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Current Opinion in Behavioral Sciences

RNAseq-ing a more integrative understanding of animal behavior

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Burgeoning sequencing technologies are driving a genomic revolution that provides the potential for a deeper and more integrative understanding of animal behavior. Behavioral traits are likely to result from epistatic relationships among genes, and techniques like RNA sequencing (RNAseq) can bring us closer to understanding these systems. RNAseq can reveal all mRNA produced at a specific sampling point, exposing correlations between genes or genetic networks and behavioral phenotypes. This technology is becoming increasingly available to biologists studying nontraditional model organisms and can unearth important species-specific genetic regulators and interactions that may have been previously overlooked with only candidate gene investigations. Initial description of gene expression associated with a given behavior, followed by hypothesis-driven approaches and experimental manipulations, can now provide one of the most in-depth explorations of bidirectional interactions between genes and animal behavior.

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Current Opinion in Behavioral Sciences 2015, **6**:65–68

This review comes from a themed issue on **Integrated study of animal behavior**

Edited by **Dustin Rubenstein** and **Hans Hofmann**

http://dx.doi.org/10.1016/j.cobeha.2015.09.007

The sequencing of the human genome [1,2] costing $3 billion dollars, paved the way for the advancement of less expensive genomic tools that have revolutionized the depth and breadth at which researchers may study mechanistic phenomenon. Now, for as little as a few thousand dollars, it is possible to obtain the genome and transcriptome sequence of any organism from which nucleic acids can be obtained. Initially, applications of this sequencing technology were limited to biomedical fields. For example, DNA sequencing has been used to identify genetic indicators of diseases [3] and to develop effective methods of treating them [4,5]. Now, these technologies, including RNA sequencing, have been applied in many other fields of biology, including the study of animal behavior, and are becoming critical resources for researchers interested in gaining a deeper, mechanistic understanding. The volume of human and non-human genomic data is growing exponentially, with large consortium-based projects (e.g. The 5000 Insect Genome Project, 1000 Fungal Genomes Project, NSF Plant Genome Research Program, Genome 10K Project) driving this growth.

For a complete and integrative understanding of animal behavior (*sensu* [6•]), researchers often apply a framework developed by Nikolaas Tinbergen [7] to understand the mechanistic, ontogenic, phylogenic and adaptive significance of behavior. The mechanistic drivers of behavior generally involve complex interactions between multiple levels of biological organization, from DNA to hormones and physiology to the social and physical environment that an organism inhabits. Indeed, we now know that the genome can respond dynamically to environmental stimuli in a way that is potentially adaptive (e.g. [8,9]). Thus, emerging genomic tools like RNA sequencing (RNAseq [10•]) combined with classic animal behavior methodologies and experimental manipulations can help to unravel the mysteries of the dynamic genome, how it functions at different levels of biological organization, and how these networks influence, and are influenced by animal behavior.

How does RNAseq work?

Prior to the emergence of next generation sequencing technology (NGS), studies with goals that included understanding the genetic correlates of behavior often relied on candidate gene approaches. Although powerful, these approaches are generally limited to the examination of only a few genes. RNAseq is a technique, applicable to traditional model and non-traditional model systems alike, that allows the gene expression of all transcripts (mRNA) in a given tissue to be estimated simultaneously [11], therefore offering significant advantage over the candidate gene approach. The process begins by the extraction of RNA from an appropriate tissue (e.g. the preoptic area of the brain in a study of the neuromechanisms of a particular reproductive behavior). A sequencing library is created to retain all transcripts, including those that have not yet been described. Sequence data are generated on a high-throughput sequencing platform that has the ability to yield hundreds of millions of sequence

reads per run. This enables the detection and quantification of both very rare and very abundant transcripts. These raw sequence data are assembled into a reference set of transcripts (e.g. *de novo* transcriptome assembly) whose abundance is then estimated [13]. Although patterns of expression are often tightly linked and responsive to environmental conditions, a vibrant new field of study — one that links patterns of expression to expression quantitative trait loci (eQTL) [14] — is allowing researchers to explore variation in gene expression. This new and exciting paradigm presents the opportunity to link a behavior with both heritable and non-heritable patterns of gene expression.

Comparative genomic approaches yield new discoveries

By contrast to studies that utilize RNAseq, comparative genomic approaches have typically quantified the expression of a limited number of specific genes of interest and leveraged these genetic data against distantly related model organisms (e.g. *Homo*, *Mus*, or *Drosophila*). Now, the advent of cheaper methods of sequencing DNA and RNA has allowed researchers to produce whole genome or transcriptome data (the collection of *all* transcripts expressed in a given tissue) directly for both study organism and related species, thus enabling a comparative genomics approach. Consequently, the mechanisms driving animal behavior can now be investigated at a level of inquiry previously reserved solely for humans, rodents and flies (Table 1). Indeed, any species now has the potential to be a 'model', with an array of tools allowing for study of genomic and physiological processes [15•]. For example, Schunter *et al.* [16•] used RNAseq to elucidate mechanisms associated with alternative reproductive strategies in the black-faced blenny (*Tripterygion delaisi*). Some males of this species maintain territories on which they attract females and guard nests, while other 'sneaker' males mimic female behavior, sneaking into nests and copulating with females. Schunter *et al.* [16•] identified differentially expressed genes according to mating tactic and sex. Importantly, these genes differed from the candidate genes previously used to study alternative mating tactics in other species of fish [16•]. Thus, although certain genes and functions can be conserved across species, important species-specific genetic regulators may have been overlooked by studies adopting the traditional candidate gene approach. By examining all changes in expression occurring in a given tissue(s), specific genes can still be tested in a targeted, hypothesis-driven approach. Moreover, this approach will facilitate the potential identification of new gene regulators and enable the creation of more complete species-specific genetic toolkits with which to study the mechanisms of behavior.

Complex traits require a systems biology approach

The candidate gene approach is powerful for many types of investigations, but applying functional genomics to behavioral traits may require a systems biology approach. Behavioral phenotypes are typically coded by regulatory gene networks (e.g. [17,18]) which likely are influenced by epistatic interactions. Although it is currently difficult to characterize the entire complement of genetic mechanisms for a particular behavioral phenotype, the ability for us to do this may be in our near future. Already, data gathered by RNAseq can be used to mathematically determine how genes form complex networks that generate behavioral phenotypes [17]. Indeed, exciting discoveries have been made by studying co-expressed gene sets (i.e. modules; [19–21]), though it is not completely clear how to best describe these relationships and their role in behavior and its evolution. Moreover, the relationship of genetic modules to physiological systems must also be considered, particularly given the advent of genomic editing and manipulation. How do genes form networks with hormones, the immune system, and other physiological variables to shape or respond to behavior? How do they work through neural circuitry to influence and be influenced by behavior? These questions present

Table 1

A Tinbergian approach to studying animal behavior using genomic tools. Nikolaas Tinbergen highlighted four main categories of inquiry that would together lead to an integrative understanding of proximate and ultimate causes of behavior. Here, we adapt these questions to incorporate genomic tools like RNAseq.

	Classic Tinbergian questions	Questions now possible with genomic tools like RNASeq
Mechanism	What is the mechanism that elicits behavior X?	Do differences in gene expression, as opposed to or in relation to other physiological (like endorcine) drivers, elicit behavior X?
Ontogeny	How does behavior X change within an individual during development?	Behavior X varies through development, but does the expression of key genes drive this? Do we see different behaviorally-relevant genes being expressed differently during development?
Adaptation	How does behavior X affect the organism's chances of survival and reproduction?	Can we see signatures of molecular adaptation in transcripts that underlie adaptive behaviors?
Evolution	How did/does behavior X evolve?	Are the transcripts or patterns of gene expression underlying behavior X in species 1 the same ones underlying the behavior in species 2, 3, 4, 5?

the next big challenge in our path toward obtaining the most integrative understanding yet of the biological mechanisms that explain animal behavior.

A more integrated understanding of animal behavior

Technologies like RNAseq offer researchers a direct means of integrating their level(s) of biological questioning and analyses with disciplines previously considered distinct from the field of animal behavior, such as molecular biology [22] and computational biology [23]. Genomic data collected during studies of behavioral phenomenon may also allow researchers to address questions related to population genetics (e.g. natural selection, mutation, and drift), itself an exceptionally vibrant and active area of research [24–26]. Statistical rigor (i.e. appropriate replication and controls) in such studies will allow comparisons of gene activity among individuals and populations to be made, as well as the identification of differential expression in gene sets or networks in response to experimental conditions, or ecological, behavioral, and temporal changes [20].

This newfound ability to collect genome-wide data on patterns of expression in relation to behavior has provided the opportunity to pose and answer questions that were previously impossible. A descriptive 'natural history' of the genome(s) of interest is often a critical first step to understanding the genes that underlie a behavior (e.g. [20,27]). Indeed, the integrative study of animal behavior embraces the need for hypothesis generation *in addition to* classic hypothesis testing [6•]. Once genomic candidates or networks have been identified, inter-species and intra-species comparative studies of the behavior of interest, or of the behavioral response to environmental manipulation, can yield expression profiles that illuminate the function of the organized network, be it gene, endocrine, or other. Armed with such knowledge, other emerging technologies may further help to disentangle correlation from causation (e.g. RNA interference, RNAi, to silence genetic expression [28], or CRISPR/Cas9 to edit parts of the genome [29] corresponding to genes critical for regulation of a given pathway).

Despite the potential advantages of NGS technologies, the utility of enormous genomic datasets has been debated. Do technologies such as RNAseq lead to a better understanding of animal behavior, or as researchers like Zuk and Balenger [30] question, are they 'a new kind of mechanism, illuminating more details but possibly distracting us from a more process-driven approach'? Has the current data-deluge resulted in researchers becoming 'lost in the map' [31], a figurative description of all behavior-linked mechanisms, rather than truly enhancing our understanding of the mechanistic underpinnings of behavioral phenotypes? These questions are valuable as they remind us that it is necessary to proceed with caution, both in terms of project design and data analysis and interpretation. However, it is clearer now that rather than obfuscating our position on the map, the use of genomic tools has increased the size and resolution of the map, leading us toward a more integrative understanding behavioral ecology [32,33•], phenotypic plasticity [34], behavioral evolution [35], the evolution of personality [36], behavioral development, adaptation and life-history theory [37,38]. Synthesizing more information undoubtedly creates new and various challenges. However, it also creates unprecedented and integrative opportunities to enhance our understanding of animal behavior (Table 1).

Conflict of interest statement

Nothing declared.

Acknowledgments

The authors thank Dustin Rubenstein and Samuel Díaz-Muñoz for their discussion of this manuscript, as well as two anonymous reviewers for their invaluable comments and suggestions. Funding was provided by NSF IOS 1455957 (to RMC and MDM).

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