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Predictable gene expression related to behavioral variation in parenting

Kyle M. Benowitz,[a] Elizabeth C. McKinney,[b] Christopher B. Cunningham,[c] and Allen J. Moore[b]

[a]Department of Entomology, University of Arizona, Tucson, AZ 85721, USA,

[b]Department of Entomology, University of Georgia, Athens, GA 30602, USA,

[c]Department of Biosciences, Swansea University, Swansea, SA2 8PP, UK

Address correspondence to A.J. Moore, Department of Entomology, University of Georgia, 137 Cedar St., Athens, GA, 30606, USA. Telephone: +17065422151. E-mail: ajmoore@uga.edu.

Running title: "Predicting genes related to variable behavior"

Differential gene expression has been associated with transitions between behavioral states for a wide variety of organisms and behaviors. Heterochrony, genetic toolkits, and predictable pathways underlying behavioral transitions have been hypothesized to explain the relationship between transcription and behavioral changes. Less studied is how variation in transcription is related to variation within a behavior, and if the genes that are associated with this variation are predictable. Here we adopt an evolutionary systems biology perspective to address two hypotheses relating differential expression to changes within and between behavior. We predicted fewer genes will be associated with variation within a behavior than with transitions between states, and the genes underlying variation within a behavior will represent a narrower set of biological functions. We tested for associations with parenting variation within a state with a set of genes known *a priori* to be differentially expressed between parenting states in the burying beetle *Nicrophorus vespilloides*. As predicted, we found that far fewer genes are differentially expressed related to variation within parenting. Moreover, these were not randomly distributed among categories or pathways in the gene set we tested and primarily involved genes associated with neurotransmission. We suggest that this means candidate genes will be easier to identify for associations within a behavior, as descriptions of behavioral state may include more than a single phenotype.

Key words: **behavior genetics, evolutionary systems biology, gene expression, *Nicrophorus vespilloides*, parental care**

INTRODUCTION

Ever since the description of the "phenotypic gambit" (Grafen 1984), evolutionary biologists in general (Travisano and Shaw 2013) and behavioral ecologists specifically (Stamps 1991; Zuk and Balenger 2014) have struggled with the questions of if and when it is necessary to understand the mechanisms underlying behavior. The development of transcriptomic, metabolomic, and proteomic approaches have provided us with new tools to address mechanisms (Moore et al. 2010), and advocates of a genomic approach to behavior have argued that finding the genes underlying behavior can lead us to important insights into the nature of pleiotropy and constraint, selection, and evolutionary convergence (Fitzpatrick et al. 2005; Rittschof and Robinson 2014; Bengston et al. 2018). Furthermore, understanding details of behavioral mechanisms may provide insight into whether behavior plays a unique role in evolution (Bailey et al. 2018). However, what precisely does it mean for a gene to underlie a behavior? Most studies have asked what genes are responsible for producing discrete changes in behavior and have focused on gene expression changes between two behavioral states (Harris and Hofmann 2014; Rittschof and Robinson 2016), particularly on a genome wide scale (Calisi and MacManes 2015). These studies have led to predictions of the types of genes that will be differentially expressed (DE). Two hypotheses have been generally supported to explain change in behavior. First, changes in timing of gene expression (heterochrony) will be associated with behavioral evolution (Linksvayer and Wade 2005). Second, the genes DE will be associated with a "genetic toolkit" (Rittschof and Robinson 2016; Toth and Rehan 2017). The toolkit hypothesis states that specific genes may differ between organisms, but the underlying pathway or category altered will be shared across animals displaying

similar behavior. These hypotheses are applied to the generation of novel behavioral states and are therefore well-suited to answer questions about the evolutionary origins of behavior and the tradeoffs involved with its production.

Behavior also displays abundant variation within a state, which also must have some mechanistic basis. The genes related to this type of behavioral variation should be more important for understanding responses to contemporary selection; if selection acts to refine behaviors, it should similarly refine levels of transcription associated with specific behavioral values. Less attention has been paid to mechanisms responsible for this type of variation. For instance, the extent that differential gene expression within and between behavioral states overlaps is unknown (Bengston et al. 2018). Many genes undergo massive expression changes when behavior shifts, indicating that behavioral transitions also involve concurrent suites of major physiological changes. We do not know to what degree the same genes influence variation within state, possibly with less extreme differences in expression. Some have argued that the mechanisms controlling the maintenance of specific behavioral phenotypes are likely to be different than those controlling transitions between behaviors (Cardoso et al. 2015). Alternatively, others have predicted more overlap between these mechanisms (Duckworth 2015), perhaps reflecting the expectation that behavioral plasticity and behavioral evolution are mechanistically similar (Pfennig et al. 2010). However, existing data explicitly comparing transitions with variation for a single behavior are sparse. One example comes from Bell et al. (2016), who found notable but limited overlap between mechanisms of induction of aggression and variation in aggression in sticklebacks. They proceed to

suggest that the magnitude of such overlap may be less important than the identity of the common genes.

Theory from developmental biology may bear on our expectations for the distinction between transitions between behavior states and variation within a behavior and provide an addition to the toolkit and heterochrony hypotheses. Wagner's (2014) makes a distinction between the origin of novel types and modification of existing types. Wagner predicts that the former evolves via sweeping changes in transcriptional programs leading to novel regulatory networks, whereas the latter evolves within existing networks through the modification of expression of "realizer" genes, which are directly related to phenotypic variation. Adapting Wagner's hypothesis to behavior, we make two predictions. First, we propose that only a narrow subset of genes DE in changes between states will be associated with variation within that state. Changing behavioral states involves complex and multifaceted environmental changes, and therefore the need for changes in multiple coordinated sensory, physiological, and neurological processes, while variation within a state occurs in a single social and more uniform biotic and abiotic environment where only a single phenotype may be different among individuals. Therefore, fewer transcriptional changes should be needed. Second, we suggest that this subset of genes influencing variation within a state will fall into functional categories typically displaying causal links to behavior; i.e., neuropeptides (Chandrasekaran et al. 2011). Presumably, if the behavior in question is heritable, *cis*-regulatory variants in such causal genes will allow them to vary transcriptionally without generating broad rewiring of transcriptional networks as occurs during transitions (Chandrasekaran et al. 2011).

Parental care in the burying beetle *Nicrophorus vespilloides* presents an opportunity with which to test these predictions. Adult beetles rear their offspring on prepared vertebrate carcasses (Eggert and Müller 1997; Scott 1998), and the transition from a non-parenting to a parenting state is phenotypically complex and multifaceted. Parents undergo changes in immune function (Cotter and Kilner 2010; Palmer et al. 2016; Ziadie et al. 2019), physiology (Benowitz et al. 2017a), chemical status (Steiger et al. 2008; 2009) and multiple behaviors (reviewed in Scott 1998; Royle and Hopwood 2017). Reflecting this, an RNA-seq study comparing non-parenting and parenting individuals identified broad transcriptional differences (> 700 genes) between the two states (Parker et al. 2015). Transcriptional differences have also been defined for responses to social context and for plasticity in male parenting (Cunningham et al. 2019). Thus, we have a clear phenotype with well characterized DE associations.

Within the burying beetle parenting state, the primary individual social behavior is provisioning, which consists of direct regurgitation of partially digested food from parents into the mouths of begging offspring (Pukowski 1933; Milne and Milne 1976). The amount of parental provisioning exhibited in *N. vespilloides* is highly variable (Benowitz et al. 2016a), heritable (Walling et al. 2008) and important for offspring development and fitness (Eggert et al. 1998; Lock et al. 2004). Variation in the extent of provisioning has been investigated in an RNA-seq study comparing genome wide transcriptional variation between ten high-caring and ten low-caring parents (Benowitz et al. 2017b). This study broadly supported the expectation of overlap between genes involved in transitions and within-state variation but could not specify the number or the identity of the genes affecting the variability of parental provisioning (Benowitz et al.

2017b). This likely reflects a basic limitation of using RNA-seq to investigate subtle behavioral differences; if small phenotypic changes are accompanied by small expression changes, important signals may be swamped by noise and costly multiple corrections.

Here, to further probe our hypotheses in *N. vespilloides*, we take a complementary approach to Benowitz et al. (2017b) but with an *a priori* candidate gene method rather than RNA-seq, creating a more focused and therefore more powerful test. We examine specific candidates shown to be significantly DE when switching from a non-parenting to a parenting state in earlier studies (Table 1, S1), and ask which of these genes also display differential expression between very high and very low caring mothers. Furthermore, we specifically chose to examine genes spanning a range of well-documented functions, including several neurotransmitters, in order to ask whether the genes associated with behavioral variation are more likely to display certain functionalities. There are certainly more genes than these involved; however, most DE genes identified in transcriptomic studies are unannotated and therefore the function is unknown (Parker et al. 2015).

METHODS

Nicrophorus vespilloides were collected and maintained as an outbred colony as described by Cunningham et al. (2014). For this study, our sample consisted of 57 female adult beetles on 19-21g mouse carcasses in a uniparental context. We observed parental provisioning, defined as mouthpart-to-mouthpart contact by parents and offspring, on the first day after larvae hatched. Following Benowitz et al. (2016) we made 80 scan samples for provisioning behavior over the course of an 8-hour period. Within the entire dataset,

parental care was roughly uniformly distributed (Benowitz et al. 2016a) with a mean and standard deviation of 47.53 ± 21.52 observations of feeding (out of 80 scans) (Benowitz et al. 2016b). Immediately after observations we removed the heads, which were flash-frozen in liquid nitrogen and stored at -80°C.

Following the method of Benowitz et al. (2017b) we selected the 12 highest and 12 lowest caring female parents for RNA extraction. Mean and standard deviation of parenting was 11.67 ± 8.33 observations of feeding for the low group and 72.42 ± 2.18 observations of feeding for the high group, indicating substantial quantitative behavioral differences (Benowitz et al. 2016b). We analyzed head tissue, which contains both brain and fat body, following previous studies that identified differential expression of genes associated with parenting in females (Parker et al. 2015; Cunningham et al. 2014, 2016, 2019; Roy-Zokan et al. 2015; Benowitz et al. 2017a,b). We performed phenol-chloroform extractions using Qiagen RNeasy Lipid Kits (Qiagen, Venlo, Netherlands) and synthesized cDNA using qScript (Quantabio, Beverly, MA) reverse transcriptase (Parker et al. 2015; Roy-Zokan et al. 2015). We designed quantitative real-time PCR (qRT-PCR) primers for 23 genes (Table S1) identified in other independent experiments to be DE in transition from non-parenting to parenting *N. vespilloides* females. Seventeen of these genes were identified in an RNA-seq experiment comparing parenting and non-parenting *N. vespilloides*, while the other six were identified in qRT-PCR experiments making the identical comparison (Cunningham et al. 2016; 2017; unpub. data). These genes have functional annotation and can be categorized into neurotransmission, energy acquisition and usage, immunity, and hormones (Table 1). This is not an exhaustive identification of any genes differing between parental states in *N. vespilloides*, but rather

those that are annotated, DE when comparing parenting and non-parenting states, and fall into different known functional categories. Therefore, this set is coarsely representative of the types of genes DE between states, and most importantly allows us to test our hypotheses. We performed qRT-PCR using each primer pair on each of the 24 samples with a Roche LightCycler 480 (Roche, Basel, Switzerland) with *alpha-tubulin* (Table S1) used as an endogenous reference gene. We calculated gene expression for each sample as $2^{-(Cp_{exp} - Cp_{ref})}$, where Cp_{exp} is the average cycle number of three technical replicates of each experimental gene and Cp_{ref} is the average cycle number of three technical replicates for *alpha-tubulin*. Following this, DE was calculated between high and low care using separate one-way ANOVAs for each experimental gene. Because each gene represents a distinct, *a priori* specified hypothesis, conservative multiple corrections are inappropriate (Rice 1989). We used a Fisher's Exact Test to test whether genes *a priori* classified as having roles in neurotransmission were more likely to be DE than genes with other functional roles. The data are publicly available in Dryad (Benowitz et al. 2018).

RESULTS

Four of seven neurotransmission genes (*serotonin receptor 2*, *octopamine/tyramine receptor 2*, *tachykinin*, and *glutamate receptor*) show statistically different expression between high vs. low caring *N. vespilloides* mothers (Table 1). Furthermore, the direction of differential expression followed *a priori* expectations based on our previous studies; genes that were upregulated during the transition into parental care were also upregulated in high caring mothers, and vice versa. However, of the other genes we

examined, which included hormones and genes related to energy, immunity, and general behavior, only one (*fatty acyl-CoA synthetase*) of 16 was DE between high and low caring mothers (Table 1), and in the opposite direction as the change with a transition to parenting. Neurotransmission genes were statistically more likely to be associated with provisioning variation than other genes ($p = 0.017$).

DISCUSSION

Differences in gene expression are suggested to be fundamental contributors to the evolution of social behavior (Calisi and McManes 2015; Rittschof and Robinson 2016; Toth and Rehan 2017; Kronauer and Libbrecht 2018). Despite this, we currently lack a nuanced understanding of how transcription is related to selectable variation in social behavior. Here, we examined how gene expression relates to quantitative variation in parental provisioning behavior involving direct regurgitation of food in the burying beetle *Nicrophorus vespilloides*. Rather than examining transcriptional differences associated with transitions between behavioral states (e.g., Harris and Hoffmann 2014; Rittschof and Robinson 2016), we determined which of the genes previously associated with this transition in *N. vespilloides* parenting were also related to variation in parenting within a state. Transitions often involve large changes in physiology, feeding, aggression and reproduction (Kronauer and Libbrecht 2018), and so many different pathways and genes should be involved. We specifically tested our predictions that far fewer genes will be involved in variation within a behavioral state, and for behavior they will be involved in neurotransmission rather than other physiological processes. Focusing on those genes that we know are associated with changes in parenting state provides a powerful test of

the hypothesis. That is, we bias our examination toward genes known to change, rather than looking for associations of genes and behavior, thereby reducing extraneous correlations and irrelevant tests. What we cannot determine from this approach is the contribution to variation of genes that do not see transcriptional changes across behavioral transitions. Behavioral syndromes theory (Sih et al. 2004) predicts the existence of large suites of genetically correlated behaviors, suggesting that common regulators may control behavior in a non-specific fashion, and therefore might not be involved in behavioral transitions. If such genes exist and are important, they may be difficult to identify by either *a priori* or genome-wide approaches.

Four of seven neurotransmitter genes associated with changes in parenting state were also associated with variation in parenting, while only one of 16 not associated with neurotransmission also influenced parental variation. This result suggests that many immune, energetic, and hormonal processes altered upon induction of parental behavior are not further changed in association with behavioral variation. We suggest this pattern is generalizable, and that this methodology eliminates many of the confounding transcriptional effects produced when comparing between different behaviors. Here, we predicted fewer transcriptional differences within parenting because the number of changes required to transition from a non-parenting to parenting state include those associated with changing biotic and abiotic conditions as well as changes in parenting. This highlights a further problem studying behavior: terminology. The labels widely used to group behaviors into categories (e.g. parenting) actually describe composites that are necessarily more complex than the individual phenotypes that are contained within them (Rittschof and Robinson 2016). However, it is these individual phenotypes that are

actually quantified, and often considered to be proxies for the entire category, which may lead to an oversimplification (Wenzel 1992).

The neurotransmission genes related to provisioning variation included serotonin, octopamine, glutamate, and tachykinin signaling genes. These pathways are all known to influence behavior, and expression of an octopamine/tyramine receptor has previously been shown to affect behavior quantitatively in *C. elegans* (Bendesky et al. 2011). Furthermore, modification of neural signaling pathways leading to variable brain function is predicted to be a general mechanism for the evolution of social behavior (Baran et al. 2017). Interestingly, the single other gene found to be differentially expressed was the lipid metabolism gene *fatty acyl CoA synthetase*, which was upregulated in high caring parents. This raises the possibility that body condition could be linked to variation in parenting, presenting precisely the sort of confound our study was designed to avoid. Because plastic changes of this nature likely require additional gene expression changes in order to incorporate environmental inputs into behavior, it will be interesting to see in the future whether plastic variation is more transcriptionally complex than genetic variation. We hypothesize that this will be the case, and therefore that more heritable behaviors will be associated with fewer DE genes.

There have been several attempts to provide a framework for predicting specific genes or pathways underlying behavior given the accessibility of modern molecular approaches, from heterochrony (Linksvayer and Wade 2005) to genetic toolkits (Toth and Rehan 2017), to hypotheses derived from ethological principles (Cunningham et al. 2016, 2017; Kronauer and Libbrecht 2018). We suggest that molecules predicted from an evolutionary systems biology approach (Chandrasekaran et al. 2011; Wagner 2014)

coupled with predictions from ethology, allowing targeted transcriptional comparison between individuals with quantitatively different behavioral phenotypes, presents a promising methodology for finding genes that may be related to behavioral variation. The identities of these molecules, and their regulation in other behavioral contexts, will help inform important questions on the constraints and possibilities of behavioral evolution.

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AUTHORS' CONTRIBUTIONS

K.M.B., E.C.M., and A.J.M. conceived the study and analyzed the data, E.C.M. and C.B.C. collected the data, and K.M.B. and A.J.M wrote the paper. All authors approved the version to be published and agree to be accountable for all aspects of the research.

DATA ACCESSIBILITY

Analyses reported in this article can be reproduced using the data provided by Benowitz et al. (2018).

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297 **COMPETING INTERESTS.**

298 We declare no competing interests.

299

300 **ETHICAL STATEMENT**

301 All institutional, national, and international guidelines for the care and use of insects for

302 research were followed.

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Table 1. Classification of genes and their patterns of differential expression between high and low cari

mothers.

Gene	Functional Role	Evidence for DE in transition between parenting states	Expression in high (mean ± sd)	Expression in low (mean ± sd)	Comp of exp in high low ca
Thaumatin	Immune	Parker et al. 2015	1.177 ± 1.051	1.143 ± 1.787	($F_{1,22}$ = p = 0.9
Peptidoglycan recognition protein SC2-like	Immune	Parker et al. 2015	3.447 ± 2.743	2.926 ± 3.519	($F_{1,22}$ = p = 0.6
Peptidoglycan recognition protein A	Immune	Parker et al. 2015	2.578 ± 1.136	2.742 ± 0.995	($F_{1,22}$ = p = 0.7
Defensin	Immune	Parker et al. 2015	9.564 ± 4.923	9.219 ± 5.237	($F_{1,22}$ = p = 0.8
Prophenoloxidase	Immune	Parker et al. 2015	4.293 ± 2.098	3.942 ± 1.585	($F_{1,22}$ = p = 0.6
Serine protease 93	Immune	Parker et al. 2015	0.885 ± 0.663	0.660 ± 0.638	($F_{1,22}$ = p = 0.4
Beta glucosidase	Digestion/Immune	Parker et al. 2015	4.294 ± 2.822	3.881 ± 5.626	($F_{1,22}$ = p = 0.8
Fatty acyl-CoA synthetase	Lipid synthesis	Parker et al. 2015	0.00927 ± 0.00401	0.00495 ± 0.00273	($F_{1,22}$ = **p = 0.0**
Vitellogenin 1	Lipid transport/Hormone	Parker et al. 2015; Roy-Zokan et al. 2015	135.800 ± 85.387	113.003 ± 38.542	($F_{1,22}$ = p = 0.8
Hexamerin 3	Energy storage/Hormone Transport	Parker et al. 2015	0.0201 ± 0.00863	0.0253 ± 0.014	($F_{1,22}$ = p = 0.2

Hexamerin 4	Energy storage/Hormone Transport	Parker et al. 2015	0.0677 ± 0.104	0.0238 ± 0.0240	($F_{1,22}$ = p = 0.1
Apolipophorin-III	Lipid transport	Parker et al. 2015; Benowitz et al. 2017b	135.430 ± 55.438	117.294 ± 25.408	($F_{1,22}$ = p = 0.3
Yellow 3	Hormone/Pigment/Immune	Parker et al. 2015	0.802 ± 0.277	0.628 ± 0.302	($F_{1,22}$ = p = 0.1
Insulin like peptide 3	Hormone	Parker et al. 2015	0.0598 ± 0.0136	0.0589 ± 0.0175	($F_{1,22}$ = p = 0.8
Neuropeptide F	Neurotransmission	Cunningham et al. 2016	0.0735 ± 0.0212	0.0916 ± 0.0601	($F_{1,22}$ = p = 0.3
Serotonin receptor 7	Neurotransmission	Unpub. data	0.400 ± 0.108	0.454 ± 0.0941	($F_{1,22}$ = p = 0.2
Serotonin receptor 2	Neurotransmission	Unpub. data	0.0233 ± 0.00336	0.0286 ± 0.00742	($F_{1,22}$ = **p = 0.0**
Glutamate receptor	Neurotransmission	Parker et al. 2015	0.179 ± 0.0261	0.0257 ± 0.0679	($F_{1,22}$ = 13.97 **0.001**)
Natalisin	Neurotransmission	Cunningham et al. 2017	0.00964 ± 0.00509	0.0208 ± 0.0259	($F_{1,22}$ = p = 0.1
Octopamine/Tyramine receptor 2	Neurotransmission	Unpub. data	0.0250 ± 0.00637	0.0326 ± 0.00931	($F_{1,22}$ = **p = 0.0**
Tachykinin	Neurotransmission	Unpub. data	0.431 ± 0.183	0.292 ± 0.136	($F_{1,22}$ = **p = 0.0**
Takeout	Circadian behavior/Feeding	Parker et al. 2015	0.0499 ± 0.0321	0.0435 ± 0.0338	($F_{1,22}$ = p = 0.6
Pickpocket	Sodium channels/Olfaction	Parker et al. 2015	0.0834 ± 0.0655	0.0559 ± 0.0176	($F_{1,22}$ = p = 0.1

Table S1. Primers and accession numbers of the genes used in this study.