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RESEARCH ARTICLE

A practical approach to RNA interference for studying gene function in a refractory social insect (on a limited budget)

A. Walton[1] · E. Flores[2] · A. Guinness[2] · R. Fortune[2] · M. J. Sheehan[3] · A. L. Toth[2,4]

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Abstract

With powerful functional genomics tools, including RNA interference (RNAi), the study of gene function is moving from simpler phenotypes in model organisms to more complex traits in non-models. However, optimizing RNAi-mediated gene knockdown for a novel system can be time consuming and expensive, so researchers interested in initiating an RNAi study for functional genomics in a non-model organism will want to consider the successes and failures of RNAi in related species and similar phenotypes. Yet, sub-optimal and negative knockdown results are rarely reported alongside positive ones, so choosing the best methodological parameters can be difficult. Furthermore, social insects pose extra challenges to designing functional genomics studies, as the traits we measure are often part of the extended phenotype of a primary reproductive, emerge from the collective phenotypes of many individuals, and may be the result of a gene taking on a novel functional role in a social system. We attempt to mitigate these challenges for other social insect biologists by reporting our own successes and impediments to optimizing RNAi for the gene *vitellogenin* in the paper wasp *Polistes fuscatus*. We discuss the many factors that can affect RNAi experimental design and which parameters worked optimally for our study, as well as compile a list of working parameters from previous RNAi studies in social Hymenoptera. We highlight the difficulties of RNAi that are specific to social Hymenoptera, including the potential differences in RNA efficacy across behavioral caste, and share results on our optimized parameters as a potential roadmap for other social insect biologists.

Keywords RNAi · Paper wasp · *Polistes fuscatus* · Functional genomics · Ethological genomics

Introduction

Establishing a causal link between a gene and a phenotype is a fundamental goal in biology. Interrogating a gene's function can be especially challenging for social insect biologists, as the phenotypes we study are often part of the extended phenotype of a primary reproductive individual or emerge from the collective biology of multiple individuals. Furthermore, the social nature of our systems means that a gene's function may not easily be inferred from its putative roles in solitary model organisms (such as *Drosophila melanogaster*) as ancestral genes may take on novel functions in eusocial species (Amdam et al. 2003a, b; Berens et al. 2015; Jandt and Toth 2015). In recent years, functional genomics has introduced an increasingly powerful toolbox of techniques to draw causal links between genes and their phenotypes (London 2020). One such tool is RNA interference (RNAi), which uses an organism's anti-viral immune response to double stranded RNA to "knock down" expression of a target gene. By observing the trait changes caused by an RNAi-mediated knockdown of gene expression, we can measure the downstream effects of a gene on the phenotype. Importantly, RNAi-mediated gene knockdown affects expression, but does not alter the genotype nor affect the germline (as opposed to gene "knockout" and CRISPR-Cas9 gene editing). The transient (and often localized) effect of RNAi also makes it a powerful tool for understanding a gene's function in vivo and in the wild (reviewed in Walton et al. 2020).

✉ A. Walton
arwalton@ualberta.ca

[1] Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada

[2] Department of Ecology, Evolution, and Organismal Biology, Iowa State University, Ames, IA, USA

[3] Department of Neurobiology and Behavior, Cornell University, Ithaca, NY, USA

[4] Department of Entomology, Iowa State University, Ames, IA, USA

RNAi has the potential to aid in functional genomics of non-model organisms (Walton et al. 2020). An RNAi experiment does not require well-established lab lines, short generation times, or even a sequenced genome. Although an understanding of an organism's natural history and general biology greatly aids in the design and optimization of an RNAi experiment, the essential requirement is the transcribed sequence of the gene of interest (Mello and Conte 2004). This makes RNAi especially advantageous to the many social insect biologists that study species that cannot be easily maintained or bred in the lab, or with genomes that have yet to be sequenced.

RNAi has been implemented in several social insect systems (highlighted in Fischman et al. 2011; Scharf et al. 2008; see Supplemental Table 1 for a comprehensive list of RNAi studies in social Hymenoptera). In particular, RNAi has been widely used in functional genomics studies in the honey bee *Apis mellifera* (Amdam et al. 2003a, b; Dearden et al. 2009; Evans et al. 2013; Scharf et al. 2008). For example, honey bee RNAi studies have helped illuminate the function of genes related to sex determination (Beye et al. 2003), development (Costa et al. 2016; Maleszka et al. 2007; Medved et al. 2014), age polyethism (Amdam et al. 2006; Marco Antonio et al. 2008; Nelson et al. 2007), immune function (Aronstein and Saldivar 2005; Brutscher and Flenniken 2015; Hunter et al. 2010; Li et al. 2016; Schlüns and Crozier, 2007) pheromone biosynthesis (Wu et al. 2020), and the possible role of DNA methylation in caste-related alternative splicing (Li-Byarlay et al. 2013). RNAi has been similarly important for many functional genomics studies of ant biology (Bonasio et al. 2012; Du and Chen 2021; Glastad et al. 2020; Liutkevičiūte et al. 2018; Lu et al. 2009; Moreira et al. 2020; Rajakumar et al. 2018; Ratzka et al. 2013) and termite caste determination (Zhou et al. 2008; Zhou et al. 2006a, b; Zhou et al. 2006a, b). However, functional genomics research on "primitively eusocial" species has had sparser success (Supplemental Table 1; highlighted in Walton et al. 2020). Yet, the promising examples of RNAi studies in these systems, such as paper wasps (Hunt et al. 2011; Weiner et al. 2018) and bumblebees (Deshwal and Mallon 2014; Du et al. 2019; Hu et al. 2010; Kim et al. 2009), exemplify the potential for this technique to address gene function across a wide swathe of social insects. Supplemental Table 1 in the Supporting Materials summarizes RNAi studies that have been successfully implemented in social Hymenoptera. Even within species and caste, RNAi methodology differs from study to study. In *Apis mellifera*, studies vary in RNAi delivery method (feeding, abdominal injection, and ocellus injection), dosage (for both volume and interfering RNA concentration), and timing between treatment and sampling (ranging from 1 to 480 h post-treatment). Furthermore, although some studies report negative results (Amdam, Simões et al. 2003a, b; Hamilton et al. 2019; Li et al. 2016; Moreira et al. 2020), it is not standard practice to publish methods that do *not* yield a knockdown.

Despite the successes in implementing RNAi in social insects, one of the largest challenges that remains is the repeated need to develop optimized protocols for RNAi each time a new species is studied, especially as related to brains and behavior. The "brain problem" presents oft-acknowledged logistical challenges, while at the same time brain and behavior related genes are among the most interesting to many social insect biologists.

In this study we report our successes with (and impediments to) developing RNAi for the paper wasp *Polistes fuscatus*. *Polistes* paper wasps are an exemplary social insect system for developing and implementing RNAi. There is a strong international base of biologists who study *Polistes* and make up the latest incarnations of a long historied lineage of devoted paper wasp scientists. Yet, we are a part of a relatively modestly sized scientific coterie when compared to the prodigious research community that studies, for example, honey bees. As such, we lack many of the conveniences of working within a highly populated sub-field, such as access to optimized molecular tools, well-vetted husbandry techniques, and an inexhaustible body of literature. Among

Table 1 RNAi treatments tested

Caste	siRNA type	Dosage	Injection site	Timing	Tissue with significant knockdown		
					Brain	Head	Abdomen
Foundress	Standard	5 µM	Ocellus	24 h			
Foundress	Standard	50 µM	Ocellus	24 h			
Foundress	in vivo	10 µM	Aorta	24 h		✓	
Foundress	in vivo	10 µM	Aorta	48 h		✓	
Worker	Standard	10 µM	Ocellus	24 h	✓		
Worker	Standard	10 µM	Aorta	24 h			✓
Worker	in vivo	10 µM	Ocellus	24 h	✓		
Worker	in vivo	10 µM	Aorta	24 h			

Check marks refer to significant knockdown of *vitellogenin* expression when compared to *gfp*-treated wasps

social insect biologists, this predicament is not unique to those of us who study *Polistes* paper wasps. Thus, the target audience of this report is not solely *Polistes* researchers, but the broader community of scientists that work on less often studied Hymenoptera.

In Walton et al. (2020), we promoted an integration of functional genomics and behavioral ecology we termed "ethological genomics" and advocated RNAi as the primary tool of this new approach. Following publication of Walton et al. (2020), we received multiple inquiries from both behavioral biologists and social insect scientists looking for guidance as they designed their own RNAi experiments. Although Walton et al. (2020) includes a general outline of how to carry out an RNAi experiment, researchers that contacted us were looking for more specific parameters and advice. There are many factors to consider when designing an RNAi experiment and each may require optimization to yield a successful gene knockdown. These include but are not limited to delivery method, timing between administration and sampling, interfering RNA dosage, and life stage/age of the organism. Simultaneous optimization of multiple factors is a daunting task, and it can be difficult deciding where to start. In this article we describe our own endeavor to optimize RNAi in the paper wasp *Polistes fuscatus*, describing both what worked and what did not. It is our hope that this article will provide a starting point and potential roadmap to success for other social insect researchers eager to incorporate RNAi and ethological genomics approaches to their research programs. We highlight the challenges of RNAi that are specific to our study systems, namely the potential for a difference in efficacy of RNAi in different behavioral castes, and share results on our own optimized parameters as a starting point for other social insect biologists.

Methods

Wasp collection and housing

We collected paper wasp nests at various sites in central Iowa throughout the Spring and early Summer of 2019 ($n = 46$) and 2020 ($n = 43$). Nests were in the "foundress phase" of the colony lifecycle (before the emergence of workers) (Matthews and Ross 1991). This ensured that 1) the foundress could be identified and 2) that the foundress had not yet progressed to the "queen" life stage, which is accompanied by a major shift in behavioral repertoire (Hunt 2007). Collections were performed in the early evening or during cloudy days when foundresses are most likely to be present on the nest. We briefly chilled foundresses at 4 °C, which slows movement and halts flight, to mark them with Testors enamel paint (Testor Corporation, Rockford, IL). We affixed nests to cardboard squares by applying hot glue to the base of the pedicel. Then, we taped these cardboard squares to the top of manufactured Plexiglas wasp boxes (30 cm × 30 cm × 30 cm), with the nests facing inward, and placed the marked foundress back on her nest. We maintained colonies in an indoor rearing facility in Bessey Hall at Iowa State University (Ames, IA, USA). Full-spectrum lights were set to a day–night cycle, with lights turning on at 6:00 and turning off at 20:00. Temperature was maintained at 27 °C. We provisioned colonies with construction paper (to maintain and expand their nests), water, and sugar cubes (Domino brand sugar) ad libitum. Twice weekly, we provided nest boxes with fresh prey consisting of *Galleria mellonella* waxworms, *Tenebrio molitor* mealworms, and *Manduca sexta* hornworms (Rainbow Mealworms, Bonsall, CA) (Tumulty et al. 2021; Walton and Toth 2021). Wasp nests were maintained in the laboratory for approximately 14 days before foundress knockdown experiments and between 30 and 60 days prior to worker knockdown experiments (to allow for workers to emerge and mature). Wasps were sampled 24 h or 48 h after injection, depending on treatment timing (see below).

Target gene and anatomical region

We targeted the yolk precursor gene *vitellogenin (vg)* for RNAi-mediated knockdown. We selected this gene for RNAi optimization in paper wasps because of its role as a major regulator of insect physiology and social insect behavior. Thus, successful knockdown of *vg* expression should result in apparent and observable effects on behavior. Additionally, previous studies showed RNAi can effectively knock down *vg* expression in honey bees with clear phenotypic effects and without major mortality (Amdam et al. 2006; Amdam et al. 2003a, b; Marco Antonio et al. 2008; Nelson et al. 2007). We designed small-interfering RNA construct sequences (see below *Type of interfering RNA*) based on a putative *vg* transcript (GDFS01048000.1) from the *Polistes fuscatus* transcriptome shotgun assembly (Berens et al. 2016), which we checked against the *P. fuscatus* genome to confirm the presence of a single ortholog (Miller et al. 2020) and vetted by performing a reciprocal BLAST of *vg* against the *Drosophila melanogaster* and *Apis mellifera* genomes.

We attempted to knock down *vg* expression in wasp heads because previous research on honey bees has shown localization of vitellogenin in the fat body that surrounds the brain (Seehuus et al. 2007). In honey bees, fat-derived vitellogenin has been proposed as a regulator of behavior via signaling to the brain or neurosecretory tissues in the head (Amdam and Omholt 2003; Guidugli et al. 2005). In *Polistes* paper wasps, *vg* expression in the head has been previously shown to be associated with nutritional state (Daugherty et al. 2011) and aggression (Manfredini et al. 2018; Uy et al. 2021), though

a causal link between head fat *vg* and *Polistes* behavior has yet to be established (Walton et al., *in prep.*).

Optimization variables

There are many factors to consider when designing an RNAi experiment and each may require some level of optimization. We tested the efficacy of different treatment levels of several variables (Table 1). Ideally, we would have been able to test every combination of every variable at each treatment level. This is, of course, not a practical option for many social insect researchers. The quantity of animal specimens and chemical reagents necessary to perform a complete survey of all the potential variable combinations would be enormous, as would the cost and time required to administer interfering RNA and verify knockdowns at that scale. With these constraints, we tested the variable combinations we construed to be most successful and tweaked the treatment levels in each subsequent experiment based on our findings from the preceding one until we detected a significant knockdown. Although less thorough than a full factorial design, this method of selecting a priori a subset of treatment combinations follows the statistical practice of implementing planned contrasts that have increased power against Type II errors (Chatham 1999; Wang 1993). The following is a description of each variable we considered in our optimization experiments.

Type of interfering RNA: There are different kinds of interfering RNAs that are used for RNAi-mediated knockdown and selecting the correct one depends on its convenience and efficacy for a particular system or study (Walton et al. 2020). These include double stranded RNA (dsRNA), small-interfering RNA (siRNA), and microRNA (miRNA). In this study we use dicer short interfering RNA (DsiRNA), which are siRNA preprocessed for use in RNAi. We purchased DsiRNA from IDT (Integrated DNA Technologies, Iowa City, IA). We used IDT's "Custom Dicer-Substrate siRNA Design Tool" (www.idtdna.com) to design 27mer *vg* siRNA duplexes. We selected 3 DsiRNA designs that were targeted to different regions of the *vg* sequence (Supplemental Table 2). We resuspended DsiRNA in Nuclease-Free Duplex Buffer (IDT) and mixed the 3 DsiRNA construct solutions in equal parts into a cocktail before administering (see "Dosage" below). Additionally, we added the transfection agent X-tremeGENE (Roche) with a 10:1 DsiRNA solution to transfection agent ratio (Sun et al. 2018).

In all experiments we administered *gfp* DsiRNA to control wasps. These interfering RNA target the green fluorescent protein gene *gfp*, which is not present in the paper wasp genome. We used *gfp* as a control because IDT sells prefabricated *gfp* DsiRNA due to its common use as a reporter gene in molecular biology experiments (Phillips 2001). We used siRNA that targets a non-endogenous gene (*gfp*) as a treatment to control for the effects of handling, injection, and immune system changes associated with the presence of foreign RNA. As with *vg*-targeted DsiRNA solutions, we added transfection agent for a 10:1 *gfp* DsiRNA solution to transfection agent ratio. We note there is some concern of non-target effects of *gfp* knockdown in honey bees (Nunes et al. 2013a, b) on gene expression. Inclusion of multiple controls is highly desirable but not always logistically possible; the main purpose of including the *gfp* DsiRNA group in this study was to control for 1) injection and handling effects and 2) effects of stimulation of the anti-viral immune system.

In addition to standard DsiRNA, IDT offers specially modified DsiRNA that are ostensibly more stable when administered in vivo (Morrissey et al. 2005). We compare the efficacy of IDT's standard DsiRNA and in vivo-modified DsiRNA (referred to throughout this study as "in vivo-modified", noting that all our manipulations were performed on wasps in vivo).

Dosage: To begin with a ballpark estimate of an appropriate dosage, we looked at prior studies with honey bees (Guo et al. 2018; Hamilton et al. 2019) and then adjusted the concentration of DsiRNA for the average mass of the paper wasp. Throughout these experiments we tested 3 different concentrations of siRNA: 5 µM, 10 µM, and 50 µM (see Table 1 for which experiments used which siRNA concentrations). We diluted siRNAs to desired concentration in nuclease-free water (IDT) and stored them in a − 20 °C freezer until use.

Injection site: In selecting a site to inject interfering RNA, we considered the proximity of the target tissue (the fat surrounding the brain), the invasiveness of injection, and previous RNAi successes in social insects (Hamilton et al. 2019; Jarosch and Moritz 2011; Rein et al. 2013). As established in previous research, we tried injecting siRNA just behind the median ocellus (Guo et al. 2018). Before injection, we placed wasps in individual 15 mL centrifuge tubes and anesthetized them on wet ice. Next, we made a small incision just superior to the wasp's median ocellus with the tip of a syringe. Although similar protocols in other social insects did not require an incision (El Hassani et al. 2012; Guo et al. 2018), the paper wasp cuticle is too rigid for a glass needle to penetrate. We injected 500nL of siRNA solution into this incision with a Nanoject III microinjector (Drummond Scientific Company, Broomall, PA) equipped with a pulled glass capillary needle.

Separately, we also tested the aorta as an alternative injection site. Hemolymph is pumped through the aorta and into the head of the insect, then dissipates back through the thorax and abdomen (Klowden 2013). To administer siRNA to the head via aorta, we injected 500nL of siRNA solution into the wasp's cervix (the neck-like integumentary cylinder that connects the head and thorax). Although this siRNA

delivery method was untested in social insects, it is seemingly less invasive than the ocellus injection, as it does not require an additional incision. After siRNA injection (either at the ocellus or aorta), we once more briefly chilled wasps on wet ice before individually housing them in 16 oz. polypropylene deli cups (Fabri-Kal, Kalamazoo, MI).

Timing: The effect of RNAi is usually transient, with peak knockdown occurring some time (often within hours or days) after administration and ultimately returning to baseline expression (Burand and Hunter 2013). Thus, an important challenge is determining how long after RNAi delivery to collect samples; long enough for the knockdown to occur, but not so long as to miss the effect entirely. Based on RNAi studies on honey bees (Guo et al. 2018; Hamilton et al. 2019; Nunes et al. 2013a, b; Rein et al. 2013), we tested for gene knockdown at 24 h and 48 h after siRNA injection.

Knockdown localization: The effects of RNAi can vary in how local or systemic of a knockdown occurs (Aigner 2006). We targeted the fat tissue that sits on the brain, which is known to express *vitellogenin* in honey bees (Corona et al. 2007; Seehuus et al. 2007). Although both our injection sites were near the target tissue, there was potential for RNAi treatment to cause a reduction or possibly even a compensatory increase in *vg* expression in other parts of the wasp, either from siRNA traveling to other tissues or due to a systemic RNAi response (Aigner 2006; Mello and Conte 2004). To test the tissue-specific effects of each RNAi treatment, we measured *vg* expression in the dissected brains, head capsules (i.e., the head with the brain removed, heretofore referred to as "heads"), and abdomens.

Behavioral caste

We tested the efficacy of RNAi in both workers and foundresses. Foundresses and workers differ in fat stores, ovary size, and behavior (Hunt 2007; Toth et al. 2009) and can have widely differing levels of baseline *vg* expression (Corona et al. 2007; Hartfelder and Engels 1998; Manfredini et al. 2018). Because differences in social role and their associated differences in physiology and behavior could have consequences on the effectiveness of RNAi at knocking down *vg* expression, we tested *vg* RNAi separately in each of these behavioral castes (workers: $n = 55$; foundresses: $n = 58$). All wasps of both castes survived until they were sampled.

Knockdown confirmation

To assess the efficacy of expression knockdown, we sampled wasps 24 h or 48 h after injection (depending on the timing treatment), and immediately after phenotyping. Phenotyping consisted of being housed in a petri dish at room temperature with an unfamiliar wasp for 1 h, during which aggressive interactions were observed and recorded. These aggression assays were part of another study (Walton et al., *in prep*) and results will not be described here. We then sampled wasps on dry ice, placed their heads and abdomens in separate tubes, and stored them in the − 80 °C until further processing. We freeze dried wasp heads at 300 mTorr and − 85 °C for 60 min in a lyophilizer. We dissected out brains over dry ice, saving the head capsule for molecular processing as well. We removed brains by chipping away at the head exoskeleton with a microscalpel starting at the antennal sockets, until the brain was exposed enough to carefully lift it out of the head capsule. Separately, we extracted RNA from abdomen, head capsule, and brain tissue using a Qiagen RNeasy Mini Kit and protocol (Qiagen) and treated RNA with DNaseI (Ambion). We used 200 ng of extracted RNA as a template for cDNA synthesis with the SuperScript III First-Strand Synthesis System (Invitrogen).

We measured expression of *vg* with RT-qPCR. We used 2 µl of cDNA in 10 µl volume reactions (ran in triplicate) of the 2X SYBR Green PCR Master Mix (Thermo Fisher) with the CFX384 TouchTM Real-Time PCR Detection System. We used a previously established (Berens et al., 2016) internal reference gene *(rp49)* to normalize gene expression data, which was not significantly differently expressed across *vg* and *gfp* RNAi treatments (ANOVAs: Worker abdomens: $F = 1.18$, $df = 1, 54$, $p = 0.28$; worker heads: $F = 1.95$, $df = 1, 54$, $p = 0.17$; worker brains: $F = 0.12$, $df = 1, 54$, $p = 0.74$; Foundress abdomens: $F = 0.006$, $df = 1, 57$, $p = 0.94$; foundress heads: $F = 0.08$, $df = 1, 57$, $p = 0.78$; foundress brains: $F = 0.53$, $df = 1, 57$, $p = 0.47$).

Statistics

We performed statistical analyses using *R* version 4.0.3 (R Core Team 2020). For all tissue samples, we calculated delta-Ct values of gene expression. Delta-Ct values are defined by the number of qPCR cycles that occur before an exponential replication growth threshold is reached (Ct = cycle threshold) for the internal reference gene (here *rp49*) subtracted from the cycle threshold of the gene of interest (*vg*) (Livak and Schmittgen 2001). Hence, the delta-Ct value is normalized to account for differences in gene expression detection caused by potential differences in gross RNA quantity that persist even after RNA and cDNA amounts were standardized (see above *Knockdown confirmation*). Note that because samples with smaller quantities of initial gene product take more qPCR cycles to reach the cycle threshold, large Ct and delta-Ct values signify *lower* gene expression and low values signify *high* gene expression. Although many studies report gene expression as fold change differences by centering a control treatment to 1, we report gene expression as delta-Ct values to avoid suggesting equivalence of all control treatments, since each *gfp* RNAi control treatment employs a unique combination of parameter levels specific to each *vg*

RNAi treatment. For knockdown confirmations we did not correct for multiple comparisons because each experiment involved only 2–4 contrasts (Bustin et al. 2009); we also used a planned contrast approach to limit the number of total comparisons across experiments (as opposed to a post hoc "fishing expedition" where uninformed contrasts can increase the chance of Type I errors) (Barnett et al. 2022; Chatham 1999).

Vg is already known to be highly expressed in abdomens compared to heads and thoraces (Corona et al. 2007). Because we were targeting heads, we wanted to know if individual differences in levels of *vg* in the abdomen are reflected in the levels of *vg* in other body parts. To do this, we investigated how individual wasp *vg* expression correlates across the three body parts examined (brain, head capsule, and abdomen) in control wasps (i.e., *gfp* RNAi-treated). To calculate average individual cross-body-segment correlations of *vg* expression, we calculated correlation coefficients and probability values with the "corr.test" function in the R package "psych" (Revelle 2021), with a Benjamini–Hochberg correction (Benjamini and Hochberg 1995). We calculated correlations with a dataset that combined dCt values of *vg* expression of *gfp*-treated wasps from all experiments. We trimmed this dataset to exclude wasps with missing dCt values for any of the three body segments (missing values are due to technical issues that can arise throughout the sample processing pipeline, such as dissection mishaps, low quantity of RNA after extraction, or failure to amplify during qPCR).

Results

Below we present the results for combinations of optimization variables that yielded a successful knockdown of *vg* expression. Table 1 summarizes which parameters resulted in significant knockdown. See Supplemental Table 3 for the results and statistics for all experiments.

Vitellogenin knockdown in workers

Of the several combinations of optimization variables we tested with workers, we recorded significant knockdown of *vg* expression in three. Brain gene expression of *vg* was significantly lower in *vg* RNAi-treated wasps than *gfp* RNAi-treated wasps when sampled 24 h after delivery of 10 µM siRNA into the median ocellus, whether we used standard siRNA (Fig. 1a; linear model: $F = 4.29$; $df = 1$, 12; $p = 0.01$; $n = 8$ *gfp*, 6 *vg*) or in vivo-modified siRNA (Fig. 1b; $F = 8.96$; $df = 1$, 14; $p = 0.01$; $n = 9$ *gfp*, 7 *vg*). We also observed a non-local knockdown of *vg* expression in the abdomens of workers when we sampled wasps 24 h after injecting 10 µM *vg* siRNA into the aorta (Fig. 1c; linear model: $F = 4.89$; $df = 1$, 28; $p = 0.035$; $n = 14$ *gfp*, 16 *vg*), though there was not a knockdown in the brain (Fig. 1c; linear model: $F = 0.03$; $df = 1$, 12; $p = 0.87$; $n = 7$ *gfp*, 7 *vg*), nor the head capsule (Fig. 1c; linear model: $F = 1.70$; $df = 1$, 22; $p = 0.21$; $n = 12$ *gfp*, 12 *vg*).

Vitellogenin knockdown in foundresses

We recorded significant knockdown of *vg* expression, in only two of the combinations of optimization variables we tested on foundresses, and in head capsules alone. After administering 10 µM in vivo-modified *vg* siRNA into foundress aortas, we observed a significant reduction of *vg* head expression 24 h post-injection (Fig. 2a; linear model: $F = 5.72$; $df = 1$, 19; $p = 0.03$; $n = 11$ *gfp*, 10 *vg*) and 48 h post-injection (Fig. 2b; linear model: $F = 20.15$; $df = 1$, 20; $p = 0.0002$; $n = 12$ *gfp*, 10 *vg*).

Cross-body-segment correlations of vitellogenin expression

To better characterize general patterns of *vg* expression in *Polistes fuscatus*, we investigated how *vg* expression correlates across body segments, without a knockdown (i.e., in control treatment wasps). This accomplished two separate goals: 1) It allowed us to document general patterns of *vg* expression across tissues in two different castes, and 2) it allowed us to combine all our data from *gfp*-treated control wasps in all experiments to see if in a larger meta-analysis of our own data there were general trends in body-wide patterns of *vg* expression. Related to RNAi, this information helps provide context about body-segment specificity of *vg* expression; this is relevant because RNAi effects, while often systemic, can sometimes be tissue-specific (Ghanim et al. 2007; Xia et al. 2002).

In workers, expression levels of *vg* were positively correlated across abdomens and heads in control *gfp* siRNA-treated workers (Fig. 3; Pearson correlation: $r = 0.44$; $p = 0.02$). However, there were no correlations of either body segment with brain expression levels (Fig. 3; Pearson correlations; abdomens: $r = 0.09$. $p = 0.62$; heads: $r = -0.08$, $p = 0.66$).

In foundresses, expression levels of *vg* were also positively correlated across abdomens and heads in control *gfp* siRNA-treated foundresses (Fig. 3; Pearson correlation: $r = 0.89$; $p < 0.001$). Expression of *vg* in the brain did not correlate with either other body segment (Fig. 3; Pearson correlations; abdomens: $r = -0.36$, $p = 0.07$; heads: $r = -0.33$; $p = 0.07$).

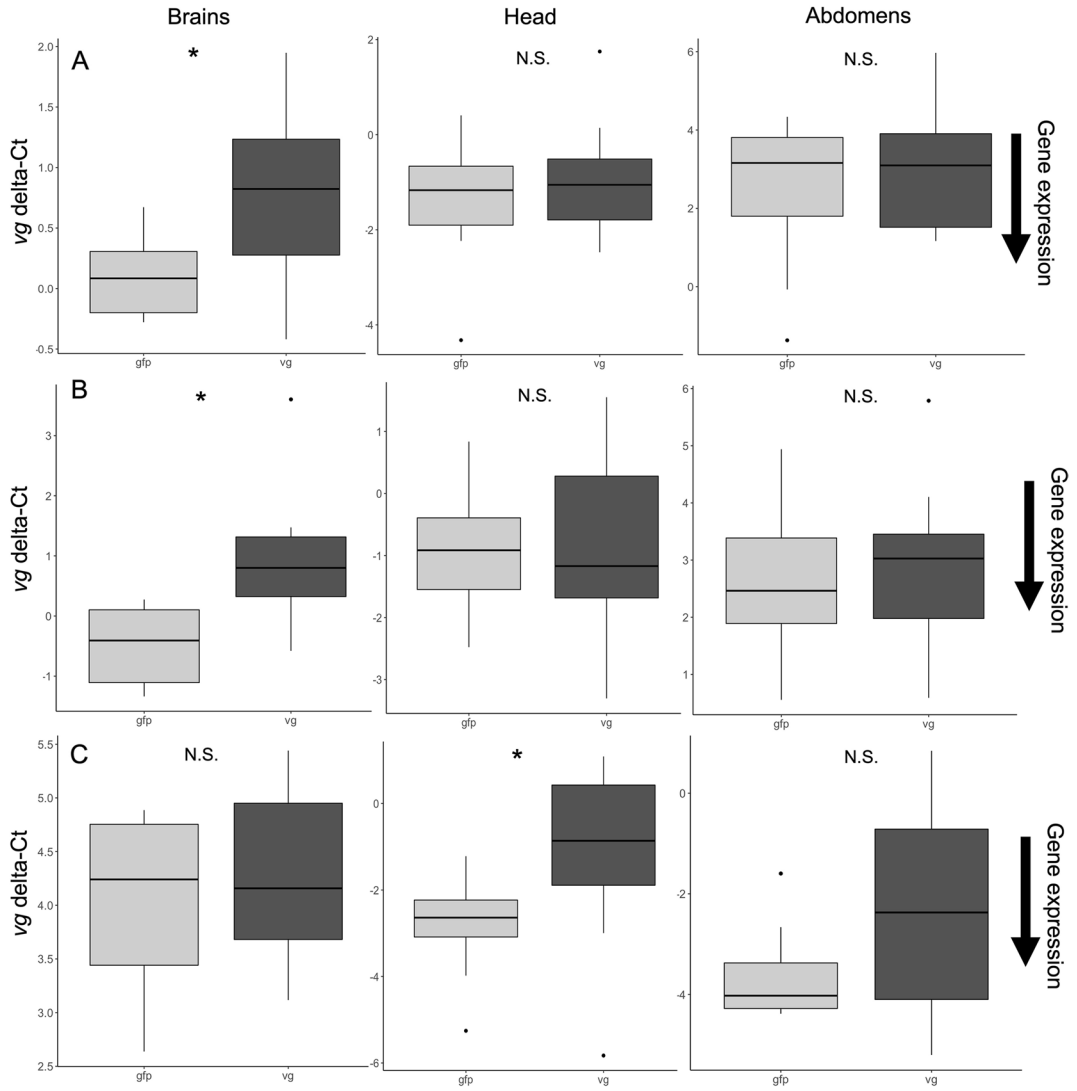

Fig. 1 *vitellogenin* knockdown in workers. **A** Workers treated with standard 10 µM *vg* siRNA injected at the median ocellus and sampled 24 h post-injection: *vg* was significantly knocked down in brains ($F=4.29$; $df=1, 12$; $p=0.01$; $n=8$ *gfp*-treated wasps, 6 *vg*-treated wasps), but not in heads or abdomens (see Supplemental Table 3 for non-significant statistics). **B** Workers treated with standard 10 µM *vg* siRNA injected into the aorta and sampled 24 h post-injection: *vg* was significantly knocked down in abdomens ($F=4.89$; $df=1, 28$; $p=0.035$; $n=14$ *gfp*-treated wasps, 16 *vg*-treated wasps), but not in brains or heads (see Supplemental Table 3). **C** Workers treated with in vivo-modified 10 µM *vg* siRNA injected at the median ocellus and sampled 24 h post-injection: *vg* was significantly knocked down in brains ($F=8.96$; $df=1, 14$; $p=0.01$; $n=9$ *gfp*-treated wasps, 7 *vg*-treated wasps), but not in heads or abdomens. (see Supplemental Table 3)

Discussion

The publication of negative results, especially in the context of methods development, is increasingly recognized as an important contribution to science (Schooler 2011). Here, we report a full complement of both positive and negative results obtained during a series of experiments to optimize RNA interference in a social insect with a moderately sized research community, with the goal of aiding other researchers in utilizing this technique in a broader range of species.

In this study, we successfully implemented RNAi to knock down expression of *vitellogenin* in paper wasps. In workers specifically, we recorded statistically significant knockdowns in *vg* brain gene expression 24 h after injecting 10 µM *vg*-targeted siRNA solution into worker ocelli. With these conditions fixed, we compared the effectiveness of the standard DsiRNA sold by Integrated DNA Technologies (IDT) versus their in vivo-modified DsiRNA and found no significant difference in efficacy. This is a valuable finding, as the in vivo-modified DsiRNA costs about three times the price of standard DsiRNA. Yet, for both standard and

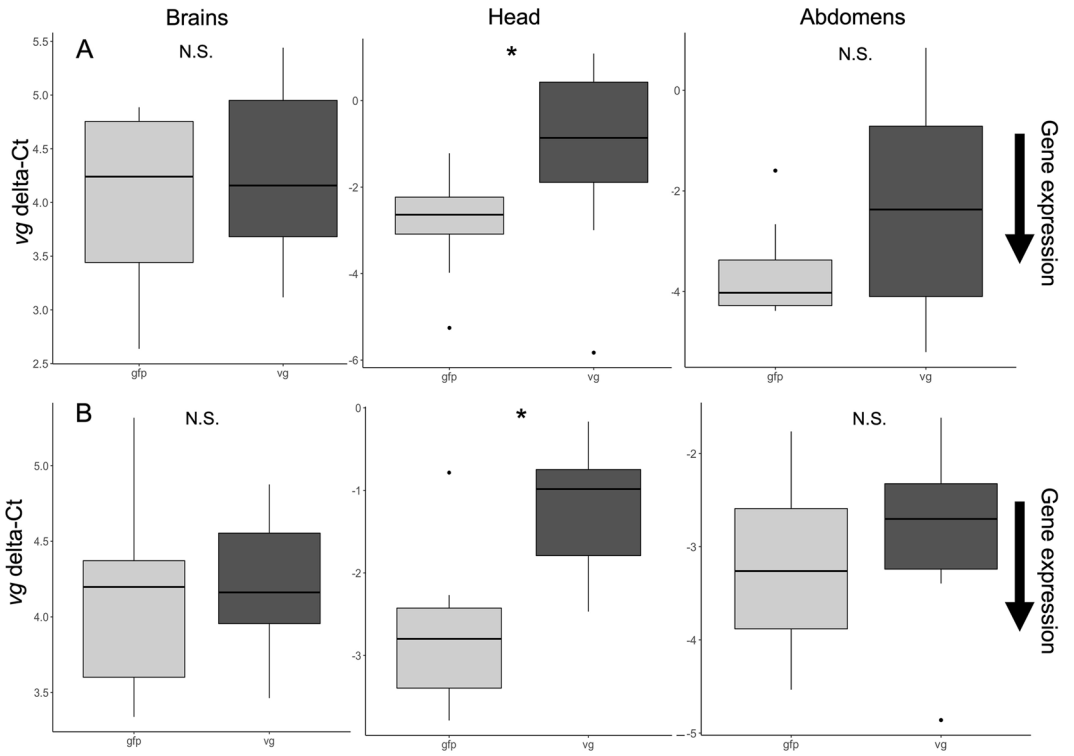

Fig. 2 *vitellogenin* knockdown in foundresses. **A** Foundresses treated with in vivo-modified 10 µM *vg* siRNA injected into the aorta and sampled 24 h post-injection: *vg* was significantly knocked down in heads ($F=5.72$; $df=1, 19$; $p=0.03$; $n=11$ *gfp*-treated wasps, 10 *vg*-treated wasps), but not in brains or abdomens (see Supplemental Table 3 for non-significant treatment statistics). **B** Foundresses treated with in vivo-modified 10 µM *vg* siRNA injected into the aorta and sampled 48 h post-injection: *vg* was significantly knocked down in heads ($F=20.15$; $df=1, 20$; $p=0.0002$; $n=12$ *gfp*-treated wasps, 10 *vg*-treated wasps), but not in brains or heads (see Supplemental Table 3)

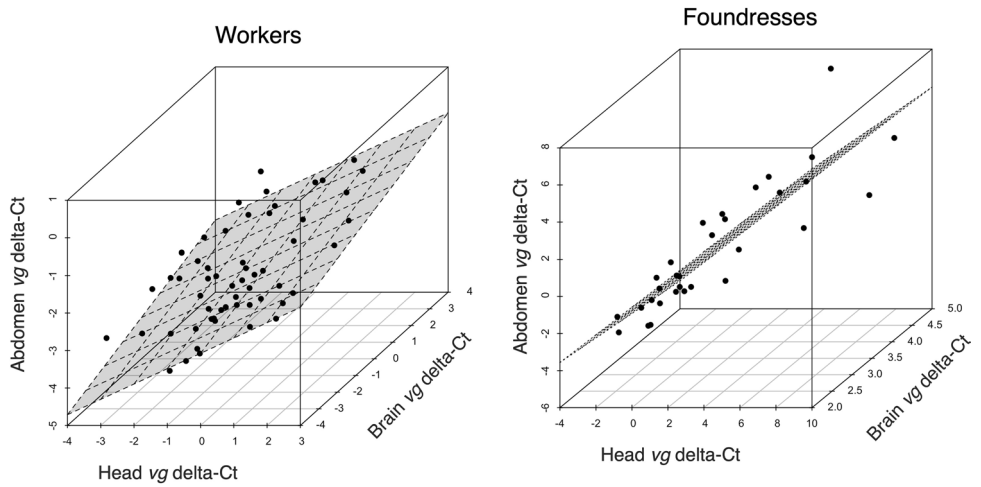

Fig. 3 Cross-body-segment correlation of *vitellogenin* expression. In workers, expression levels of *vitellogenin* were positively correlated across abdomens and heads in control *gfp* siRNA-treated workers (Pearson correlation: $r=0.44$, $p=0.02$). However, there were no correlations of either body segment with brain expression levels (Pearson correlations; abdomens: $r=0.09$, $p=0.62$; heads: $r=-0.08$, $p=0.66$). In foundresses, expression levels of *vitellogenin* were also positively correlated across abdomens and heads in control *gfp* siRNA-treated foundresses (Pearson correlation: $r=0.89$, $p<0.001$). Expression of *vg* in the brain did not correlate with either other body segment (Pearson correlations; abdomens: $r=-0.36$, $p=0.07$; heads: $r=-0.33$, $p=0.07$)

in vivo-modified DsiRNA, a concentration of 10 µM produced a significant knockdown. Moreover, we observed a significant knockdown of *vg* expression in the abdomens (and not the brains nor heads) of workers when we administered 10 µM standard *vg*-targeted siRNA into the aorta. Because our goal was to knock down expression of *vg* in the fat that encases the brain, this suggests a more non-localized effect. This result could mean that injecting siRNA into the aorta, though more efficient and less invasive, may not be as precise as intra-ocellus delivery.

We used RNAi to knock down expression of *vitellogenin* in paper wasps in two distinct behavioral castes. Even though these wasps show relatively subtle caste differences with no morphological differences, we nonetheless found caste-based differences in RNAi efficacy. Although we did not record any knockdown in foundress brains or abdomens, we observed significantly reduced *vg* expression in foundress head capsules at both 24 h and 48 h after injecting in vivo-modified *vg*-targeted siRNA into the aorta. This contrasts with our results from workers, which exhibited successful *vg* knockdown (in the brain, not the head capsule) from standard siRNA and injection into the ocellus. The reason for this discrepancy in location of knockdown (brain vs. head) between behavioral castes is not clear. It may be due, in part, to inherent differences in the quantity and location of fat in the heads of foundresses and workers (Toth et al. 2009), or differences in baseline *vg* expression between the castes. In foundresses, we had higher success with in vivo-modified siRNA even though this was not true for workers. After failing to knock down *vg* in foundresses with siRNA in the first year of optimization experimentation, we switched to in vivo-modified siRNA at a concentration of 10 µM (after observing a successful knockdown at this concentration in workers) and tried a new and less invasive injection site (the aorta). Because of the perennial problem of limited foundress sample sizes, it was unfeasible to design a full factorial comparison of all treatment combinations. So, the question remains as to whether an injection of standard siRNA into the aorta would have yielded a similar knockdown in foundresses at a third of the price of in vivo-modified siRNA.

In addition, we examined general trends in *vitellogenin (vg)* expression across *Polistes fuscatus* brains, heads, and abdomens of control wasps injected with *gfp* DsiRNA. We found that *vg* expression in the head is positively correlated with *vg* expression in the abdomens of both workers and foundresses (in control *gfp*-treated wasps). However, brain expression of *vg* was not significantly correlated with expression in either heads or abdomens in either caste. The detection of *vg* expression in head fat body, which is adjacent to the brain, potentially adds to growing evidence that the functional roles of *vg* in social insect workers includes coordinating behavior (Amdam and Omholt 2003; Guidugli et al. 2005; Nelson et al. 2007). Because abdominal *vg* expression levels are linked to reproductive potential in insects in general (Hagedorn and Kunkel 1979), and social insect workers specifically (Lin et al. 1999; Lin and Winston 1998; Richards 2019), the correlation of *vg* expression across abdomen and heads suggests a coordination in fat body signaling between a worker's reproductive status and her behavior. For future RNAi investigations of the phenotypic effects of *vg* RNAi on social phenotypes (Walton et al. *in prep)*, this information also provides insight for understanding the potential regulatory role of head fat body *vg* expression.

Bringing together all of the aforementioned results from *Polistes fuscatus* and informed by our collection of the results of a large number of prior studies on social Hymenoptera (Supplmental Table 1), we conclude with the following recommendations.

Do not underestimate caste differences. The differentiation between reproductive and workers is a defining characteristic of eusociality (Michener 1969). The differences between these castes can include behavioral, physiological, and morphological traits, so it is unsurprising that the efficacy of RNAi could differ between castes as well. However, our results illustrate this point well, as even in *Polistes fuscatus*, where castes are flexible and monomorphic, optimal RNAi conditions were not uniform between foundresses and workers.

Balance trade-offs between precision and harm when selecting an RNAi delivery method. An RNAi-mediated gene knockdown can range from broadly systemic to localized effects (Aigner 2006). So, it is important to consider the desired specificity of the knockdown and to select an appropriate delivery method. The precision of the delivery procedure should be balanced against the damage it might cause the organism, however. It is important to consider how an invasive RNAi delivery method could cause off-target effects, such as behavioral or immune responses. Additionally, it is ethical practice to reduce harm to the experimental organisms where possible. In this study we compared the efficacy of two injection sites: the median ocellus and the cervix (aorta). Although we did not empirically test which delivery method is more harmful, the median ocellus injection requires an incision prior to injection whereas the cervical injection does not. However, if high specificity of effect localization is important to the experimental design, an invasive delivery method may be the preferred option. Although we compare two injection sites in this study, other social insect researchers have also used abdominal injections (Li-Byarlay et al. 2013), as well as other delivery methods, such as feeding (Hunt et al. 2011; Weiner et al. 2018).

Consider previous successful studies and/or start with our "best recipes" when designing an RNAi experiment. Supplemental Table 1 summarizes the methodological parameters used in 54 RNAi studies of social Hymenoptera. This can serve as an initial guide when considering

the appropriate dosage, timing, and delivery method for a given caste (though you will need to account for an organism's mass if you are adapting RNAi to a new study system). In this study, we tested several combinations of several methodological factors and recorded successful *vg* knockdowns in some. Although your own RNAi experiment is likely in a different species and targeted to a different gene, the conditions that worked for us may work for you. These "best recipes" methods should, at least, provide a starting point from which changes can be made to optimize your own system.

As biological research continues to progress in the era of functional genomics, more social insect scientists may wish to include RNAi-mediated knockdown of gene expression in their methodological arsenal. The adaptability and universality of RNAi makes it an especially useful tool for researchers whose systems lack the genomic resources and streamlined husbandry practices available to scientists who study model organisms (Walton et al. 2020). Yet, developing RNAi for a novel system can be challenging. There are several factors to consider when designing an RNAi experiment, and if no significant knockdown of gene expression occurs, determining which variable needs adjusting can be difficult. As such, employing an RNAi experiment in a new system can seem daunting. This study suggests RNAi is feasible even in insect species refractory to RNAi, and especially encourage social insect biologists to consider the factors and parameters summarized above as a starting point for developing RNAi for their own system.

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Author contribution AW, ALT, MJS, and AG designed the research; AW and RF collected and dissected wasp samples; AW and AG performed RNAi experiments; AW and EF carried out sample processing and molecular lab work; AW analyzed the data; AW and ALT wrote the manuscript; ALT and MJS acquired funding and supervised the research.

Data availability Data will be made available upon request.

Declarations

Conflict of interest The authors declare no competing interests.

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