studies focused on morphology.

3.1 Genomic Advances Facilitate Behavioral Genetic Research

Traditional approaches for identifying individual genes that pattern morphology during development are not easily applied to the comparative study of natural variation in complex behaviors. Genomics tools have greatly advanced understanding of the relationship between genes and behavior, especially for nonmodel organisms studied under natural conditions (Harris & Hofmann, 2014; Kültz et al., 2013; Rittschof & Robinson, 2014). Behavioral genomics studies take a range of approaches to identify behaviorally relevant genes. This field largely employs functional genomics approaches to assess the genomic correlates of a behavior of interest, often without knowledge of whether sequence variation or epigenetic or feedback mechanisms lead to this functional variation (reviewed recently in Harris & Hofmann, 2014). With increased availability of new genomes, studies utilizing DNA sequence-level comparisons to identify genes for behavior are also growing. Taken together, these studies have uncovered many ways in which genome content and function influence behavioral phenotypes across diverse species.

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3.1.1 Functional Genomics Approaches

Transcriptomes can be easily assessed across an array of organisms and tissue types, and even short-term shifts in behavior often are associated with a transcriptomic response (reviewed earlier). Functional genomics approaches have been used to evaluate behavioral genetic toolkits across a range of behavioral contexts, including the repeated evolution of divergent swimming behavior in sympatric fish species pairs (Rogers et al., 2002; Whiteley et al., 2008), the physiological basis of division of labor in insects (Ament et al., 2008; Ferreira et al., 2013; Patalano et al., 2015; Toth et al., 2008), the evolution of eusociality (reviewed in Kapheim, 2016), the molecular basis of male dimorphism in fish (Schunter, Vollmer, Macpherson, & Pascual, 2014), the molecular basis of aggression (Kelstrup, Hartfelder, Nascimento, & Riddiford, 2014; Rittschof et al., 2014), and the evolution of personalities or behavioral syndromes across vertebrate and invertebrate species (Bell & Aubin-Horth, 2010). Most of these studies find some evidence for behavioral genetic toolkits, as well as evidence that species-specific genes play a substantial role in modulating behavioral phenotypes.

Functional genomics studies typically compare the transcriptomes in a particular tissue for individuals in a species occupying two different behavioral states; they identify differentially expressed genes, and then compare those genes to those identified using similar approaches in another species. For example, to search for a genetic toolkit for postmating changes in female behaviors (which include cessation of mating activities and eventually egg-laying behaviors), Kocher et al. (2008) compared the brain gene expression differences between virgin and mated honey bee queens with the whole-body expression profiles of mated and unmated fruit flies (a previously published study; McGraw, Gibson, Clark, & Wolfner, 2004). Despite major differences in physiology, behavior, and mechanisms of sexual conflict across these two species, there was some evidence of overlap in genes whose activities are regulated by the experience of mating (Kocher et al., 2008). In this study, however, there was not enough cross-species overlap to assess statistical significance. Several other studies have found additional support for the existence of a genetic toolkit for mating-induced changes in behavior among social insects (Grozinger et al., 2007; Manfredini et al., 2015; Woodard et al., 2014), including genes associated with vision, immunity, stress response, alternative splicing, and metabolism.

In vertebrates, several studies have evaluated gene expression patterns among dimorphic or polymorphic males within a single species, a

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phenomenon that has evolved a number of times across animal lineages (Brockmann & Taborsky, 2008). These gene expression studies provide intriguing evidence to suggest that there may be behavioral genetic toolkits for male polymorphism generally, or even for specific forms of male polymorphism. For instance, in the black-faced blenny, dominant and sneaker males show more extreme differences in gene expression when compared to one another than if either morph is compared to females (Schunter et al., 2014). Similarly, in salmon, there are substantial differences in brain gene expression comparing sneaker males to immature males that are destined to mature into the nonsneaker male morph; moreover, sneakers show greater similarity in brain gene expression when compared to immature females vs immature males (Aubin-Horth et al., 2005). In both the blenny and salmon, morph-dependent variation in brain gene expression implicates genes associated with reproduction, development, and neural plasticity, although the direction of expression changes differed across species (Aubin-Horth et al., 2005; Schunter et al., 2014). Though there was no formal statistical assessment of toolkit presence in these studies, the results generally suggest there may be behavioral genetic toolkits for male dimorphism in fish. Such toolkits could be compared even more broadly, including additional species for which there are genomic signatures of male polymorphism (Pointer, Harrison, Wright, & Mank, 2013).

One interesting pattern illustrated by the blenny and salmon comparison above is that similar genes can be implicated in a shared behavior across species while showing opposite directions of expression in relation to the behavioral phenotype. This pattern has emerged in other studies as well (Rittschof et al., 2014), with several possible explanations. For example, the genes showing expression variation in correlation with the behavior of interest could belong to a larger gene network that is showing some overall shift in activity that is comparable across species, despite divergent expression of specific genes. Another possibility is that certain pathways are linked to a behavior consistently, but that the valence of the relationship depends on other aspects of behavioral and molecular state that may or may not be comparable across species under all circumstances. For example, in humans, aggression and violence are associated with inflammation, immune function, and metabolism in both positive and negative ways under different conditions (Copeland, Wolke, Angold, & Costello, 2013; Felitti et al., 1998; Granger, Booth, & Johnson, 2000). These variable relationships could easily manifest at the transcriptomic level for cross-species comparisons.

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3.1.2 DNA Sequence-Level Approaches

DNA sequence-level comparisons can also be used to identify evidence of behavioral genetic toolkits. Such studies have examined evidence of DNA or protein sequence convergence at the species level (Foote et al., 2015; Parker et al., 2013), compared rates of evolution for orthologous vs novel genes within a single species (Harpur et al., 2014; Kapheim et al., 2015), looked for similarities in genome structure or function across species (Kapheim et al., 2015), and examined population-level similarities in quantitative trait loci associated with convergently evolved phenotypes (Kowalko et al., 2013).

Studies in vertebrates have used sequence-level approaches to assess the molecular basis of a range of convergently evolved phenotypic traits, including monogamy in rodents (Turner et al., 2010), and adaptations to cave living in populations of a fish species (Kowalko et al., 2013). Parker et al. (2013) used a DNA sequence-level approach to determine whether echolocation capability in mammals, which has evolved convergently twice in bats and once in the bottlenose dolphin, shows evidence of evolutionary convergence at the genetic level, which would suggest a genetic toolkit. The authors found that genes encoding three characteristics of echolocating mammals, hearing, vision, and blindness, showed evidence for sequence convergence, with hearing-related genes showing the strongest patterns (Parker et al., 2013). This study supports the hypothesis that sequence convergence plays an important, and perhaps underappreciated role in the evolution of complex traits (Parker et al., 2013; but see also Thomas & Hahn, 2015). Similarly, Foote et al. (2015) compared genomes of the killer whale, bottlenose dolphin, walrus, and manatee to evaluate the molecular basis of adaptation to marine living. The authors found widespread evidence of sequence convergence at the level of nonsynonymous substitutions to protein-coding genes across all three lineages. Many of these genes were under positive selection in at least one lineage, which suggests they may be adaptive changes associated with marine living. However, the authors also assessed sequence convergence among terrestrial sister taxa in order to establish a null expectation for convergence at the molecular level that is not associated with phenotypic convergence. They reported high levels of sequence convergence among the terrestrial sister taxa, suggesting convergent changes at the molecular level may be neutral in many cases, and perhaps not always indicative of selection-induced evolutionary convergence in phenotype (Foote et al., 2015). A recent study suggests a similar

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phenomenon may occur in the above-mentioned context of echolocation (Zou & Zhang, 2015). These studies underscore the importance of developing clear null and alternative hypotheses to the behavioral genetic toolkit when evaluating convergent evolution at the molecular level (reviewed later).

Several studies have used sequence-level approaches to evaluate whether the evolution of insect eusociality involves a behavioral genetic toolkit. Kapheim et al. (2015) analyzed genome sequences from 10 bee species, which encompass two independent evolutions of eusociality, to evaluate patterns of gene regulation and genetic novelty that accompany the origin and elaboration of eusociality in bees. This study found that eusociality is generally associated with increased regulatory capacity in the genome, including higher numbers of transcription factor binding sites for orthologous genes, increased predicted levels of DNA methylation genome-wide, and more rapid evolution of genes involved in coordinating gene regulation. Thus in bees, there is some evidence for convergence at the level of genome lability in association with the evolution of eusociality. Simola et al. (2013) observed a similar phenomenon comparing honey bees and ants. Although these studies also found evidence for unique, lineage-specific variation in gene composition and gene expression associated with different eusocial transitions, enhanced lability appears to be a general and perhaps convergently evolved feature of eusocial genomes.

Other studies of eusocial evolution have emphasized a role for putatively novel or taxonomically restricted genes. This is based on findings showing that these proteins are often more likely to be expressed in the non-reproductive worker caste; the existence of the worker caste (reproductive division of labor) is one of the hallmarks of eusociality (Feldmeyer, Elsner, & Foitzik, 2014; Ferreira et al., 2013; Johnson & Tsutsui, 2011). Similarly, by sequencing individual honey bee genomes, identifying single-nucleotide polymorphisms among individuals, and comparing nonsynonymous vs synonymous amino acid substitutions in protein-coding regions of genes, Harpur et al. (2014) showed that genes taxonomically restricted to the super family Apoidea and the genus *Apis* show high rates of positive selection compared to hymenopteran or insect-specific genes, suggesting that novel genes might be important for the elaboration of worker-specific phenotypes (Harpur et al., 2014); these genes could also be involved in processes unrelated to behavior, for example, genomic conflict. Though they are not mutually exclusive explanations for phenotypic diversity, understanding

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the roles of novel and conserved genes in the evolution of behavioral phenotypes is an important area of future research (addressed later). There are a number of theoretical issues to consider, but one important practical issue involves the definition of novelty at the genetic level; increased genomic information and improved annotations may eventually result in reclassification of at least some novel genes.

In mammals, artificial selection for domestication has led to convergent evolution of an array of behavioral and morphological phenotypes. This area of study has utilized both functional genomic approaches and DNA sequence-level information to identify the heritable molecular and physiological correlates of repeated behavioral evolution (reviewed by Trut et al. (2009)). Because certain morphological traits often accompany the behavioral response to domestication (eg, floppy ears and white spotting on the head), this area of work provides an interesting context to investigate the role of pleiotropic constraints in behavioral evolution (Trut et al., 2009). Domestication is also a unique application of experimental evolution to the study of behavioral genetic toolkits.

At the molecular level, there appears to be a role for both heritable epigenetic modifications (which manifest as gene expression changes) and DNA sequence-level changes in mediating domesticated phenotypes (Trut et al., 2009); moreover, studies find some evidence for behavioral genetic toolkits (Martin & Orgogozo, 2013). For example, there is a consistent role for the hypothalamic-pituitary axis and serotonergic systems in mediating the increased tameness and sociability that characterizes domesticated mammals, and certain regulatory changes are shared across domesticated dogs and tame foxes (Trut et al., 2009). Moreover, genes under positive selection in domesticated dogs are also under positive selection in humans, suggesting a behavioral genetic toolkit for sociability, which could be linked to domestication in other species (Wang et al., 2013). Because domestication is often associated with changes in developmental rate, these kinds of studies provide a possible means to link classical Evo-Devo approaches focused on tissue development and the timing of developmental transitions, to behavioral and molecular variation that manifests during the adult stage; the majority of behavioral genetic toolkit studies focus on the adult stage only (but see later). From a theoretical perspective, increased continuity across life stages may lead to a better understanding of the types of genes and processes that serve as behavioral genetic toolkits, and whether these genes maintain toolkit status across a range of timescales for behavioral variation.

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3.2 Neuro-Evo-Devo

In contrast to the genomics approaches discussed earlier, Neuro-Evo-Devo (reviewed in O'Connell, 2013) specifically investigates conserved genes and gene networks involved in patterning the brain, and thus offers the most direct comparison to traditional Evo-Devo approaches (Holland et al., 2013). Similar to the Evo-Devo framework, primary questions of interest in Neuro-Evo-Devo revolve around understanding the number of origins of the nervous system across animal phyla (Holland et al., 2013), and determining how diversity in brain structure and function (and thus behavior) arises despite the maintenance of evolutionarily conserved gene networks that guide brain development across species. Like the general body plan or the development of the eye, certain genes regulating central nervous system developmental patterning are broadly shared across both invertebrate and vertebrate lineages (O'Connell, 2013; Tessmar-Raible et al., 2007). These patterning genes are largely transcription factors (Tessmar-Raible et al., 2007), including *Bmp* (*Dpp* in insects), which specifies neural vs nonneural tissue, and *Nk2.2*, *Gsx*, *Msx*, and *Pax6* and their insect orthologs, which specify patterning along the mediolateral axis of the brain (O'Connell, 2013). In addition, the expression patterns of these genes during development are used to infer genetically homologous regions of the brain, which may also show structural or functional homology in some cases (Strausfeld & Hirth, 2013).

Work in Neuro-Evo-Devo supports the general idea that variation in timing of developmental gene expression is essential for brain structural variation within and among species (O'Connell, 2013). Variation in the onset of neurogenesis (which is a function of cell cycle progression) can lead to variation in neural structure or size. Delaying neurogenesis or changing the length of the period of neurogenesis has been shown to have structural and behavioral effects in diverse species including vertebrates and invertebrates. For example, *Fgf2* delays neocortical cell cycle exit and is associated with increased neocortical volume in rats, primates, and birds (reviewed in O'Connell, 2013). Although there may be genetic toolkits for brain structure, the link between neural developmental phenotypes and behavioral phenotypes is not always clear. Like other areas of Evo-Devo, Neuro-Evo-Devo can be limited in part by the analytical tools available to certain organisms. However, this theoretical approach has been applied broadly across animals systems at different analytical levels associated with behavioral phenotypes (Strausfeld & Hirth, 2013; Tessmar-Raible et al., 2007).

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The scope of behavioral genetic toolkit studies spans life stages from development to adulthood, and in many ways encompasses the range of behaviors represented in behavioral ecology and evolution research. These studies not only suggest the presence of genetic toolkits for a range of behaviors, but they also bring to light some of the challenges of comparing genes and gene networks for complex phenotypes across diverse species. In the following section we review some of these challenges.

4. GENES AND GENE NETWORKS: LINKS TO BEHAVIOR AND EVOLUTION ACROSS SPECIES

In previous sections, we highlighted two types of challenges inherent to behavioral genetic toolkit study. These include (1) establishing links among genotype, neural function, and behavioral phenotype, and accommodating vast differences in knowledge of these relationships among species, and (2) inferring behavioral similarity, homology, and homoplasy across a broad phylogenetic range. In this section, we broaden this discussion, addressing the complexity of identifying, or even defining, genetic similarity across species. These challenges, which are rooted in the complexity of gene networks and their evolution, provide the foundation for a broader discussion about the different conceptual viewpoints in evolutionary and developmental genetics that converge at the toolkit idea. Incorporating these different perspectives may help elucidate hypotheses associated with the behavioral genetic toolkit, providing directions for future research.

4.1 Evolutionary Relevance: Genetic Homology and Gene Network Structure and Evolution

Defining genetic orthologs, which are two genes descended from the same DNA sequence and separated from one another by a speciation event (Moreno-Hagelsieb & Latimer, 2008), can be as difficult as identifying similarities at the level of the behavioral phenotype. However, determining relationships among behaviorally relevant genes across species is central to the behavioral genetic toolkit concept, and comparative genomics more generally. In practice, orthology can be defined using a number of methods that differ in stringency, or the amount or type of evidence used to distinguish orthology from paralogy (which arises in cases of gene duplication

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events; Heidelberg et al., 2002). For example, reciprocal best protein blast hits identify the closest protein matches across species, based solely on protein sequence (Moreno-Hagelsieb & Latimer, 2008). Because sequence similarity can result from evolutionary conservation or convergence, other protein phylogeny-based methods (eg, OrthoDB; Waterhouse, Tegenfeldt, Li, Zdobnov, & Kriventseva, 2013 and OrthoMCL; Doerks, Copley, Schultz, Ponting, & Bork, 2002) incorporate evolutionary relationships within and among gene families, which provides more definitive information about the evolutionary history of the gene and therefore whether or not it can be considered a true ortholog. For certain categories of genes, eg, transcription factors, specific types of sequences, like the DNA-binding domains, can also be used to assess functional similarity across species (Marchler-Bauer et al., 2015; Mathelier et al., 2014; Portales-Casamar et al., 2010). Determining whether and how genes are related among species is central to understanding gene evolution, assessing gene function, and implicating genes as taxon specific and thus novel in a particular species (at least based on the limits of current genomics databases).

The type of approach used to define orthology, and the stringency of the definition, should depend in part on the purpose of assessing the relationship. In functional genomics research, particularly in nonmodel organisms, orthologous relationships are used to hypothesize the functions of genes of interest, since there is typically more experimental information available for certain model organisms (Sanogo, Band, Blatti, Sinha, & Bell, 2012; Wheeler & Robinson, 2014). Increasingly, databases are incorporating data from diverse species to provide a repository of possible functional roles for uncharacterized genes (Blast2GO; Conesa et al., 2005, PANTHER; Mi, Muruganujan, & Thomas, 2013). In the context of behavioral genetic toolkits, identifying orthology primarily serves to determine whether genes associated with a behavioral phenotype are indeed the same across species (Fitzpatrick et al., 2005). As such, it seems at first that strict orthology should be a fundamental requirement for toolkit genes. However, aside from the practical limitations of applying such a stringent definition of genetic similarity (ie, such an approach greatly limits the number of genes that can be compared across broad phylogenetic distances), a requirement of stringent orthology may also miss important species similarities in the molecular basis of a shared behavior, especially because behaviors are often regulated by large networks of genes (Anholt, 2004; van Swinderen & Greenspan, 2005; Yamamoto et al., 2008). Instead, it may be more appropriate to also consider genes across species that are paralogous, are members of

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the same gene network, have similar functional properties, or subserve the same physiological processes (Berens et al., 2015).

Going beyond a strict one-to-one comparison of orthologous genes evokes a multigene or network-level approach to identifying behavioral genetic toolkits. Such a perspective is in keeping with the history of the genetic toolkit concept in Evo-Devo, where even at its inception, there was recognition that single genes act in the context of larger gene networks to regulate phenotypes (Wilkins, 2014), and the structure of these networks may be labile and variable across species (Buchanan, Sholtis, Richtsmeier, & Weiss, 2009; Phillips, 2008; Tyler, Asselbergs, Williams, & Moore, 2009). Thus while the discovery of single "toolkit" genes was surprising, the more puzzling pattern is that shared genes, or sometimes paralogs of these genes, maintain an association with a phenotype over evolutionary time despite major variation in surrounding gene networks. This same puzzle is relevant in the context of behavioral genetic toolkit research. Although theoretical treatments of behavioral genetic toolkits take a single-gene approach (Fitzpatrick et al., 2005), in practice, studies often incorporate a multigene perspective (Kocher et al., 2008; Toth et al., 2014) as a result of the common genome-wide analytical approach (reviewed earlier). Given the theoretical and practical importance of network-level approaches in the context of behavioral genetic toolkits, here we review the various definitions of a gene network, and the ways in which these concepts have been used in behavioral genetic toolkit research. Considering how work already utilizes a network-level approach may point toward easy avenues of future research.

4.2 Identify Gene Networks

There is no single definition of "gene network"; the term is used in a variety of ways depending on context. Here we consider a very broad definition of a network as a group of genes that is related or connected structurally or functionally (Abouheif, 1999); such networks can be considered on the scale of relatively small sets of genes (Monteiro, 2012; Olson, 2006) or at the genome scale (O'Brien, Lerman, Chang, Hyduke, & Palsson, 2013). A liberal concept of gene network (in terms of the type and scale of connections) is useful for the study of behavioral genetic toolkits, because of the complexity of the connections between genotype and phenotype. Most behavioral genetic toolkit studies incorporate network-level information, defined conceptually in a variety of ways.

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4.2.1 Regulatory Networks

Perhaps the most widely recognized conception of "gene network" refers to transcription regulatory relationships among genes (Chesler et al., 2005; Davidson, 2006; Portales-Casamar et al., 2010; Wilson, Charoensawan, Kummerfeld, & Teichmann, 2008). Regulatory relationships can be determined experimentally (Agoston et al., 2014; Monteiro, 2012; Valouev et al., 2008) or inferred using coexpression analyses (Ament, Blatti, et al., 2012; Chandrasekaran et al., 2011; Edwards et al., 2009; Filteau, Pavey, St-Cyr, & Bernatchez, 2013; Iancu et al., 2013). Variation in transcription factor-regulated gene expression emerged early on in Evo-Devo as an important source of phenotypic variation, and a similar legacy is present in behavioral genetic toolkit research.

A focus on gene regulation, particularly large-scale gene regulatory networks, provided a framework for hypotheses about the evolution of novelty and diversity in phenotype and gene content. For example, in Evo-Devo, two related hypotheses that emerged in this context were that changes in genes' regulatory regions, rather than protein-coding regions, play a significant role in the evolution of diversity, and these changes influence the timing of gene expression during development (Carroll, 2000, 2005; Hoekstra & Coyne, 2007). Other hypotheses emerged based on the observation that genes in a network also differ in their degree of connectivity, or number of connections to other genes (Barabasi & Oltvai, 2004); for example, transcription factors are highly networked genes, leading to a prediction that these genes show constrained changes in sequence and function over evolutionary time due to pleiotropy (Carroll, 2005, 2008). There is evidence that highly connected genes may be both more or less likely to exhibit variation in sequence or function (Jovelin & Phillips, 2009; Kapheim et al., 2015; Tyler et al., 2009).

Some of these classic hypotheses have been addressed specifically in the context of behavioral genetic toolkit research (eg, O'Connell (2013)). For example, Molodtsova, Harpur, Kent, Seevananthan, and Zayed (2014) evaluated the relationship between a gene's connectivity and adaptive evolution in protein-coding regions, using a model of a honey bee brain transcription regulatory network for behavior (Chandrasekaran et al., 2011). The authors found that adaptive evolution in protein-coding regions was more common for genes at the network periphery than more centrally connected genes. In contrast, the strength of selection on regulatory mutations did not vary as a function of connectivity, suggesting changes in coding sequence may be more dependent on network position than changes in expression patterns (Molodtsova et al., 2014).

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Studies take several approaches to implicate particular regulatory gene networks in the expression of a behavioral phenotype. Evaluating activity of immediate early genes (transcription factors that show changes in expression levels quickly after a stimulus), or other key transcription factors, is one approach (Ament, Wang, et al., 2012). A second approach involves evaluating differentially expressed genes associated with a behavioral phenotype for significant enrichment for certain transcription factor binding sites (Ament, Blatti, et al., 2012; Rittschof et al., 2014; Sanogo et al., 2012). This strategy can implicate a particular transcription factor without the transcription factor itself showing evidence of differential expression. This approach reflects the fact that the timing of expression change for different parts of a gene network can vary and that some but not all components of networks may be shared for shared behaviors. Interestingly, this observation, that networks may overlap in association with a shared behavior across species while individual regulatory genes may not, is the converse of the original network puzzle posed by the genetic toolkit concept; it underscores the relevance of gene networks vs single genes in modulating behavioral phenotypes.

Gene expression data can be used to construct coexpression networks, which, when stable across broad evolutionary distances, likely reflect regulatory relationships (Stuart, Segal, Koller, & Kim, 2003). Stuart et al. (2003) built a gene network from coexpression relationships for reciprocal-best-hit orthologs conserved across humans, flies, worms, and yeast (these groups of four orthologs were called metagenes). This network consisted of 12 major groupings of highly connected metagenes involved in similar biological processes. While some groupings, eg, those associated with ribosomal function, represented ancient modules containing metagenes with highly conserved protein-coding regions and coexpression connections, other groupings showed greater change over evolutionary time. One such grouping contained metagenes associated with neuronal function, suggesting a relatively high degree of change over evolutionary time for this behavior-related metagene grouping, consistent with the idea that behavior is labile compared to other phenotypes. This type of analysis could also be performed at a finer scale to determine if there are specific types of behaviors that are more or less likely to show lineage-specific evolutionary patterns, and thus evidence of behavioral genetic toolkits.

Gene regulatory network analyses, particularly those based in experimental evidence of relationships between transcription factor binding and downstream effector gene expression, have the advantage of providing a functional framework for linking evolutionarily relevant DNA sequence

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changes to variation in molecular and behavioral phenotypes. For example, changes in regulatory regions could affect transcription factor binding affinity and thus the degree of expression of downstream genes. However, transcriptional regulatory relationships are combinatorial, complex, and even labile within the life span of an individual, and relationships to effector genes are not all known or easily deduced experimentally. Despite this complexity, transcriptomic data can be analyzed for regulatory network information fairly easily, as exemplified by coexpression analyses, making a gene regulatory network perspective tractable for behavioral genomics studies.

4.2.2 Biochemical and Signal Transduction Pathway Networks

Gene networks can be described in ways other than specific transcription regulatory relationships. Well-defined biochemical, cellular, and signal transduction pathways can be considered networks as their gene members are interconnected (Abouheif, 1999; Hahn, Conant, & Wagner, 2004; Tyler et al., 2009). These networks can be approached similarly to gene regulatory networks, though some of the network properties may fundamentally differ (Albert, 2005). These networks can be regulated at the transcriptional level, as well as at the level of protein–protein and metabolite–protein interactions (Ideker et al., 2001). A change in the structure or function of one protein in a biochemical pathway can have overall consequences for pathway function, and possibly even indirect effects on gene transcription through regulatory feedback. Examples include energy metabolic pathways (Chavali, Whittemore, Eddy, Williams, & Papin, 2008; Ideker et al., 2001; Schuster, de Figueiredo, Schroeter, & Kaleta, 2011), insulin/target of rapamycin signaling pathways (Ament et al., 2008; McGaugh et al., 2015), stress response pathways in vertebrates (Schwartz & Bronikowski, 2013), the juvenile hormone signaling pathway in insects (Konopova, Smykal, & Jindra, 2011; Zhou, Tarver, & Scharf, 2007), and the JAK-STAT pathway involved in immune response (Papin & Palsson, 2004). Physiological pathways can be considered on a fine scale, and they often possess a well-defined (and sometimes highly conserved) set of components with clearly measurable outcomes (eg, production of particular metabolites). One advantage to this functional conception of a gene network is that it can be used to define endophenotypes for behaviors. Endophenotypes are discrete functional phenotypes at a level of organization between a genotype and a behavior; these endophenotypes, which represent significant functional components of the behavior itself, provide a tractable unit for evaluation of evolutionary lability (Gottesman & Gould, 2003)

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or experimental manipulation for causality. The biochemical pathway conception of gene network focuses on regulation at multiple levels of organization, not just transcriptional regulatory relationships.

4.2.3 Gene Ontology and Ad Hoc Networks

Many behavioral genetic toolkit studies already incorporate network-level inferences through the use of Gene Ontology (GO) analyses, which provide functional information about a gene or set of genes associated with a behavioral phenotype, based on orthology to genes from species in which functional analyses have occurred. GO terms are largely species independent (Gene Ontology Consortium, 2000), and so behaviorally relevant terms can be compared across species. GO terms provide convenient higher order classifications for genes and proteins that allow comparisons among species or contexts without requiring specific orthologous genes to be shared or even directly compared, and as such, they serve as ad hoc gene networks. GO terms have nested levels of specificity; for example, a gene can belong to the molecular function "receptor activity," which contains the nested term "retinoic acid receptor activity." As a result, the degree of functional information derived from a GO analysis varies depending on the level at which similarities among species are identified. Though GO terms may identify broad classes of genes that may be difficult to interpret, in other cases, GO terms point to specific biochemical pathways that can be considered as physical networks (eg, "glycolytic process"). Such networks may be comparable to biochemical networks discussed earlier. In other cases, GO terms may point to a particular type of receptor or ion channel that is involved in behavioral variation. These findings do not always implicate a network, but instead suggest a similar repeated evolutionary event at the molecular level. In behavioral genomics studies, GO terms often provide the best evidence that some form of a behavioral genetic toolkit exists, because higher order processes may overlap even if the exact genes modulating a behavior of interest are not shared (Berens et al., 2015; Kapheim et al., 2015). Future work should probe the implications of this "low-resolution" overlap at the genetic level (Martin & Orgogozo, 2013).

One advantage of identifying candidate functional pathways (using GO or some other type of analysis) is that they can be manipulated genetically or pharmacologically to demonstrate causal relationships with behaviors of interest. Alaux et al. (2009) used GO analyses to associate decreased brain oxidative phosphorylation activity with increased aggression in the honey bee. Subsequent studies showed that this pattern extends to the enzyme

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activity level, and used enzyme inhibitors and genetic manipulations to test causal and cell type-specific associations between energy metabolism and aggression in the honey bee and fruit fly (Alaux et al., 2009; Li-Byarlay, Rittschof, Massey, Pittendrigh, & Robinson, 2014; Rittschof, Grozinger, & Robinson, 2015). Thus using GO to identify discrete "candidate processes" associated with a shared behavioral phenotype provides an opportunity to explore gene–behavior associations empirically and at different levels of biological organization (Rittschof & Robinson, 2014).

4.3 Network Lability and Variation in Phenotypic Expression

The activity of genes in combination does not always show a consistent relationship to phenotypic variation, a phenomenon that is generally referred to as network lability or flexibility (van Swinderen & Greenspan, 2005). Some understanding of network robustness and lability is essential to determine how a sequence change in a single gene, or the addition of a novel gene to a network, contributes to variation in the phenotype of interest.

The extent of gene network lability and its relationship to the expression and evolution of even simple behavioral phenotypes is not well understood. For example, using an experimental epistasis approach, van Swinderen and Greenspan (2005) assessed the lability of the gene network associated with a locomotor phenotype in *D. melanogaster*. They determined interactions among 16 members of the gene network known to contribute quantitatively to the locomotor phenotype. Relationships were evaluated in the presence or absence of an additional mutation in a gene (*Syx1A*), which is involved in secretion and synaptic transmission. This experimental analysis showed that interactions among these 16 genes were highly labile depending on activity at this single additional locus. In a genome-scale context, Chandrasekaran et al. (2011) modeled the regulatory associations between transcription factors and target genes using brain expression patterns across behavioral contexts in honey bee workers. The authors found that the relative strength of association between a transcription factor and a putative target gene varied across different behavioral contexts, evidence of network lability associated with naturally occurring variation in behavioral phenotype. These results suggest that variation in network connections not only defines species-level differences in behavior, but it also has a role in modulating behavior within a single species.

There are other examples of changes in gene network interactions with consequences for behavioral phenotypes. Different mouse lines selected for

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"drinking in the dark" showed low convergence in terms of differentially expressed genes, but many similarities in changes in network connections that were associated with the expression of this behavioral phenotype (Iancu et al., 2013). In contrast, Oldham, Horvath, and Geschwind (2006) set out to determine whether species-level variation in cognitive ability among humans and chimpanzees could be explained by variation in network connectivity, given the high level of sequence homology across these species. They generated microarray data from six brain regions across chimpanzees and humans, used weighted gene coexpression network analysis (WGCNA) to identify modules of coexpressed genes corresponding to functionally relevant brain regions, and then compared these networks across species. Particular gene modules corresponded to major subregions in the brain for both species. However, the degree of network conservation between humans and chimpanzees was brain region dependent. Cerebral cortex networks (a region greatly expanded in humans) showed the weakest cross-species conservation. Similar to the mouse example earlier, some genes showed similarities in gene expression across species but differed greatly in network connections. However, in general genes showing higher degrees of differential connectivity showed a higher degree of differential expression across species. At the sequence level, these genes also showed higher rates of evolutionary change in protein-coding sequences. Thus variation in connectivity among genes reflects multiple processes, including evolutionary changes in protein-coding regions and/or changes in gene regulation (Oldham et al., 2006).

The physical properties that underlie variation in network function are still poorly understood. For example, even at the level of DNA–protein interactions, transcription regulatory relationships are affected by enhancers that can be far from the regulatory region of interest, as well as combinatorial relationships among transcription factors themselves. The different conceptions of gene networks reviewed earlier contain elements of lability that operate across different levels of organization. One goal of network research in the future should be to connect these levels of biological organization toward a better understanding of changes in network properties under different conditions, and the consequences of these changes at physiological and behavioral levels. For example, how does a behaviorally relevant perturbation to a biochemical pathway at the protein level influence network regulatory relationships or predict sequence-level changes in regulatory regions of genes? Such approaches, which combine variation in gene expression with feedback at other levels of organization, are being implemented in

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systems biology research (Herrgard, Lee, Portnoy, & Palsson, 2006; Ideker et al., 2001), and could be applied to behavioral work in order to better understand network robustness.

5. EMERGING IDEAS AND FUTURE DIRECTIONS

At its inception within the field of Evo-Devo, the genetic toolkit concept was grounded in the observation that similar genes govern similar phenotypic processes across species. Behavioral genetic toolkit research has expanded to address a number of fascinating questions associated with evolutionary conservation, convergence, novelty, and diversity at both the genotypic and phenotypic levels. As the scope of research in this area is expanding, it is becoming increasingly important to clearly define theoretical objectives embodied in toolkit research, as well as the underlying assumptions of the diverse analytical approaches used to address these objectives. Doing so will enable researchers to articulate alternative and null hypotheses to the toolkit hypothesis, determine whether these hypotheses are indeed mutually exclusive, and develop ways to assess the degree to which these various processes at the molecular level contribute to the behavioral variation we observe within and among species.

To aid in the choice of how to use the genetic toolkit concept in behavioral research moving forward, we present a set of the broad research questions and corresponding hypotheses the concept can evoke (Table 1). Though these questions are not mutually exclusive, they clarify the scope of toolkit research and the existing puzzles, and the potential alternative hypotheses that remain to be addressed (discussed later). Some of these concepts and hypotheses arose from Evo-Devo studies using classical developmental genetics approaches, while other concepts emerged with the expansion of genomics tools. As such, it is not always clear how concepts derived using classical developmental genetics will translate into hypotheses that are tractable for behavioral genomics, and vice versa. However, within the framework of current whole-genome behavioral genetic toolkit research, several of these hypotheses can indeed be addressed. For example, hypotheses about network placement, structure, lability, and conservation can be addressed using transcriptomic data and coexpression network analyses (Oldham et al., 2006). Insights from genome-scale transcriptomic analyses can also be used to target discrete pathways or genes of interest, which can be further evaluated for gene content and regulatory sequence variation in a comparative framework (Turner et al., 2010). Importantly,

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Table 1 Summary of Research Questions That Converge at the Genetic Toolkit Concept

Evolutionary Pattern	Questions	Relevant Hypotheses and Phenomena	Examples
Novelty and diversity at the phenotypic level	How does *diversity* arise in spite of conserved developmental toolkits?	Diversity results from *regulatory changes* in toolkit genes that influence patterns of gene expression (eg, changes in expression timing)	O'Connell (2013)—Neuro-Evo-Devo Turner et al. (2010)—Evolution of monogamy Robinson and Ben-Shahar (2002)—Social behavior
	How does *novelty* evolve, and does it utilize conserved toolkits or new genes?	Diversity or novelty can be attributed to the addition of *new genes* to existing networks, or the expansion and diversification of gene families	Harpur et al. (2014)—Honey bee worker phenotypic novelty Ferreira et al. (2013)—Wasp social evolution
		Diversity or novelty arises from *rewiring* existing networks, including changes in direction of regulation, expansion of a network to incorporate existing genes, and/or changes in sequence that influence expression levels	van Swinderen and Greenspan (2005)—*Drosophila* locomotion Oldham et al. (2006)—Human cognitive ability Patalano et al. (2015)—Wasp and ant social evolution Jasper et al. (2014)—Honey bee worker behavior
		Degree of *network connectivity* and *network placement* influences gene evolution	Liao, Weng, and Zhang (2010)—Morphological vs physiological mouse mutants Oldham et al. (2006)—Human cognitive ability Molodtsova et al. (2014)—Honey bee behavioral regulation
		Alternative splicing allows conserved genes to assume novel functions	Parker, Gardiner, Neville, Ritchie, and Goodwin (2014)—*Drosophila fruitless* gene

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Table 1 Summary of Research Questions That Converge at the Genetic Toolkit Concept—cont'd

Evolutionary Pattern	Questions	Relevant Hypotheses and Phenomena	Examples
Conservation at the genetic level	What evolutionary processes *conserve* core toolkit genes over evolutionary time?	Toolkit genes have *pleiotropic effects* in a range of tissue types—mutations could be strongly selected against	Carroll (2008)—General review Wittkopp and Beldade (2009)—Pleiotropy associated with pigmentation in insects
		Gene networks are plastic over time and across tissues, which could mean few changes are required to accommodate environmental variation over evolutionary time	Dimas et al. (2009)—Human gene expression variation across tissues Jasper et al. (2014)—Comparison of the behavior of conserved and novel genes across tissue types
Conservation at the phenotypic level	How are *conserved* phenotypes regulated at the genetic level?	*Conserved networks of genes* underlie conserved phenotypes	Null hypothesis
		There is drift in the gene regulatory systems that underlie conserved traits over time (*Developmental Systems Drift*)	True and Haag (2001)—Conceptual overview Johnson and Porter (2007)—Interactions of pleiotropy and developmental systems drift Lynch (2009)—Review of animal models
Divergence at the phenotypic level and conservation at the genetic level	Can conserved gene networks give rise to different phenotypes?	*Phenologs*—shared genes that correspond to different higher order phenotypes across species—higher order phenotypes can be united by phenotypic modules at lower levels	Woods, Singh-Blom, Laurent, McGary, and Marcotte (2013)—Phenologs across animal species
		Gene networks are *retained* even if phenotype is not always expressed	Rajakumar et al. (2012)—Soldier caste evolution in ants
		Existing genes and *gene networks are coopted multiple times* (eg, through novel mutations that influence gene expression patterns)	Monteiro (2012)—Eye modules used for color spots in butterflies True and Carroll (2002)—Review

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Convergence at the phenotypic level and/or genetic level	How do *convergent* phenotypes evolve?	There are *evolutionary hotspots* that are sites of repeated de novo mutation associating a particular gene with a repeatedly evolved phenotype	Martin and Orgogozo (2013) and Stern (2013)—Reviews of genetic hotspots Haag and True (2001)—"Phylomimicry" in the context of experimental evolution Marden (2013)—Metabolic gene reuse
		A polymorphic allele present in a shared ancestor evolved the same way independently (*collateral genetic evolution*)	Stern (2013) and Martin and Orgogozo (2013)—Reviews
		Completely *independent genetic changes* give rise to similar phenotypes, presumably driven by *shared ecological conditions*	Null hypothesis

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tracing transcriptomic data to DNA sequence-level variation is not always straightforward. However, the fact that there are examples in which within and among species behavioral variation is associated with similar transcriptomic patterns (which are presumably driven by sequence-level variation in the latter case but not the former; Alaux et al., 2009), suggests translating transcriptomic information to sequence-level variation may be productive. Insights derived from functional genomics within a species require individual-, population-, or species-level comparisons to pinpoint the critical sequence-level variation responsible for the emergence of a behavioral trait.

In addition to the logistical considerations of comparing insights from classical developmental genetics with behavioral genomics, it is not yet clear the degree to which general rules about phenotypic evolution elucidated for morphology are directly applicable to behavior. For instance, morphological traits show different underlying gene network structure compared to physiological traits (Liao et al., 2010), and genes involved in neuronal function show lower levels of evolutionary conservation compared to other processes like ribosomal function (Stuart et al., 2003). Thus it is reasonable to assume that processes underlying behavioral variation could be fundamentally different from morphology in terms of the role of genetic toolkits or the types of genes that make up toolkits. However, there is likely also extensive conceptual overlap (Robinson & Ben-Shahar, 2002), and Table 1 illustrates that the genetic toolkit concept has been used in a variety of ways to evaluate general principles of complex phenotypic evolution in the context of behaviors.

5.1 Investigating Alternative Hypotheses

One topic that deserves study is to compare and contrast predictions of the genetic toolkit concept with those from null and alternative hypotheses. For example, one prominent alternative hypothesis already under investigation is that novel genes contribute more significantly to phenotypic diversity than conserved genes, as discussed more fully below (Harpur et al., 2014). Though such hypotheses are not necessarily mutually exclusive with respect to the toolkit hypothesis, evaluating them may enable theoretical extensions to the toolkit idea. In this section, we highlight some of these emerging hypotheses and approaches.

5.1.1 Incorporating Null Hypotheses

Genetic toolkit studies are often missing a statement or assessment of a null expectation as a basis of comparison for experimental results. For instance,

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when evaluating genomic changes associated with a marine life style, Foote et al. (2015) used a comparison of terrestrial species to determine the null expectation for nonadaptive sequence convergence. Another way to devise a null hypothesis would be to incorporate molecular comparisons among species showing behavioral conservation, instead of focusing solely on examples of convergence. A third approach could be to evaluate the extent of gene network similarities for divergent traits across species (phenologs; McGary et al., 2010; Woods et al., 2013) as a basis of comparison for convergent traits. Establishing null expectations for highly complex genotype–phenotype relationships is difficult, but could provide additional theoretical grounding to strengthen evolutionary genomic studies of behavior.

5.1.2 Novel Genes and the Toolkit Hypothesis

Behavioral genetic toolkit research has made strides in identifying genes and groups of genes that show a similar relationship to behavioral phenotypes across diverse species. However, there is ample evidence that novel taxon-specific genes also play an important role in behavioral evolution (Ferreira et al., 2013; Harpur et al., 2014; Jasper et al., 2014). A role for novel genes has emerged as an alternative to the genetic toolkit hypothesis. However, rather than being two mutually exclusive hypotheses, it is more likely that both conserved and novel genes regulate shared behavioral phenotypes. Defining novel genes with certainty is difficult (discussed earlier) but moreover, understanding the relative contributions of novel and conserved genes to the expression of a behavioral phenotype is a challenge. For example, because genes make up networks, and these networks are somewhat robust to perturbation (Albert, 2005), the phenotypic consequences of adding or subtracting a gene will depend on its placement in the network as well as the function of the gene itself. Furthermore, molecular genetics recognizes that even conserved phenotypes show evidence of shifts in underlying gene networks (True & Haag, 2001); little is known about the extent of these shifts, despite the fact that this "moving baseline" may provide the best null hypothesis for expected levels of gene network change over time and functional consequences for behavioral expression. Modular approaches to behavior and gene networks, and comparisons at variable evolutionary distances (discussed later), may help resolve some of these challenges.

5.1.3 Testing Among Genetic Toolkit Hypotheses

Functional genomics approaches in the context of behavioral genetic toolkits may be even more valuable if they can be used to evaluate

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competing hypotheses about the features, or modules, of particular behaviors that show evidence of genetic toolkits. For example, Toth et al. (2014) used a functional genomics approach to investigate whether genes involved in the expression of reproductively dominant (queen) or subordinate (worker) phenotypes are similar across social insect species that establish and maintain dominance with physical aggression (*Polistes* wasps) or chemical signals (honey bees). To do this, the authors compared the brain transcriptomes of different types of dominant and subordinate *Polistes* individuals, identified differentially expressed genes, and compared those genes with genes associated with caste differences or queen pheromone exposure (which communicates dominance) in honey bees. This study found no significant overlap in genes associated with dominance across the two species, which does not support the hypothesis of a behavioral genetic toolkit for reproductive dominance. However, a comparison of dominance-related genes in *Polistes* with genes associated with aggression in honey bees and fruit flies showed significant overlap, suggesting some evidence for a behavioral genetic toolkit for aggression instead (Toth et al., 2014). Thus functional genomics studies at the whole-genome scale may provide adequate data to evaluate behavioral genetic toolkits from multiple viewpoints.

5.2 Emerging Experimental Approaches

5.2.1 Modular Gene Networks and Behaviors

The vast majority of behavioral genetic toolkit research has implemented whole-genome, comparative approaches to evaluate the genetic basis of complex behaviors. These studies have pinpointed a large number of specific gene networks and modules associated with particular behavioral phenotypes across species. More targeted analyses of these smaller gene networks could provide new insights into phenotypic evolution. For example, a small network of genes can be probed experimentally or with modeling approaches to understand the relative contributions of conserved and novel genes to behavioral variation. Smaller gene networks are also more tractable for analyses of the links between behavioral variation and changes in epigenetic markers, connectivity, and gene content, and thus could be used to evaluate evidence for convergence and conservation at multiple genetic levels. Furthermore, this type of modular approach, which connects gene networks to distinct physiological processes linked to a behavior of interest, may provide general rules about the types of processes and thus behaviors that are associated with high vs low degrees of gene network plasticity (Liao et al., 2010).

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It may also be useful to apply the concept of modularity to behavioral phenotypes themselves (Barron & Robinson, 2008; Scharff & Petri, 2011). Such an approach may identify particular subunits of a behavioral phenotype that are highly similar, providing a stronger experimental basis for the assessment of behavioral genetic toolkits (Scharff & Petri, 2011). Some behavioral phenotypes, well studied in terms of genetic toolkits, already contain very specific criteria for inclusion that successfully span broad phylogenetic distances. For example, sociality can be defined in a variety of ways, but the most extreme form, eusociality, has three basic requirements: reproductive division of labor, overlapping generations, and cooperative brood care (Gadagkar, 1994). Despite this specificity, these criteria are applicable to both eusocial insects and mammals (Jarvis, 1981). If not clearly defined, partial similarities for complex behavioral phenotypes can lead to miscommunications about the role of genetic conservation or novelty in governing a trait of interest. For example, aggression is a ubiquitous behavioral phenotype that has likely evolved convergently across animal species. However, comparing within lineages, there are novel features of the aggressive phenotype. For example, many bee species sting, but honey bees lose their stinger in self-sacrifice during an aggressive response (Winston, 1987). Stinger loss is a novel trait within certain bee lineages, while territorial aggression is likely homologous among bees, and perhaps homoplasious across broader species comparisons.

Along similar lines, few behavioral genetic toolkit studies have evaluated behavioral phenotypes with explicit criteria for similarity. Such an approach may provide new fruitful insights about behavioral regulation and evolution, but moreover, it may provide a more robust basis of comparison between behavioral genetic toolkit research and existing behavioral ecology theory. For example, earlier we discussed how various types of male polymorphisms could have specific underlying genetic toolkits. Such an idea could apply in other contexts of repeatedly evolved substrategies, eg, the various strategies males use to obtain multiple mates (ie, defending multiple females themselves, or defending resources that attract females; Emlen & Oring, 1977). Identifying behavioral and genetic modules could provide an interesting way forward for examining behavioral genetic toolkits at the network level with increased specificity.

5.2.2 Evolutionary Distance

Future studies could benefit from using evolutionary distance more explicitly as an analytical tool in behavioral genetic toolkit research. Examining phenotypic and genetic similarities across species at variable evolutionary

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distances strengthens or weakens assumptions about evolutionary relationships among traits. In cases where behavioral genetic toolkits have been identified, species at different distances can be compared to evaluate variation in gene network composition and function. Mapping this variation onto behavioral trait variation may be one way to determine the relative contributions of particular network changes to phenotypic expression, which could be relevant to assessing hypotheses about different genetic sources of phenotypic variation (eg, the relative importance of the addition of novel genes vs a change in connectivity or expression among conserved genes, which are likely not mutually exclusive). Finally, closely related species can be used to evaluate the predicted relationship among gene networks for conserved behavioral phenotypes (discussed later).

5.3 Why Are Some Genes Toolkit Genes?

Given the vast diversity of genes that have been implicated in behavioral genetic toolkit studies, determining why certain genes emerge as toolkit genes remains an open question. For instance, in a recent study of genes involved in social evolution, Zhou et al. (2015) found that expansion of chemoreceptor gene families is associated with the transition to eusociality in hymenoptera (Zhou et al., 2015), presumably due to selection on these genes in the context of pheromone social signaling. Other studies more generally emphasize the role that sensory systems play in the evolution of behavioral variation (McGrath, 2013). Examining mutant strains of mice, Liao et al. (2010) found that genes involved with morphological vs physiological phenotypes were fundamentally different; the former involved many regulatory genes, which tended to be pleiotropic, while the latter involved channels, transporters, enzymes, and receptors. These gene types also had different degrees of tissue specificity (Liao et al., 2010).

Behavior involves a variety of endophenotypes that span the morphology and physiology continuum. The types of genes showing toolkit evidence may depend on the behavior assessed, or the means of assessment. For instance, immediate early genes represent a category of genes that show a rapid increase in expression in neurons in response to an acute stimulus. There are some examples of immediate early gene orthologs consistently involved in certain types of behavioral response across diverse species, perhaps due to their generalized role in neural function. For example, *Egr-1* is associated with learning and response to novelty across vertebrates and invertebrates (Lutz & Robinson, 2013). Immediate early genes could

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represent an interesting class of behavioral toolkit genes; though many are transcription factors, they can encode a variety of products (Guzowski et al., 2005). Thus perhaps it is the environmental responsiveness of these genes, not their position in particular gene regulatory networks that predisposes them to behavioral toolkit status. Importantly, the temporal sensitivity of these genes provides an unusual opportunity to investigate the spatiotemporal dynamics of the toolkit gene signal as it propagates through the brain in response to a stimulus across diverse species.

Alternatively, the general involvement of immediate early genes in neural activation may create the appearance of a toolkit, when expression of these genes may simply be the effect, not the cause, of differential neural activity resulting from an unrelated genetic difference causing variation in the behavior of interest. Another possibility is that there are mutations causing evolutionary changes in expression patterns of immediate early genes, but these mutations lie in an upstream gene rather than in the immediate early genes themselves. If the causal mutation lies in the coding region of such an upstream gene, then this gene may not be detected through differential expression in comparative transcriptomics studies. As with other putative toolkit genes, additional causal experimental validation is required before strong conclusions are possible.

There is evidence that certain genes are repeatedly implicated in convergently evolved phenotypes because they represent mutational hotspots in the genome (Martin & Orgogozo, 2013). Haag and True (2001) observed a similar phenomenon in the context of experimental evolution, finding that mutagens sometimes recapitulated patterns observed naturally in other species, both in terms of phenotype and underlying genetic mechanism; they termed such events "phylomimicry." There is some evidence of similar phenomena within species as well, ie, genes that tend to be associated with plasticity at different time points throughout life (Sweatt, 2001). For example, in a recent study assessing behavioral genetic toolkits associated with aggression across vertebrate and invertebrate species, one surprising result was that the behavioral response to an acute aggression-inducing stimulus was associated with activity of genes canonically associated with brain development (Rittschof et al., 2014). Thus these genes are not only associated with plasticity in brain structure during an early time point in life, they are also related to plasticity in neural function at a later time point. The possibility that this is a general pattern raises some interesting questions. First, what are the features that make these genes plasticity genes, and is this related to genome structure or some other property of the genes themselves—are they highly

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environmentally responsive, responsive to endocrine signals, or do they occupy a particular network position? Second, how can these genes show highly canalized expression during development and highly variable and environmentally sensitive expression during adulthood? Future research should focus on identifying the common properties of toolkit genes.

5.4 Cumulative Evidence and Knowledge Gaps

The application of the genetic toolkit concept to behavior expanded the phenomenon beyond the scope originally defined by Evo-Devo, which focused on a relatively small set of transcription factors. This difference in toolkit definition may reflect fundamental differences in the evolution of behavior vs morphology at the genetic and phenotypic levels. However, given the complexity of behavioral phenotypes, the apparent conceptual difference could simply reflect predominant experimental approaches and the current state of the field of behavioral genomics relative to evolutionary developmental biology. So far, few behavioral genetic toolkit studies explore causal relationships or evaluate the DNA sequence-level changes underlying observed variation at either the molecular or behavioral levels. As such, it is possible that future work could narrow the definition of behavioral toolkit gene to be more similar to the classical definition. However, it seems equally possible that the wide variety of toolkit genes for behavior accurately reflects an important feature of behavioral adaptation, that convergently evolved behaviors involve similar but nonidentical underlying molecular mechanisms. This pattern could reflect, for example, the complex genetic regulation of behavior, or the relatively high evolutionary lability of behavior. Similar patterns of "low-resolution" genetic similarity (Martin & Orgogozo, 2013) have been observed for other highly environmentally responsive physiological traits, eg, the evolution of insecticide resistance (Ffrench-Constant, Daborn, & Le Goff, 2004).

Currently, the strength and type of evidence implicating a gene as a behavioral genetic toolkit differs from evidence for morphological toolkit genes. This difference reflects a combination of the complex and polygenic nature of behavioral phenotypes, and the typical approaches used to implicate genes in behavior. In some cases, eg, the relationship between *FoxP2* and vocalization, there is causal evidence linking a single gene to variation in a phenotype of interest for certain focal species. For *FoxP2*, there is also extensive information about the patterns of expression and putative functional roles of the protein at the neural level (Scharff & Petri, 2011), which,

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though not causal evidence in all cases, provides a better understanding of functional associations with behavior. At present, however, a correlative relationship between a large set of genes or even a GO term and behavior, observed across multiple species, is considered sufficient to invoke the genetic toolkit hypothesis. Such evidence is clearly only the first step toward understanding the role of convergent molecular evolution in the evolution of convergent behavioral phenotypes.

Experimental validation is necessary to demonstrate a causal relationship between a gene of interest and a behavioral phenotype, and this represents a logical next step for many behavioral genetic toolkit studies. Because many studies implicate a large number of genes, experimental validation could take many forms, from specific genetic manipulations to pharmacological manipulations that target entire physiological pathways. New technologies for high-throughput genetic manipulations and behavioral assessment may be necessary to enable experimental validation to match the pace of discovery genomics. Advances for model organisms like fruit flies and mice may offer comparative systems with high-throughput abilities at both the molecular and behavioral levels (Branson, Robie, Bender, Perona, & Dickinson, 2009).

Assuming that putative toolkit genes show causal relationships to behavior across species of interest, further studies are needed to connect a particular genetic change to the ecologically relevant adaptive behavioral response. For example, manipulating one of many genes and pathways downstream of a critical change in DNA regulatory sequence could affect phenotypic expression even though the upstream change is the most evolutionarily relevant event. This type of validation goes beyond the requirements for demonstrating evolutionary conservation in the context of traditional genetic toolkit research. Determining and comparing which genetic changes are both essential to the expression of the behavioral phenotype and shared across species may require more fine-grained analyses aimed at identifying behaviorally relevant genetic changes among sets of closely related species (eg, Glassford et al., 2015) or among populations of a single species. These genetic changes can then be evaluated in distantly related taxa. Experimental evolution approaches, including domestication studies and adaptations to anthropogenic changes like pesticide resistance (Ffrench-Constant et al., 2004), provide other avenues to address such questions. These more fine-scale approaches may also enable researchers to address a broader range of research questions associated with the behavioral genetic toolkit (as outlined in Table 1).

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6. CONCLUSIONS

The behavioral genetic toolkit hypothesis continues to provide surprising insights into the evolution of behavioral phenotypes. Perhaps more importantly, work in this area has elevated comparative and integrative approaches in the study of long-standing evolutionary questions in behavioral ecology. Though the comparative study of behavior at the phenotypic and molecular levels is undoubtedly complex, behavioral genetic toolkit research has made progress in this area through the use of diverse and creative approaches. As reviewed here, applying the toolkit concept to the study of behavior has prompted the field to tackle the puzzles associated with the evolution of behavioral phenotypes with increased nuance, articulating and incorporating new hypotheses, perspectives, and analytical approaches, and illuminating exciting challenges for future work.

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