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Going wild for functional genomics: RNA interference as a tool to study gene-behavior associations in diverse species and ecological contexts

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ABSTRACT

Identifying the genetic basis of behavior has remained a challenge for biologists. A major obstacle to this goal is the difficulty of examining gene function in an ecologically relevant context. New tools such as CRISPR/Cas9, which alter the germline of an organism, have taken center stage in functional genomics in non-model organisms. However, germline modifications of this nature cannot be ethically implemented in the wild as a part of field experiments. This impediment is more than technical. Gene function is intimately tied to the environment in which the gene is expressed, especially for behavior. Most lab-based studies fail to recapitulate an organism's ecological niche, thus most published functional genomics studies of gene-behavior relationships may provide an incomplete or even inaccurate assessment of gene function. In this review, we highlight RNA interference as an especially effective experimental method to deepen our understanding of the interplay between genes, behavior, and the environment. We highlight the utility of RNAi for researchers investigating behavioral genetics, noting unique attributes of RNAi including transience of effect and the feasibility of releasing treated animals into the wild, that make it especially useful for studying the function of behavior-related genes. Furthermore, we provide guidelines for planning and executing an RNAi experiment to study behavior, including challenges to consider. We urge behavioral ecologists and functional genomicists to adopt a more fully integrated approach which we call "ethological genomics". We advocate this approach, utilizing tools such as RNAi, to study gene-behavior relationships in their natural context, arguing that such studies can provide a deeper understanding of how genes can influence behavior, as well as ecological aspects beyond the organism that houses them.

1. Introduction

Wilson (1975) suggested that animal behavioral traits, specifically social ones, are "the class of phenotypes furthest removed from genes". Indeed, behavioral phenotypes result from myriad abiotic, biotic, and social inputs, received by multiple sensory systems, integrated in the brain with numerous elements representing an animal's internal state, and finally manifest as a complex series of motor outputs. In addition, behavioral traits can be challenging to measure and quantify due to the inherent complexity of behavioral sequences and the context-specificity of many behavioral traits. Thus, the prospect of pinpointing the genetic basis of even the simplest behavior can be a somewhat daunting enterprise. It is therefore not surprising that the study of the molecular genetic basis of behavior has lagged behind the study of other phenotypes such as development, morphology, and physiology (Robinson et al., 2005). One major impediment to progress in behavioral genetics has been the difficulty in establishing causal relationships between individual genes and behavior, especially in an ecologically relevant context (Rittschof and Robinson, 2014). Establishing causality may be especially challenging for behavioral traits, due the highly polygenic nature of many forms of complex behavior and the highly environmentally responsive nature of many behavior-related genes (Clayton, 2000; Clayton et al., 2019). In recent years, research teams working in diverse organisms have been successfully using functional genomic methods, such as RNA interference (RNAi) and CRISPR/Cas9 gene editing to establish links between individual genes and complex behaviors in a growing cadre of diverse organisms (London, 2020).

Despite recent progress, a true integration of behavioral genomics with fundamental tenets of ecology and evolutionary biology has not yet been achieved. Ethology has classically focused on studying animal behavior in natural environments, and its sister field behavioral ecology has been highly productive in providing numerous fundamental

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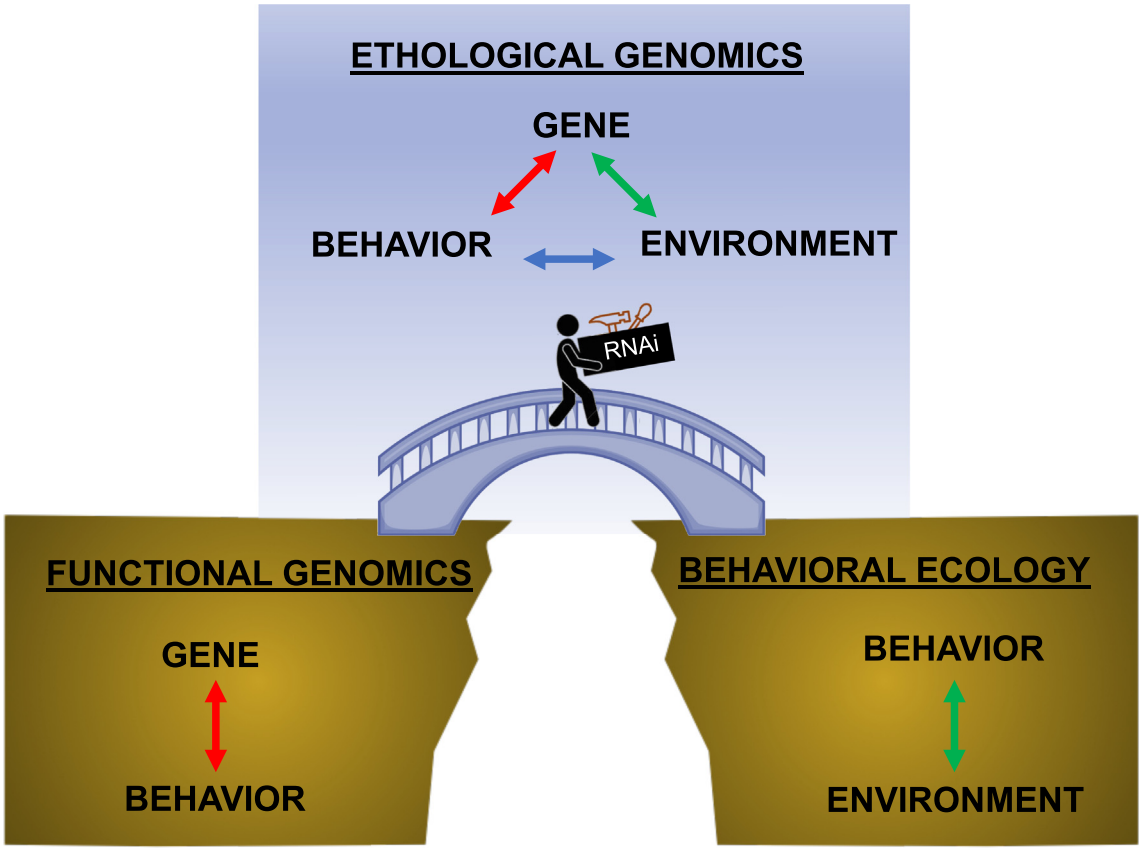

Fig. 1. Functional genomics is concerned with elucidating the relationships between genes and phenotypes, such as behavior. Behavioral ecology is concerned with understanding the adaptive value of behavior in an animal's natural environment in response to ecological pressures. The rift between functional genomics and behavioral ecology can be bridged by studying gene function under natural conditions, which we term "ethological genomics". RNA interference (RNAi) can be an extremely useful part of the researcher's toolbox for ethological genomics, utilizing experiments conducted in the field or under semi-natural conditions for a more comprehensive understanding of the complex interrelationships between genes, the environment, and behavior.

insights into the adaptive function of animal behavior in the face of ecological pressures (Krebs and Davies, 2009). At the same time, there is a large behavioral genetics literature from model organisms, such as mice and fruit flies, with the successful identification of numerous genes that can influence behavior under laboratory conditions (Anholt and Mackay, 2009). There are clear advantages to studying behavior in the laboratory, including precise control of environmental conditions, greatly simplified tracking of individuals, and ease of observation and large numbers of individuals of a controlled genetic background. The vast majority of functional genomic studies on behavior-related genes have been conducted in the laboratory (Anholt and Mackay, 2009), but these conditions may not recapitulate important aspects of an animal's abiotic, biotic, and social environment. There is a growing appreciation in biology that genes cannot be understood apart from the environment in which they evolved (Lewontin, 2001), and behavioral phenotypes may be particularly prone to gene-by-environment interactions and indirect genetic effects (Bailey et al., 2018). Given these important considerations, there still exists a large gap between modern behavioral functional genomics and behavioral ecology (Fig. 1). This is important, because gene function is intertwined with the ecological and evolutionary environment in which the gene was selected (Zaidem et al., 2019). For example, a recent study in social wasps found social status-related gene expression was strongly perturbed by placing animals in the laboratory compared to the field (Jandt et al., 2015), thus lab-based studies of gene-behavior relationships in systems such as this could lead to incomplete or misleading interpretations of gene function. However, at this time, there have been exceedingly few studies of behavioral gene function in the wild, partly due to technical challenges of gene manipulation and functional assessment.

The time is right for a fuller integration of ethology with functional genomics (Fig. 1). With the advent of new tools for functional genomics, the study of behavioral genomics is gradually moving from simpler behaviors in model organisms to more complex behaviors in non-models. There has been increasing interest in the application of CRISPR/Cas9 gene editing technology to non-model species, including for studies of gene-behavior relationships (Yan et al., 2017; Trible et al., 2017). While germ-line modification-based methods such as CRISPR/Cas9 hold much promise in this realm, a major hurdle is that it is not ethically feasible to release these modified organisms into the wild to study gene function. Thus, it is important to broaden the outlook for ecological and evolutionary functional genomics beyond CRISPR/Cas9. By doing so, behavioral genetics has the opportunity to return to its ethological "roots" so that the genetic basis of behavior can be studied in natural or semi-natural contexts, allowing for a deeper understanding of gene-behavior relationships in their ecological context, potentially allowing a more accurate assessment of true gene function.

In this review, we aim to highlight the use of one tool for ethological genomics, RNA interference (RNAi). Because RNAi does not alter the germ line, is transient, and silences gene expression (Mello and Conte, 2004), it has unique properties that make it highly useful for ecologically relevant studies of behavioral functional genomics, including for use in free-living wild animals. Here, we describe the use of RNAi technology for gene silencing, point out the attributes that we believe make it especially useful for behavioral genomics, and provide examples from prior studies that highlight unique advantages of RNAi to facilitate the study of behavioral phenotypes. We then address some of the challenges and technical considerations associated with the use of RNAi. Overall, we argue RNAi has a unique role to play, and heretofore

Box 1
Taxonomy of RNAs used in RNAi.

RNAi (RNA interference) is the process by which RNA regulates gene expression in a specific and targeted manner, often through degradation of mRNA (though evidence of reduced transcription has been reported as well). By inducing RNAi mechanisms, researchers can cause gene knockdown in a temporal and tissue-specific manner in many species.

mRNA (messenger RNA) is the mature RNA that is intermediate to the genetic information coded by the DNA and the amino acid sequence that is eventually translated.

miRNA (microRNA) are small non-coding RNA molecules that bind to X' region of mRNA and downregulate gene expression through a variety of mechanisms including mRNA degradation and interference with ribosomes. miRNAs are endogenously produced in a variety of plants and animals.

siRNA (small-interfering RNA) are short (~20–24 bp) double stranded RNA molecular with overhangs on both ends. siRNAs are generated from longer double-stranded RNA or short hairpin RNAs by Dicer, an RNase III enzyme, and can complex with the RNA Interference Silencing Complex (RISC). The siRNA sequence acts as a template for degradation of mRNA with complementary sequences, providing the potential for highly targeted gene silencing.

dsRNA (double-stranded RNA) is targeted by the Dicer enzyme for processing into siRNA. Endogenous RNA in eukaryotes is single-stranded and the presence of dsRNA is indicated of an RNA virus.

shRNA (short hairpin RNA) are artificially designed single-stranded RNA molecules that bind with themselves creating a hairpin-like structure. The doubled-over RNA molecule is processed by the Dicer enzyme to generate siRNA that will cause gene silencing when bound to the RISC complex. shRNA are generated in cells using plasmid or viral vectors.

DsiRNA (Dicer short interfering RNA) are preprocessed dsRNA molecules that can be used as the vehicle for RNAi.

untapped potential, as a tool for researchers in bridging the gap between ethology and functional genomics across a wide diversity of animal taxa (Fig. 1).

2. What is RNAi and how does it work?

RNAi takes advantage of pathways within cells to degrade RNA and as a result effectively lower the abundance of a target transcript. The purpose of an RNAi experiment in the context of functional genomics is to silence or “knock down” the expression of a target gene of interest in the focal species, and then to examine the resulting effects on the organism's phenotype. The goal of this section is to provide readers a brief overview of the research history and mechanism of various RNAi techniques, as well as citations pointing to additional reading for those interested in learning more. To aid readers in following the RNAi literature, we also provide a glossary of commonly seen acronyms in the RNAi literature (Box 1).

RNA interference was first reported from experiments working on anthocyanin synthesis in petunias, where over-expression of the mRNA for enzymes that generate purple pigments instead generated white flowers (Napoli et al., 1990; Sen and Blau, 2006). Experiments showed that RNA levels, rather than being elevated as intended by the experiment, had been reduced (Napoli et al., 1990). This initial description of co-suppression of RNA led to a flurry of work and demonstrations of similar phenomena in a range of species (Guo and Kemphues, 1995; Romano and Macino, 1992), though the mechanism causing silencing was unclear. Toward the end of the 1990's researchers working on nematodes identified that it was double-stranded RNA (dsRNA) that led to gene silencing (Fire et al., 1998) and within a few years our understanding of the molecular pathways causing RNAi took shape (Sen and Blau, 2006). Longer dsRNA molecules are broken into ~22 bp fragments with overhangs on each end by the RNase III enzyme Dicer (Bellés, 2010; Kim and Rossi, 2008; Sen and Blau, 2006). The fragments can then bind with the RNA-induced silencing complex (RISC), which retains one of the strands of the dsRNA fragment and uses that as a guide. While many elements of the multi-component RISC are understood, such as the role of Argonaute proteins in targeted cleavage of RNA, the full picture of RISC is still unclear. It is thought that eukaryotes evolved mechanisms to target and degrade mRNA based on similarity to dsRNA as a defense against RNA viruses (Zambon et al., 2006). The same machinery, however, has also been shown to play a role in endogenous gene regulation via microRNAs (miRNAs), which are abundant in most eukaryotic genomes and known to play a role in gene regulation (Farh et al., 2005; Niwa and Slack, 2007).

Although dsRNA is effective at triggering RNAi responses across diverse eukaryotes, it is proven to be problematic in jawed vertebrates, where it can trigger an interferon response (Bagasra and Prilliman, 2004). A work-around for utilizing RNAi in vertebrates (which has seen the most development in mammals) is to use viral vectors to drive the expression of short-hairpin RNA (shRNA), which forms a double stranded molecule by binding to itself due to complementary sequences encoded within the length of the single strand that is then processed by the Drosha enzyme before being exported from the nucleus (Borel et al., 2014). These modified shRNA molecules are then processed by the RISC and Argonaute enzymes to cause RNAi. While viral vectors are more complicated to make than the direct synthesis of dsRNA, they provide a number of experimental advantages. Notably, viral serotypes vary in tissue specificity. For example, adeno-associated viral vectors (AAVs) are attenuated viruses that lack replication machinery and come in many different serotypes that target distinct tissues (Zincarelli et al., 2008). Expression of shRNA is driven by using a gene promoter from the experimental species (Borel et al., 2014), allowing for further cell-type specificity. For example, using an AAV-mediated RNAi it would be possible to cause knockdowns of a specific target gene only in neurons that express a particular receptor. The recent explosion of single-cell RNAseq studies documenting gene expression markers for distinct cell-types in neural tissues (Moffitt et al., 2018; Ofengeim et al., 2017) hold particular promise for targeted use of RNAi to study gene-behavior relationships.

3. Strengths of RNAi in an ecological context

RNAi is especially well-suited for use in ecologically-relevant contexts. For example, RNAi is already being experimented with extensively and utilized in practical contexts for pest control in insects in the field (Scott et al., 2013; Katoch et al., 2013; Fishilevich et al., 2016). Thus, behavioral scientists can utilize knowledge and lessons from such studies in designing and implementing experiments in a field context. One of the most important logistical barriers to performing gene manipulations involving germ-line transformation is that such organisms cannot be released into the field. There are often strong regulations/restrictions in place (Bruggemann, 1993; Xu et al., 2011; Fernandez-Cornejo, 2014) that prevent studying genetically modified organisms in the field, including in a semi-natural context in which release of these organisms into the wild is even remotely possible (Williamson, 1992; Alphey and Bonsall, 2018). Because RNAi alters levels of mRNA available for translation but does not alter the germ line (Mello and Conte, 2004), there are far fewer ethical barriers in utilizing this

technology in a wider variety of natural, semi-natural, and open field contexts than for transgenic organisms. In essence, the researcher is able to exploit "temporary" genetic modification to get at gene function, without the environmental and ethical issues associated with release of mutant organisms into natural populations.

Another benefit of RNAi is that the effects are usually transient, often reaching peak knockdown within 48 h (Burand and Hunter, 2013) and then returning to baseline within days of introduction of the interfering RNA into the organism (though knockdown timing is organism, tissue, and even gene dependent). Because many/most genes show short term or conditional expression throughout the lifetime of an organism, the subtler alterations in gene expression created by RNAi may offer a more realistic test of the true effect of a gene on behavioral development of expression. This is unlike a full knockout, which effectively removes the gene entirely from the organismal system, sometimes leading to devastating (lethal) or highly disruptive effects that may mask the gene's true biological role. RNAi, in contrast, reduces expression of a gene but does not completely turn it off. The effects of RNAi, while potentially systemic, may also be somewhat tissue specific (Ghanim et al., 2007), that is higher at the site of injection or area of introduction of the interfering RNA into the system (Xia et al., 2002). This is also more realistic considering the fact that many/most genes show tissue or body area specificity in their expression (Getzenberg, 1994; Papatheodorou et al., 2018).

Another advantage is that in contrast to germ-line modification, RNAi can potentially be performed on animals that have not been developed as a lab system. For many non-model organisms, there are large or even insurmountable barriers preventing continuous rearing, controlled breeding, or maintenance of large laboratory colonies. Rearing the animal in a lab environment from embryo to adult need not be necessary to perform RNAi knockdown, so non-model organisms and animals that are not cultured in laboratories can still be targets of RNAi experimentation. In theory, an animal at any life-stage (barring organismal obstacles to successful RNAi administration, Fig. 2) (Posnien et al., 2009) could be plucked from the wild, treated with interfering RNA, and subsequently observed in the lab or in its natural environment.

Overall, gene knockdowns represent a more realistic way to assess gene function than knockouts, because of their transient, more subtle dynamics that better reflect true fluctuations in expression and localization, and the fact that the gene is not being entirely "removed from the system". Conditional knockouts (e.g. tissue-specific or on-off switch type knockouts such as temperature induced) have been used with great success (Skarnes et al., 2011) to address some of these issues in model organisms (e.g. Lee et al., 2008), but the generation of conditional knockouts is a complex process that is likely to be inaccessible to non-model organisms. In contrast, RNAi should in theory be accessible to a wider possible range of study organisms, where experimental manipulations are feasible and ethical given their population sizes and status.

4. Promising behavioral applications of RNAi and associated case studies

In order to highlight the potential of RNAi in addressing gene-behavior relationships, below we highlight a few illustrative examples in which RNAi has been utilized in the field or laboratory as a powerful functional genomic tool to establish causality for particular genes in a variety of behaviors in several different taxa. This is not meant to be an exhaustive review of the literature on RNAi and behavior; rather, we have chosen specific examples that illustrate some of the unique advantages and potential of RNAi for behavioral genetics. Because there are so few studies that have actually utilized RNAi to study behavior in the field, most of the following examples revolve around studies that have addressed questions on the ecology and evolution of behavior by performing RNAi in the lab. These studies provide useful examples for behavioral ecologists on how effective RNAi can be for elucidating certain types of gene-behavior relationships, and may also guide future research using RNAi in more natural settings.

4.1. Elucidating the functions of master regulator genes

RNAi is a robust tool to interrogate genes near the top of a behavioral regulation hierarchy—the so-called "master regulator genes" (Ohno, 1977). Master regulator genes may include those that are highly expressed (or ubiquitously expressed), highly pleiotropic with multiple phenotypic effects, and/or central regulators of transcription of many other downstream genes. Because these genes are expected to have large phenotypic consequences, knockdown should yield clear and observable effects on manipulated organisms. Conversely, genes lower

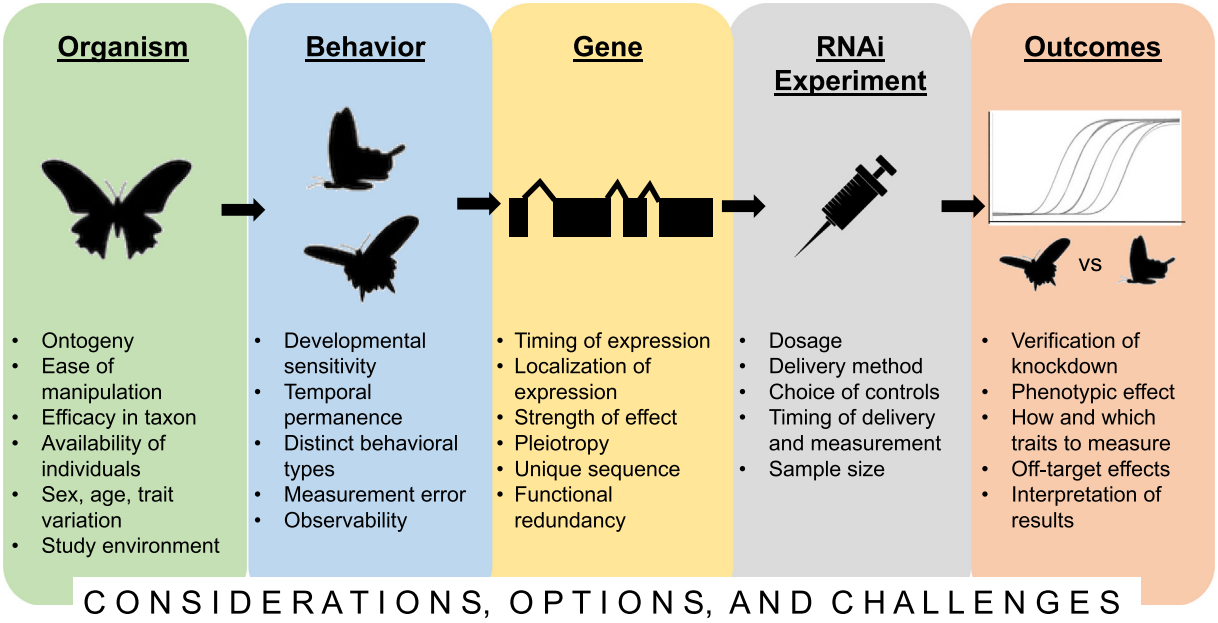

Fig. 2. Researchers are faced with many different considerations, options, and challenges in designing and implementing an RNAi experiment, starting with the study organism of choice, behavior to be studied, target gene of interest, parameters and design of the RNAi experiment, and assessment of experimental outcomes.

down in a regulatory hierarchy may yield only small, additive behavioral effects or even have their effects compensated for by other, redundant genes in the genome. For such genes, RNAi perturbance may be ineffective at causing a reliably detectable phenotypic change even if the gene is involved in influencing the phenotype. Thus, master regulator genes that orchestrate major phenotypic shifts are excellent targets for RNAi experimentation. Below we outline one such study.

The egg yolk precursor protein vitellogenin is an important regulator of reproduction in oviparous animals, but has taken on many diverse roles in honey bee (*Apis mellifera*) worker behavior and physiology. In honey bee colonies, division of labor is mediated largely by worker age, with food foraging behavior initiating around 3 weeks of life. In a study by Nelson et al. (2007), the authors investigate the role of the gene *vitellogenin* (*Vg*) in regulating this age-based division of labor. Injection with *Vg* RNAi resulted in several behavioral effects, including an earlier onset of foraging behavior and a preference for nectar forage over pollen forage. These results support a long-standing hypothesis that the regulation of non-reproductive social phenotypes (i.e., foraging for a colony) can be co-opted from reproductive pathways (West-Eberhard, 1996; Amdam et al., 2004).

vitellogenin is a master regulator gene in honey bees. Knockdown confirmed its diverse roles in life-history traits, including division of labor and longevity. Furthermore, RNAi resulted in a persistent suppression of *Vg*, as lower vitellogenin protein concentrations were recorded at 10, 15, and 20 days after injection. Most noteworthy, this study uses RNAi to examine behavior in the wild (i.e. the semi-natural setting of managed honey bee hives in an open-air apiary). This is a feat usually impeded by most of the challenges associated with RNAi (see section "Challenges and Considerations when Designing an RNAi-Behavior Experiment" below) and Nelson et al. (2007) is a singular successful example, in addition to a few others (Ihle et al., 2019; Ament et al., 2012; Antonio et al., 2008). It is notable that the only studies that use RNAi to examine behavior in the wild have been on honey bees, and specifically foraging behavior. This is likely due to the accommodating properties of the honey bee system, including the ease in which individuals can be manipulated in the lab but reintroduced to colonies, that hives are stationary, and that foraging behavior is well-understood and easily observed.

Although studies have used RNAi in the field to explore non-behavior traits (Weiner et al., 2018) and potential pest control applications (Whyard et al., 2009; Huvenne and Smagghe, 2010), RNAi has rarely been used as a tool to study behavior in the wild despite its high utility. We suggest that social insect systems in particular are promising candidates for RNAi in the field or in semi-natural mesocosms. In fact, there are several studies that have successfully used RNAi to interrogate the function of behavioral master regulator genes in social insect biology, including the neuropeptide *Corazonin* in *Harpegnathos saltator* ants (Gospocic et al., 2017), the insulin receptor substrate *irs* in honey bees (Ihle et al., 2019), and the vitellogenin-like gene *Vg-like A* in *Temnothorax longispinosus* ants (Kohlmeier et al., 2018). The next step is to understand how these master regulators operate in an ecological context. Because most social insect groups have stationary colonies, they are ideal for RNAi manipulation in natural or semi-natural conditions.

4.2. Understanding genes with effects that are time-sensitive or transient

RNAi can be especially effective if the gene of interest is expressed at a particular time in development or its effects are impermanent. For example, RNAi can be used to perturb time-dependent gene expression during development, time-dependent gene expression responses to specific environmental stimuli, or behaviors associated with circadian patterns in gene expression (as discussed below).

Circadian rhythm irregularities are often associated with psychiatric illness in humans, including bipolar disorder. To examine the genetic regulation of circadian rhythms and associated behavioral phenotypes, Mukherjee et al. (2010) used an adeno-associated virus vector of a short hairpin RNA (shRNA) to knock down *Clock*, a gene that is integral in the regulation of circadian rhythms, in the ventral tegmental area (VTA) brain region in mice. Suppression of *Clock* resulted in an increase in hyperactivity as well as an increase in depression-like behaviors. Gene knockdown via RNAi proved to be an especially effective method to investigate the role of *Clock* in mood regulation. A previous *Clock* knockout study resulted in a compensatory expression of the transcription factor Neuronal PAS domain Protein 2, which functionally substituted the role of *Clock* (DeBruyne et al., 2007). As a result, these *Clock* knockout mice exhibited normal circadian rhythms. Similarly, a previous study found that mice with a mutation in the *Clock* gene exhibited the hyperactivity behavior, but not the depression-like behavior observed in *Clock* knockdown mice (Roybal et al., 2007). Mukherjee et al. (2010), suggest that the permanence of a mutation could have led to compensatory effects during development to offset the phenotypic consequences of the mutation. The transient nature of RNAi, in contrast with the permanence of gene knockout or bred mutant lines, likely contributed to its power to illuminate this gene-behavior relationship.

In addition to genes associated with circadian rhythms, there are myriad genes with time-sensitive expression. For example, explicating which genes are associated with signaling the developmental transition to the next life-stage have benefited from RNAi (i.e. molting in cockroaches: Martín et al., 2006). Similarly, RNAi has been used to interrogate genes involved in the transition from solitary to swarm-state locusts when they are signaled by crowding (Ott et al., 2012; Guo et al., 2011). Clearly, RNAi can be a highly useful tool for understanding time-sensitive gene effects, whereas the permanence of many other germline altering gene manipulation methods provides less temporal precision.

4.3. Manipulation of simpler elicitors of complex behavioral interactions

A tractable approach to studying the mechanistic basis of complex behaviors is to study the elicitors of behavior, which may have a simpler genetic basis. For example, identifying brain-based genetic regulators of social interactions, involving quantizing the genetic regulation of behavioral interactions between two or more individuals, may be highly challenging, as this can involve complex neural integration of multiple social signals by multiple individuals. A more feasible solution can be to examine the genetic basis of the production of semiochemicals, which are chemical communication signals released by one individual and received by another, which coordinate behavioral interactions between individuals. Studying the biochemical pathways that regulate the production of semiochemicals in isolated glands or tissues may be simpler than understanding how social information is processed in the brain, making semiochemical-related genes prime targets for using RNAi to study behavior. Because semiochemical production is usually transient, life-stage specific, and tissue specific, RNAi is well-suited to investigate the relationship between the genes that regulate semiochemical production and the behaviors they elicit.

RNAi has been successfully implemented to investigate sex pheromone production and its behavioral consequences. For example, Lee et al. (2011) reduced sex pheromone production in female diamondback moths via knockdown of a pheromone biosynthesis-activating neuropeptide receptor gene (*Plx-PBANr*). This resulted in a 20—40% reduction of successful matings when treated females were exposed to males. Prior to performing RNAi, the authors comprehensively characterized where and when *Plx-PBANr* is expressed during the moth's development. They found that it is expressed only in the pheromone gland and expression begins at early adult stages. Thus, they were able to determine where best to inject dsRNA and at which life stage. This study is notable among research that implements RNAi to investigate gene-behavior relationships, as here the authors examine how expression of a gene in one individual affects the behavior of another (female gene expression influences male behavior). Here we see that RNAi can be used as an effective tool to link function at seemingly vastly separated scales– tissue-specific gene expression changes to inter-individual

behavioral dynamics.

RNAi has proved effective in investing the genetic basis and function of semiochemical production across a range of species including moths (Lee et al., 2011), flatworms (Patlar et al., 2020), and fruit flies (Chapman et al., 2003; Dembeck et al., 2015; Kubli and Bopp, 2012). While determining synthesis pathways may be best done in the lab, RNAi approaches that allow for altering semiochemical production offer new experimental tools to investigate the function and evolutionary consequences of animal communication. Selecting simpler targets of RNAi (such as genes involved in production of chemical signals or neuronal receptor activity), may be one efficient way for researchers in ethological genomics to begin to bridge the gap between gene and behavior.

4.4. Manipulating ornaments and weapons to study choice and conflict behaviors

One of the richest areas of research in behavioral ecology is understanding sexual selection and the extreme traits that can evolve as a result. Behavioral ecologists have long been interested in the developmental inputs that generate variability among individuals in sexually selected traits. Additionally, much can be learned about the function of sexually selected traits from methods that allow for manipulation of traits or de-coupling of traits and behaviors. RNAi offers new opportunities to study both of these questions in non-model organisms and in the wild.

Some of the most exciting work using RNAi to study sexually selected traits comes from beetles, where multiple groups have been successfully using the technique to study the genetic basis of horn and mandible development (Gotoh et al., 2014; Moczek and Rose, 2009; Ohde et al., 2018; Wasik et al., 2010). For example, in a recent study Gotoh et al. (2014) demonstrated that elaborated and condition-sensitive male mandible development in the stag beetle *Cyclommatus metallifer* depends on the interaction of juvenile hormone and sex-specific isoforms of *doublesex* (*dsx*). Treatment with *dsx* RNAi reduces the sensitivity of male mandibles to treatment with juvenile hormone, but increases female sensitivity. Using RNAi, the researchers generated large male beetles lacking the elaborated weapons typical of such individuals. Thus, RNAi provides a novel tool for manipulating the expression of secondary sexual characteristics in individuals that could then be studied and tracked in natural populations. This research area is ripe for follow up studies examining the behavioral impacts of sexual ornament manipulation via RNAi.

In theory, similar approaches could be used in other taxa as well to investigate the function of sexually selected or other developmentally sensitive traits. For example, AAVs could imaginably be used in birds to influence feather color production in association with a molt. RNAi offers exciting possibilities for phenotypic manipulation of traits that are relevant to sexual selection, communication, and signaling in behavioral ecology studies.

5. Challenges and considerations when designing an RNAi-behavior experiment

At each step in the process of an RNAi experiment, the researcher has many options and considerations to bear in mind. Some of the main steps in an RNAi based functional genomic experiment are as follows (Fig. 2).

5.1. Identify a target gene of interest in the focal species

Ideally, this should be based on robust, highly replicated experiments showing a strong association between a phenotype of interest and high levels of gene expression or gene activity of the target gene. In addition, genes identified as having heritable effects on behavioral phenotypic differences, even without differences in expression, can still be useful target genes for RNAi. If prior information is not available, knowledge of putative gene function and expression patterns from other, ideally closely related, species can be used as justification of a candidate target gene.

5.2. Design the RNAi experiment

The experimenter must use their knowledge of their focal species (informed by prior studies on the same species or related taxa) to decide upon which developmental stage, sex, age, etc. to treat. Other important options include delivery method (injection, oral, other), type of interfering RNA (dsRNA, siRNA, etc.), type of controls to be implemented (unmanipulated, sham, non-endogenous gene interfering RNA (to control for side effects of RNAi pathway stimulation), taking into account the genomic background of focal individuals from treatment groups to control for differences caused by genetic differences), timing of delivery, sampling, and phenotyping, and whether to conduct the study in the lab or the field.

5.3. Collect and analyze specimens to verify knockdown

This is usually accomplished by extracting RNA and measuring gene expression using real time quantitative RT-PCR, in situ hybridization, or antibody staining. Before extensive phenotyping and other experimentation, the researchers should always endeavor to perform a preliminary experiment to show that expression knockdown is possible. Although a pilot experiment of this nature is strongly encouraged, there can be limitations of working with species that are not lab-ready, are not perennial, and yield small sample sizes. Thus, meticulously executed RNAi pilot experiments may not always be logistically or financially feasible. Researchers must weigh the trade-off between these risks and investments. One way to alleviate some of these risks is to include redundant knockdown techniques, such as using a few different siRNA constructs that target different sequences of the same gene.

5.4. Phenotype individuals after RNAi delivery

The penultimate key step is to measure behavior in focal individuals. The experimenter must decide on the types and number of behaviors to measure, and the time interval after the injection to observe (note, this is usually done within a fairly short time frame, such as 1–3 days, due to the transient nature of RNAi knockdown).

5.5. Re-verify knockdown

The final step is to sample a subset of focal individuals and show that a knockdown occurred. Similar to Step 3, using real time RT-qPCR or other method is key to demonstrate that any phenotypic effect observed in step 4 is a result of knockdown.

Although there are many potential benefits and advantages of RNAi for analyzing gene-behavior relationships, in practice there are numerous technical challenges associated with the real-world application of RNAi (Fig. 2). First, at the organismal level there are many considerations and challenges of which a researcher should be aware. For example, researchers must consider ontogeny; that is, if the targeted gene is important at different times in the organism's development. If so, knowledge of the timing of the gene's effect during development, and proper delivery of interfering RNAs at the correct developmental period will be extremely important. The transience of RNAi can assist in temporally targeted knockdown, affecting development at specific stages/times. Also, some studies have reported that RNAi is more effective during early development as opposed to in adulthood, which may be related to higher dilution of RNAi molecules in a larger body (Dong and Friedrich, 2005). Also, efficacious delivery of the interfering RNAs into the organism is critical. Common methods of administration include feeding (paper wasp larvae, *C. elegans*), injection (many taxa),

or even topical application using a spray or mist (honey bees (Li-Byarlay et al., 2013)). In addition, different types of transfection reagents (usually lipophilic substances to facilitate entry of the interfering RNAs across cell membranes) are often used to improve efficacy of RNAi (Kim, 2003). It is generally thought that injection is the most direct and efficacious method of delivery, because interfering RNAs introduced through feeding may be broken down in the gut, and topical application may expose RNAs to degradation before they are able to actually enter the organism. Finally, there appear to be large differences in RNAi efficacy across taxa (Bushman, 2003; Cullen, 2005), with some taxa that are refractory to successful RNAi and others that show consistent and efficient gene silencing, with substantial variation even within taxa (such as within the insects: Whitten and Dyson, 2017). It is not always clear why certain taxa are less amenable to experimental RNAi, and negative results are rarely published, so we lack a good database of taxonomic efficacy to inform decisions about whether RNAi has a good chance of working in a particular taxonomic group compared to others. Oral RNAi delivery efficiency has been improved in some insects through the addition of gut nuclease inhibitors (Spit et al., 2017) or delivery via baculoviral vectors (Chavez-Pena and Kamen, 2018). Substantial effort has been made to improve RNAi methods in mammalian systems due to potential applications to human health, and successful RNAi in mammals has focused on the use of silencing triggers such as short hairpin RNAs (shRNAs) (Siolas et al., 2005). Similar vectors have also proved useful in other vertebrates including birds (Bhattacharya et al., 2017) and fish (Zenke and Kim, 2008), suggesting that the approach may be broadly useful among vertebrate taxa. Different taxa are likely to require different forms of RNAi implementation. We suggest researchers take cues from studies on related taxa as a starting place for the likelihood of RNAi to be efficacious in their taxon of interest.

Second, there are also considerations for successful RNAi at the phenotypic (behavioral) level. The formation of a behavioral phenotype may be more or less sensitive to perturbation at specific developmental times. RNAi is a powerful tool at dissecting when behavioral development is most sensitive. As behavioral phenotypes expressed at one life stage may be influenced by gene expression at earlier life stages, RNAi can be used to target specific times in development without disrupting expression beforehand (Tomoyasu and Denell, 2004). Similarly, behaviors often vary in how temporary or permanent they are, as certain behaviors are only performed at a particular time or life stage (ie., mating behaviors, migration, or home scouting). Thus, RNAi can be used to affect a temporary behavior at the appropriate time. Although the temporal specificity of RNAi is useful, its genetic specificity can lead to experimental challenges. For example, as many behaviors are polygenic, compensatory expression of multiple genes may mask the effects of an otherwise successful gene knockdown. So, knockdown of one gene may not be effective in altering behavior. Finally, the observability of the behavior of interest must be considered. A clear and quantifiable phenotype is necessary for any behavioral study. When testing a gene's behavioral function, this is especially true. For example, behaviors that can be recorded as presence/absence may yield clearer results than more subtle phenotypes. If performing RNAi in the wild, animals that are easy to relocate or track and observe or remotely monitor are necessary.

Third, there are challenges and considerations that must be taken into account at the molecular level. As is true for ontogeny of the organism and the developmental plasticity of the behavioral phenotype, the timing of when a gene is expressed can be highly important to the development of a behavioral phenotype. General knowledge of when a gene is expressed may be necessary to ensure the gene is knocked down during the correct window of time when expression affects behavior. Alternatively, if the goal of the experiment is to *determine* when expression affects behavior, a controlled time-series of RNAi treatments is an effective method. In addition to being time specific, RNAi methods can be location specific as well. In many cases, RNAi can be used to knock down gene expression in an organism's specific tissues (especially using viral vectors). Thus, the importance of the location of a gene's expression can be examined. This is also beneficial if the gene's expression in other locations has phenotypic effects that could confound the effects in the location of interest. Similarly, how pleiotropic the gene is must be considered. If the target gene influences multiple phenotypic elements, RNAi may be less effective at pinpointing its function in relation to the behavior of interest. Knocking down a highly pleiotropic gene can have the benefit of producing large or multiple observable phenotypic effects (i.e. Nelson et al., 2007; see section above: “Elucidating the functions of master regulator genes”), but may obfuscate the gene-behavior relationship by causing major systemic disruptions to the organism's biology. Thus, it is important to phenotype your focal individuals broadly, as there may be other non-behavioral effects of a knockdown. Another essential issue that needs to be addressed when designing an RNAi experiment is the sequence similarity of the target gene to other genes. When designing RNAi molecules, if a unique enough sequence is not chosen, other genes with similar sequences may be knocked down, leading to off target effects that cause unwanted phenotypic change. In contrast, a successful silencing of the gene of interest may not result in the expected phenotypic effect if the pathway that yields the behavior of interest is redundant (either by gene homologs or compensatory development pathways) which can mask the expression perturbation of the target gene. This is one way in which RNAi can be more effective than knockout methods, as compensatory pathways may be less effective at counteracting transient RNAi as opposed to permanent genetic changes.

6. Conclusions and future outlook

Looking forward, we suggest the next powerful application of RNAi to study the genetic underpinnings of behavior will be to do so in a more ecologically relevant context. Our knowledge of many otherwise well-studied behaviors has long been lacking robust understanding of the environmental and evolutionary context of the gene. This is, in great part due to dual constraints of the lab and the field, which each fill in only half the relationship (gene to behavior and behavior to ecology, respectively) (Fig. 1). Unlike some other functional genomic tools, RNAi is a feasible and ethical method for exploring these relationships in the wild. It bears mentioning, however, that RNAi in the field is ethical only if the researchers that employ these techniques are mindful. Although we highlight the transience of RNAi, i.e. that it should not affect the germline nor cause heritable genetic changes to the DNA sequence, we note there exists a possibility of causing heritable epigenetic changes (Castel and Martienssen, 2013). Therefore, best practices for tracking and retrieving treated individuals should be established if RNAi experiments will take place in the wild. While there are currently not regulations in place regarding the release of RNAi treated animals into the field, we believe that moving forward, ethical regulations should be established for the use of this technology that consider potential environmental impacts as well as addressing the potential of heritable (though possibly reversible) epigenetic changes.

In addition, we envision new opportunities could arise as researchers more widely adopt the use of RNAi to address gene function for behaviors that have previously been the purview of behavioral ecologists. For example, fascinating aspects of natural behavior that can only be properly studied in the field (such as migration, home range size, foraging, dispersal patterns, and species interactions) could potentially be experimentally manipulated and examined at the level of gene expression. Moreover, by contextualizing a gene within an animal's natural environment, this opens up new and exciting possibilities to examine how the behavioral effects of one gene trickle down through food chains, mutualisms, parasitism, and other types of ecological interactions. As such, ethological genomics may allow researchers to gain a better understanding of the “long reach of the gene” beyond the phenotype of the species in which it occurs (Dawkins, 1982),

influencing both biotic and abiotic aspects of its environment. In the same way that a cell-culture based approach to studying gene function provides a limited view of its role in whole organisms, lab based studies of gene function in organisms provide an incomplete understanding of the role of the gene shaping behavior in a species' ecological context. In conclusion, there are many exciting avenues for ethological genomics in the future, and the use of RNAi in the wild may help bridge the historic gap between behavioral ecology and functional genomics.

Declaration of competing interest

The authors declare no competing interests.

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