

1           **Duplication and Sub/neofunctionalization of *malvolio*, an Insect Homolog of**  
2   ***Nramp*, in the Subsocial Beetle *Nicrophorus vespilloides***

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12 Running title: Duplication and neofunctionalization of *malvolio*

13 Keywords: Behavior, burying beetle, gene expression, parenting, phylogenetics

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22 **ABSTRACT** Gene duplication has long been thought to play a facilitating role in evolution. With  
23 growing numbers of sequenced genomes, increasing numbers of duplicate genes are uncovered  
24 with unknown functions. Here we examine *malvolio*, a gene involved in heavy metal transport  
25 but that also affects behavior in honey bees and *Drosophila*. There is only one copy of *malvolio*  
26 in honey bees and *Drosophila* despite its different roles. A phylogenetic analysis in insects  
27 suggests that *malvolio* has duplicated multiple times in different orders. To test if the two  
28 copies might have different functions, we examined expression levels of *malvolio* in brain, fat  
29 bodies, Malpighian tubules, midgut, ovaries, testes and thoracic musculature in the beetle  
30 *Nicrophorus vespilloides*. We found that *mv1* was expressed in all tissues, with highest  
31 expression in fat bodies and relatively lower expression in testes, Malpighian tubules, and  
32 brain, and ovaries. Expression of *mv2* differed, with significant expression only seen in brain  
33 and midgut. Because *malvolio* has been implicated in behavior, and these beetles have highly  
34 developed parenting behavior, we next examined expression during different behavioral states  
35 including virgin, mating, preparing resources for offspring, feeding offspring and post care. We  
36 found differing expression patterns for the two copies, with *mv1* increasing in expression  
37 during resource preparation and feeding offspring, and *mv2* decreasing in these same states.  
38 Given these patterns of expression, we suggest that *malvolio* in *N. vespilloides* has experienced  
39 sub/neofunctionalization following its duplication, and is evolving differing and tissue-specific  
40 roles in behavior and physiology.

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43 The process of gene duplication is one of the primary mechanisms hypothesized to play a role in  
44 the evolution of novel phenotypes (Ohno 1970; Innan and Kondrashov 2010; Ditmar and  
45 Liberles 2010; Wagner 2011). When duplicate genes are maintained, one copy often becomes  
46 free to mutate and acquire new functions, as it is no longer constrained by the selective pressure  
47 to perform its previous role (Ohno 1970; Maere and Van de Peer 2010). This process can take  
48 two non-exclusive paths: subfunctionalization or neofunctionalization (Nadeau and Sankoff  
49 1997; Nowak *et al.* 1997; Wagner 1998; Force *et al.* 1999; Maere and Van de Peer 2010). In the  
50 former process, both genes lose a portion of their function, so that the two duplicated genes  
51 together recapitulate the function of the ancestral gene (Force *et al.* 1999; Maere and Van de  
52 Peer 2010). In the latter process, one duplicate evolves a novel function absent from the ancestral  
53 gene (Ohno 1970; Maere and Van de Peer 2010). Neofunctionalization may arise following  
54 subfunctionalization (Maere and Van de Peer 2010). Given the proposed role that gene  
55 duplication has in the production of new phenotypes it follows that more derived organisms with  
56 novel traits provide good systems for investigating the divergence of duplicated genes. Genetic  
57 influences on behavior may require neofunctionalization of gene duplications to overcome  
58 constraints that would otherwise arise through pleiotropy. For example, G-protein coupled  
59 receptors are cell surface receptors and have evolved to become diversified and specialized for  
60 behavior (Katz and Lillvis 2014). Gene duplication and neofunctionalization has been implicated  
61 in the evolution of insect behavior as diverse as *vitellogenin* influences on ant queen and worker  
62 social behavior and tasks (Corona *et al.* 2013), olfactory receptors related to shifts to herbivory  
63 (Goldman-Huertas *et al.* 2015), and opsin genes related to color vision and foraging preferences  
64 (Feuda *et al.* 2016).

65 With the advent of improved bioinformatics and sequencing, we are acquiring information

66 on genomes of non-model organisms at an accelerated pace, many of which are studied primarily  
67 for their novel traits and not for their genetic accessibility. Such genomes provide may reveal  
68 duplicated genes that may have previously been unexpected. We recently sequenced, assembled,  
69 and annotated the genome of such an organism, the burying beetle *Nicrophorus vespilloides*  
70 (Cunningham *et al.* 2015b). Burying beetles (*Nicrophorus spp.*) are unusual among beetles for  
71 their parenting behavior. Burying beetles breed on vertebrate carrion, which they shape into a  
72 ball, prepare with antimicrobial secretions and bury. The larvae then crawl onto the carcass and  
73 one or both parents care for the offspring. Parenting in burying beetles is more than provisioning  
74 food for developing offspring, as in many insects, but instead involves direct and extensive  
75 prolonged social interactions. Burying beetles not only prepare and maintain a carcass for food,  
76 they feed their offspring by regurgitating predigested carrion directly into their mouths.  
77 Parenting in this taxon is thus both complex and extensive, and strongly selected as it influences  
78 the fitness of offspring (Eggert *et al.* 1998; Lock *et al.* 2004). This behavior is highly derived,  
79 and therefore we predicted that sub/neofunctionalization could be important in the evolution of  
80 complex parenting. Therefore, we examined this genome for evidence of duplications.

81 We hypothesized that genes likely to have undergone sub/neofunctionalization in burying  
82 beetles are those with a predicted relation to their parenting behavior. Therefore, we focused on  
83 genes implicated in the social behavior of other insects. A candidate duplicate gene we found in  
84 the genome of *N. vespilloides* is *malvolio*, a heavy metal transporter and homolog of NRAMP  
85 family (natural resistance-associated macrophage proteins) in vertebrates (Folwell *et al.* 2006).  
86 *Malvolio* has been ascribed a role in behavior as well as heavy-metal transport, and is linked to  
87 the transition between nurse and forager roles in honey bees (Ben-Shahar *et al.* 2004) and food  
88 choice in *Drosophila* (Søvik *et al.* 2017). There is also ample evidence that *Nramp1* (*Slc11a1* in

89 humans) and *Nramp2* (*Slc11a2*) have subfunctionalized in mammals and fish (Techau *et al.*  
90 2007; Neves *et al.* 2011). Other insects such as honey bees and *Drosophila* have only one copy  
91 of *malvolio*. It therefore seems likely that this second copy arose in a relatively recent duplication  
92 event, and could be unique to beetles.

93 To test the hypothesis that the duplication of *malvolio* in beetles may facilitate  
94 sub/neofunctionalization and effects on behavior, we first examined *malvolio* sequences across  
95 insect species and built a gene phylogeny to determine the evolutionary history of duplication in  
96 this gene. Next, to ask whether this gene displays behavior consistent with  
97 sub/neofunctionalization, we measured gene expression of *mv11* and *mv12* across eight tissue  
98 types in *N. vespilloides*. *Malvolio* shows evidence of several gene duplications in insects,  
99 suggesting something about *malvolio* that lends itself to maintenance after duplication. In *N.*  
100 *vespilloides*, we show that while *mv11* is expressed ubiquitously and consistently across tissues,  
101 *mv12* only shows expression in the brain and midgut. Finally, we examined expression of the two  
102 *malvolio* copies in head tissue collected from beetles before, during and after they were parenting  
103 and found changes in expression during parenting in opposite directions for the two copies. We  
104 argue that these results provide evidence consistent with the process of sub/neofunctionalization  
105 of *mv1*, although it is difficult to tease the two apart. We suggest that the *malvolio* duplicates in  
106 *N. vespilloides* are in the process of evolutionary divergence, with neofunctionalization a likely  
107 endpoint .

108

## 109 **Materials and Methods**

### 110 *Biological samples*

111 We maintained *N. vespilloides* as an actively outbred colony at the University of Georgia.  
112 We founded the colony with beetles collected from the wild near the University of Exeter,  
113 Cornwall, UK and new wild individuals were introduced to the colony yearly to maintain genetic  
114 variation. We isolated individuals as larvae and housed them individually in 4 x 7 cm  
115 biodegradable circular deli containers (Eco products, Boulder, CO) filled with 2.5 cm of moist  
116 soil (FoxFarm, Samoa, CA). Individuals were kept in an incubator (Percival Scientific, Perry,  
117 IA) set at  $22 \pm 0.1$  °C, under a 15:9 light:dark cycle. Upon reaching adulthood they were fed two  
118 decapitated mealworms (*Tenebrio*) once a week.

119 For the comparison of expression across different tissues, we collected eight tissues;  
120 brain, fat bodies, hindgut, midgut, thoracic musculature, Malpighian tubules, testes, and ovaries;  
121 from 5 virgin female beetles at 26-30 days post adult eclosion (testes came from 5 males of the  
122 same age and rearing conditions). These same tissues types were previously examined for  
123 octopamine expression in Cunningham *et al.* (2015a), except for testes, but on separate tissue  
124 collections. We dissected beetles in ice cold PBS, starting with the brain and then moving on to  
125 the internal organs. We cleaned fat and connective tissue from each organ and placed them in  
126 separate 1.5 mL vials with 300  $\mu$ L of RNA later (Applied Biosystems, Foster City, CA) on ice.  
127 Dissection times for brains were 10 minutes or less, and the total time of dissections was less  
128 than 30 minutes. After dissection, we stored organs overnight in 4°C and then moved them to -  
129 20°C until RNA extraction. See Cunningham *et al.* (2015a) for further details.

130 We collected whole heads from 10 individuals in each of five behavioral states to  
131 examine changes associated with changes in behavior: virgins, individuals mated but not  
132 provided with the resource necessary to breed, mated individuals provided with a mouse carcass  
133 to prepare and that stimulates egg laying, individuals actively caring for and provisioning food to

134 begging offspring, and individuals that had completed parental care and had dispersed away from  
135 the carcass and larvae. See Roy-Zokan *et al.* (2015) and Cunningham *et al.* (2016) for further  
136 details.

### 137 ***Identification and comparison of gene sequences***

138 To verify the putative duplication that we found in the genome, we searched for putative  
139 *N. vespilloides malvolio* homologs using BLASTp (v2.3.0+; default search settings; Camacho *et*  
140 *al.* 2008) with *Drosophila melanogaster* (NP\_524425.2) and *Tribolium castaneum*  
141 (XP\_967521.1) *mvl* sequences. We obtained sequences from National Center for Biotechnology  
142 Information (NCBI) or UniProt. We BLASTed these *mvl* sequences against the proteome  
143 produced from the annotated *N. vespilloides* genome (Cunningham *et al.* 2015b). Further  
144 identification of putative *mvl1* and *mvl2* of *N. vespilloides* was done by BLASTing (BLASTp,  
145 default setting) them into NCBI's non-redundant insect protein database and by BLASTing  
146 (BLASTp, default setting) them into *Drosophila melanogaster* and *Tribolium castaneum*  
147 proteomes alone to establish if all of sequences were reciprocal best BLAST's (RBB) for each  
148 other.

149 All sequences were identified by using the gene and protein databases and the BLAST  
150 feature at NCBI (<http://www.ncbi.nlm.nih.gov/>). To visualize protein conservation across both  
151 *mvl* copies, we aligned protein sequences from *N. vespilloides*, *T. castaneum*, *H. sapiens*, and *M.*  
152 *musculus* using ClustalW and produced boxshade plots with the Mobylye@Pasteur web portal  
153 (<http://mobile.pasteur.fr>). For the phylogenetic analysis, we included all insect *mvl* sequences and  
154 *Mvl* proteins available from NCBI, except for *Drosophila spp.* Due to the large number of  
155 published *Drosophila* genomes, and to avoid redundancy, we only included *Drosophila*  
156 *melanogaster*. In order to provide a representative sample of insect species we searched for *mvl*



157 in Lepidoptera but there are currently no assembled and annotated genomes of the order that  
158 contain a copy of *mvl*. The NCBI BLAST used both the sequences for Mv11 (XP\_967521.1) and  
159 Mv12 (XP\_973779.1) from *T. castaneum*. Protein sequences were then aligned using Clustal  
160 Omega (McWilliam *et al.* 2013), and a model test was performed in Mr. Bayes v3.2 (Ronquist  
161 *et al.* 2012) to determine the most appropriate model of protein evolution, which was WAG  
162 (Whelan and Goldman 2001). A Bayesian phylogenetic analysis was conducted in Mr. Bayes for  
163 5,000,000 generations with a sample frequency of every 100 generations. The consensus tree was  
164 compiled after discarding the first 25% of trees sampled and the resultant tree was rooted with  
165 human Slc11a1 and SLC11a2, mouse Nramp1 and Nramp2, and *C. gigas* Mv1 outgroup.

#### 166 ***Comparison of gene expression***

167 RNA was extracted using a Qiagen RNeasy micro kit (Qiagen, Venlo, Netherlands) for  
168 the brain tissue and larval hemolymph and a Qiagen RNeasy lipid kit for all other tissue. The  
169 extractions were performed with 350  $\mu$ L QIAzol (Qiagen) as the lysis buffer and 150  $\mu$ L  
170 chloroform (J.T. Baker, Center Valley, PA). DNA was removed using DNase I (Qiagen)  
171 according to manufactures instructions. After the final RNA product was obtained it was  
172 quantified with the Qubit 2.0 fluorometer according to manufactures instructions. The RNA was  
173 then stored until the time of cDNA production in a freezer set to -80 °C. cDNA was created  
174 using 500 ng total RNA and the Quanta Biosciences qScript reverse transcriptase master mix  
175 (QuantaBio, Beverly, MA) following the manufactures instructions. The RNA template was then  
176 eliminated using RNase H (New England BioLabs, Ipswich, MA) and the single-strand cDNA  
177 was quantified using the Qubit 2.0 fluorometer according to manufacturer's instructions. The  
178 resulting cDNA was then stored at -20 °C.

179 Using the two *malvolio* gene sequences 8 primer pairs (4 primer pairs per gene) were  
180 produced by utilizing Integrated DNA Technology (IDT, Coralville, IA, USA) and Primer 3 v.  
181 4.0 (Koressaar and Remm 2007; Untergasser *et al.* 2012). These primer pairs were then  
182 validated by estimating PCR efficiency and observing the number of amplicons generated by  
183 each pair. The primer efficiency was determined by running a qRT-PCR reaction with stock  
184 cDNA (produced using same methods as experimental cDNA from whole body samples) diluted  
185 to 1:4, 1:16, 1:64, 1:256 and 1:1024 concentrations, while amplicons were observed in the Melt  
186 Curve Analysis. These primer pairs had efficiency levels of 1.805 (*mv11*) and 1.7852 (*mv12*).

187 The quantification of gene expression was accomplished using a qRT-PCR reaction with  
188 the Roche LightCycler 480 using Roche LightCycler 480 SYBR I Green Master Mix (Roche  
189 Applied Science, Indianapolis, IN, USA). Each biological replicate (N = 5) was run with three  
190 technical replicates using 10  $\mu$ L reactions containing 5  $\mu$ L SYBR mix, 2  $\mu$ L of 1.5ng/  $\mu$ L cDNA,  
191 and 3  $\mu$ L of an equal mixture of forward and reverse primers at 1.33  $\mu$ mol/L each. The  
192 LightCycler was run according to manufacturer's instructions for the enzyme activation step,  
193 followed by 45 cycles of amplification at 60°C and a disassociation curve step to measure the  
194 number of amplicons produced in the reaction. Each reaction included the primers (*mv11*-  
195 forward: CGACGATGACGGGAAGTTATG reverse: TTGCGATGGATCTGGTGAAG *mv12*-  
196 forward: GGTATCGTGGGAGCAGTTATC reverse: GCTGCTCTCGATGAGGTAATAG *tbp*-  
197 forward: CACCCATGACTCCAGCAGAT reverse: ACGTGCATGCAGAGCTATCTT) for  
198 *mv11*, *mv12*, and TATA binding protein, an endogenous control gene.

### 199 **Statistical analysis**

200 Differences in levels of *mv11* and *mv12* expression across tissues were quantified using -  
201  $\Delta C_T$ , or the difference between experimental and control gene expression. Because both versions

202 of *malvolio* were run on the same tissue, same plate, and using the same control gene, the  $-\Delta C_T$   
203 values allows us to qualitatively compare expression of the two genes. We made comparisons  
204 among the tissues types using ANOVA on the  $-\Delta C_T$  values, with specific pairwise comparisons  
205 made using Fisher's Least Significant Difference (LSD) test.

206 Comparisons of expression of *mvl1* and *mvl2* across different behavioral states were  
207 made as described in Roy-Zokan *et al.* (2015) and Cunningham *et al.* (2016), using the  $-\Delta\Delta C_T$   
208 method with relative expression standardized to virgins. We used virgins as the comparison as  
209 this is the behavioral/physiological state of individuals used in the tissue comparison. ANOVA  
210 on relative expression was used to determine statistically significant changes in expression.

211 All data and reagents are available on request. Data will be deposited in Dryad. All  
212 accession numbers for sequences used in the phylogenetic comparison are available in  
213 supplemental file 2.

## 214 **Results**

### 215 ***Phylogenetic analysis of malvolio across insects***

216 Boxshade plots showing sequence homology between *N. vespilloides*, *T. castaneum*, *H. sapiens*,  
217 and *M. musculus* can be found in supplemental Fig. 1. Phylogenetic analysis (Figure 1) shows  
218 that *malvolio* has undergone several independent gene duplications that have been maintained  
219 both in insects and other animals as well. Among insects, *malvolio* appears to have duplicated  
220 separately in hemipterans, the ancestor of Coleoptera and Diptera, and several times in wasps.  
221 Other than wasps, among the Hymenoptera bees and ants have only one copy of *malvolio*. One  
222 duplication appears to have been lost in Diptera.

### 223 ***Differences in expression in different tissues***

224 Expression of *mv11* in brain, fat bodies, Malpighian tubules, midgut, ovaries, testes and thoracic  
225 musculature varied across the different tissue types ( $F_{7,32} = 44.361$ ,  $P < 0.0001$ ). Expression in  
226 fat bodies was statistically significantly higher than other tissues (Figure 2a). Hindgut, midgut  
227 and thoracic musculature had moderate levels of expression, while expression was relatively low  
228 in testes, Malpighian tubules, brains, and ovaries.

229 Expression patterns across tissues of *mv12* differed from those of *mv11* (Figure 2b).  
230 Overall, there was statistically significantly different expression across the different tissue types  
231 ( $F_{7,32} = 37.420$ ,  $P < 0.0001$ ) although in all tissues expression was much lower than that of *mv11*.  
232 Expression was highest in midgut and brain, with low expression in fat bodies, hindgut and  
233 testes. Expression was negligible, and sometimes undetectable, in Malpighian tubules, ovaries  
234 and thoracic muscle (Figure 2b).

### 235 ***Differences in expression in heads across different behavioral states***

236 Expression patterns across behavioral states differed for *mv11* compared to *mv12*. Overall, there  
237 was statistically significantly difference in expression across the behavioral states in *mv11* ( $F_{4,45} =$   
238  $3.4087$ ,  $P = 0.077$ ) (Fig 4a), with a significant increase in expression in resource preparation ( $P =$   
239  $0.0044$ ) and caring for offspring ( $P = 0.0032$ ). There was no overall statistically significant  
240 difference in expression across behavioral states for *mv12* ( $F_{4,43} = 2.2682$ ,  $P = 0.077$ ) (Fig 4b)  
241 although there was a strong trend for decreased expression during resource preparation and  
242 parental care. In pairwise comparisons, resource preparation showed significantly lower  
243 expression than either virgin ( $P = 0.019$ ) or post care ( $P = 0.0285$ ). Regardless, the patterns of  
244 expression are opposite for *mv11* and *mv12* comparing behavioral states.

245

## 246 Discussion

247 Gene duplication is a major factor in evolution (Ohno 1970; Innan and Kondrashov 2010;  
248 Wagner 2011), particularly where there is neofunctionalization, as the duplicated gene can  
249 permit access to variation that may have otherwise been constrained. Here we examined  
250 *malvolio*, a gene that typically functions in heavy metal transport. Examining the genome of the  
251 subsocial beetle, *N. vespilloides*, we found that *malvolio* was duplicated in this insect. Two other  
252 factors suggested that it would be informative to examine this duplication further; first, *malvolio*  
253 also plays a role in social behavior in bees (although they have only one copy), and second,  
254 *malvolio* is the homolog of *Nramp* in vertebrates, a gene that is duplicated and subfunctionalized  
255 (Techau *et al.* 2007; Neves *et al.* 2011). Our study has two components. In our phylogenetic  
256 analysis, we found that duplication of this gene was not unique to *N. vespilloides* or insects in  
257 general; *malvolio* was often independently duplicated and maintained in multiple insect lineages.  
258 In our expression studies, we found that *mv11* and *mv12* display both tissue and behavior specific  
259 expression patterns. Moreover, level of expression in the brain depended the behavioral state of  
260 the insect, with differential expression of both copies of *malvolio*, albeit in opposite direction,  
261 associated with parenting behavior.

262 Contrary to our hypothesis that insects have only one copy of *malvolio*, our phylogenetic  
263 analysis showed that many species of insects have duplicate copies and, furthermore, that these  
264 duplications appear to have occurred in multiple lineage specific events. *Malvolio* duplicates  
265 have arisen and persisted in at least three different insect lineages (and possibly again in wasps,  
266 leading at least some to have three copies of *malvolio*). Given the tendency of duplicated genes  
267 to remain redundant and eventually be removed from the genome it suggests that *malvolio* may  
268 possess qualities that have been found to encourage persistence after a duplication event

269 (Kondrashov *et al.* 2002; Papp *et al.* 2003; Marland *et al.* 2004; Jordan *et al.* 2004; Davis and  
270 Petrov 2004).

271 *Nicrophorus vespilloides* is not a genetic model organism, although we have a sequenced  
272 genome (Cunningham *et al.* 2015b). Instead, this species is interesting for its unusually  
273 elaborate parenting and social interactions (Parker *et al.* 2015). To examine whether  
274 sub/neofunctionalization may be responsible for the maintenance of both *malvolio* duplicates in  
275 *N. vespilloides*, we examined tissue-specific expression of both genes. Our data show that *mv11*  
276 is expressed in all eight measured tissues, with relatively low variance in gene expression within  
277 a tissue. In contrast to *mv11*, expression of *mv12* was limited to only two tissues, the brain and the  
278 midgut. This pattern is roughly consistent with tissue and stage-specific data from *Tribolium*,  
279 which also shows high and ubiquitous *mv11* expression as opposed to low and inconsistent, but  
280 detectable, *mv12* expression (Dippel *et al.* 2014). This suggests that *mv11* may have maintained a  
281 conserved homeostatic role throughout the coleopteran lineage, consistent with the necessity of  
282 manganese transport on the cellular level (Culotta *et al.* 2005). Differences in expression  
283 between specific tissues may be related to other well-established functions of *mv1* and its  
284 homologues, such as intercellular immunity (Evans *et al.* 2001; Cellier *et al.* 2007). *mv12*, on the  
285 other hand, is clearly not required for basic tissue function, and thus may be released from  
286 pleiotropic constraint.

287 To further examine the possibility that the function of *mv12* has diverged from that of *mv11*  
288 in *N. vespilloides*, we examined the expression patterns of both genes in the head in relation to  
289 reproductive and parental care behavior. Previous research has shown that genes differentially  
290 expressed during parenting are detected in these samples (Parker *et al.* 2015; Roy-Zokan *et al.*  
291 2015; Cunningham *et al.* 2016, 2017). In terms of having a function in behavior, *malvolio* is

292 involved in caste differentiation in honey bees (Ben-Shahar *et al.* 2004) as well as feeding  
293 behavior in *Drosophila* (Søvik *et al.* 2017), and therefore represents a strong candidate for  
294 influencing social behavior in insects. In particular, we have hypothesized that feeding pathways  
295 are co-opted to influence parental provisioning behavior (Cunningham *et al.* 2016, 2017). We  
296 found that the two copies do show differences in expression in head tissue associated with  
297 changes in behavior and social interactions. Whereas *mv11* increases expression during parenting,  
298 *mv12* appears to decrease during the same behavioral stages. These opposing expression patterns  
299 suggest that even though both gene copies have retained roles in social behavior, these roles have  
300 clearly diverged.

301         Given the tissue and stage-specific expression patterns of *mv11* and *mv12*, it appears likely  
302 that these genes have undergone either sub- or neofunctionalization in burying beetles.  
303 However, it remains unclear which process has occurred. Data from honey bees, in which  
304 *malvolio* is not duplicated, shows that a single copy can account for both behavioral and other  
305 gene functions (Ben-Shahar *et al.* 2004), suggesting that divergence in gene function between  
306 copies could be obtained by subfunctionalization alone. However, if this were the case, we  
307 would predict that one copy would have completely lost its association with behavior in *N.*  
308 *vespilloides*. Instead, we observe the evolution of opposing gene expression patterns between  
309 copies, meaning the expression patterns of at least one gene copy must be derived. Given the  
310 highly divergent and elaborate social interactions during parenting, and extensive parenting, in  
311 this species this suggests that *malvolio* may be co-opted for further behavioral evolution.  
312 Furthermore, divergence in tissue specific expression patterns, as we observe here, is often  
313 associated with neofunctionalization (Huminięcki and Wolfe 2004, Li *et al.* 2005). Therefore,  
314 our data is consistent with neofunctionalization. It may be that the expression patterns of *mv12* in

315 the brain and midgut are still evolving, and understanding whether expression is being gained or  
316 lost in these tissues along with explicitly functional studies would help resolve this.

317 In conclusion, *N. vespilloides* produces two copies of *malvolio*, both of which are  
318 expressed, but expression depends on the tissue examined. Duplications of *malvolio* are not  
319 unusual among insects, as they appear to have arisen independently and been maintained in  
320 several other species of insects. Our expression data suggest that, in *N. vespilloides*, *malvolio* has  
321 experienced neo-functionalization following its duplication, with an enhanced role in behavior.  
322 Further functional studies are needed to eliminate subfunctionalization but our expression data  
323 suggest that the two copies are not equivalent. Finally, we further suggest that the predilection  
324 for duplicates of this gene to be maintained may reflect a tendency for sub or  
325 neofunctionalization of *mvl* in other systems as well.

326

### 327 **Acknowledgments**

328 We thank Trish Moore and Michelle Ziadie for helpful discussions. This research was supported  
329 by a National Science Foundation grant IOS-1326900 to AJM, with ECMe supported by an REU  
330 supplement.

331

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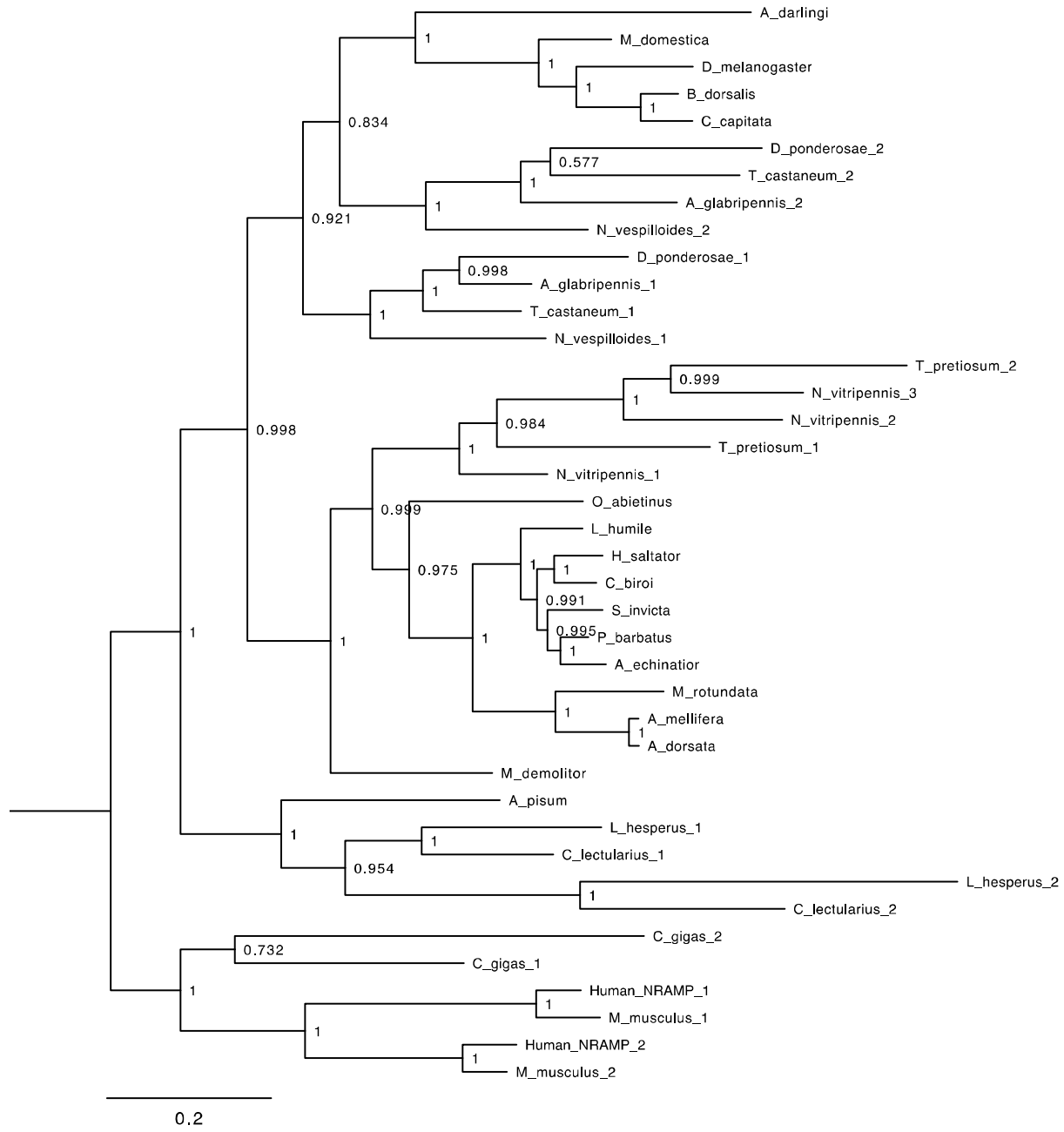
435 **FIGURE CAPTIONS**

436 **Figure 1:** Phylogenetic relationships of *malvolio*. Included in this tree are mammals (human  
437 *Homo sapiens*, mouse *Mus musculus*), an oyster (*Crassostera gigas*), a spider (*Latrodectus*  
438 *hesperus*), and several insect orders including Hemiptera (bed bug *Cimex lectularius*, pea aphid  
439 *Acyrtosiphon pisum*), Hymenoptera (bees: *Apis dorsata*, *Apis mellifera* and *Metamicroptera*  
440 *rotundata*; wasps: *Orussus abietinus*, *Microplitis demolitor*, *Nasonia vitripennis*, *Trichogramma*  
441 *pretiosum*; ants: *Acromyrmex echinator*, *Pogonomyrmex barbatus*, *Solenopsis invicta*, *Ooceraea*  
442 *biroi*, *Harpegnathos saltator*, *Linepithema humile*), Coleoptera (beetles: *Tribolium castaneum*,  
443 *Nicrophorus vespilloides*, *Anoplophora glabripennis*, *Dendroctonus ponderosae*) and Diptera  
444 (flies: *Ceratitis capitata*, *Bactrocera dorsalis*, *Drosophila melanogaster*, *Musca domestica*,  
445 *Anopheles darlingi*).

446 Figure 2: mRNA expression of *mv11* (2a) and *mv12* (2b) calculated as  $-\Delta C_T$ . Significant  
447 differences in expression are indicated by the letters above each tissue.

448 Figure 3: Relative expression of *mv11* (3a) and *mv12* (3b) in female heads across different  
449 physiological/behavioral states. Virgin females had no social experience or exposure to a carcass  
450 resource required for mating and oogenesis; mated females were placed with a male for 48 hours  
451 but not provided with a carcass; resource preparation were mated females provided with a  
452 carcass for 48 hours to prepare for reproduction and provisioning of offspring; direct and indirect  
453 care were females sampled during the most active period of direct feeding of offspring and in the  
454 act of regurgitating food to the offspring; post care females had dispersed from the carcass and  
455 had no further interactions with larvae for at least 24 hours. All individuals were 21 days of age  
456 when sampled. Significant differences in expression are indicated by the letters above each state.

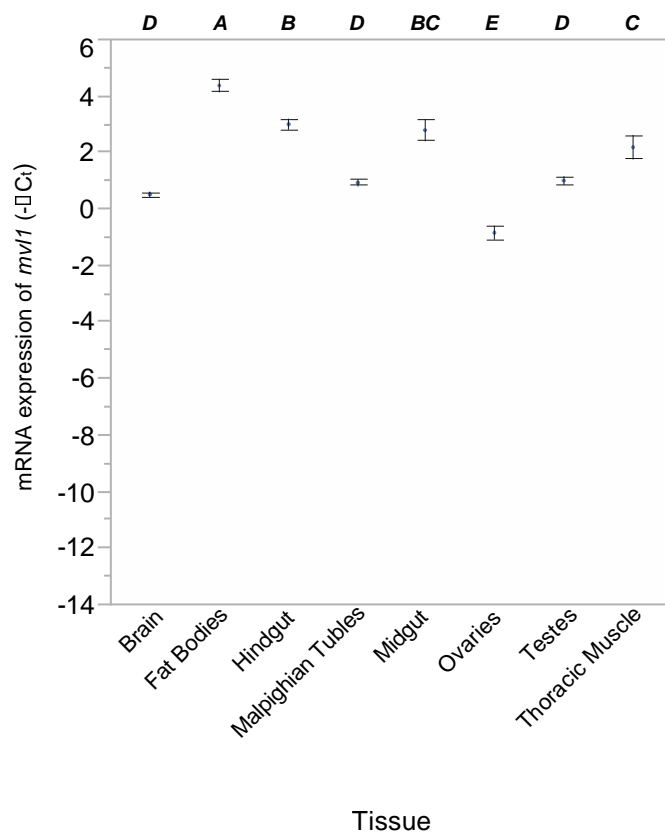
457 **Figure 1**



458

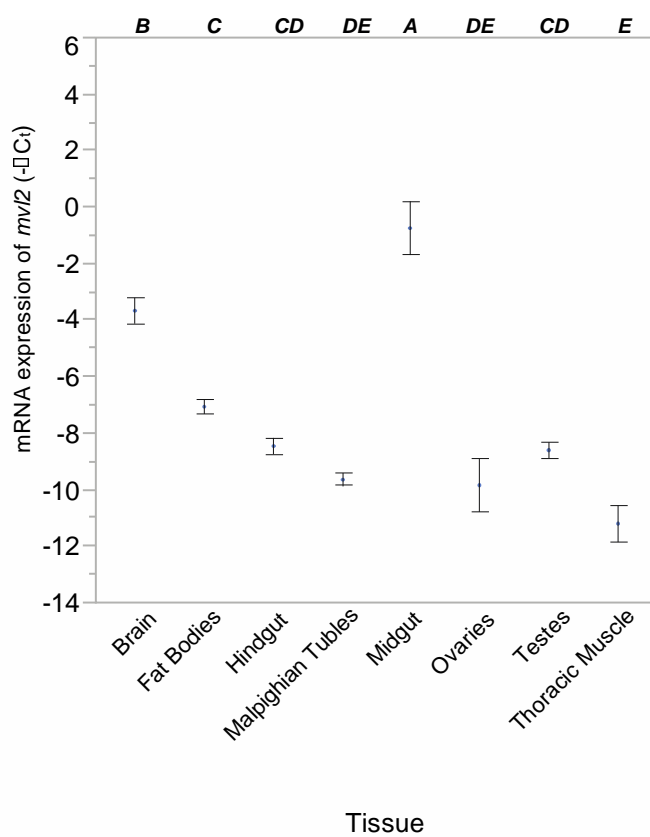
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460 Figure 2a

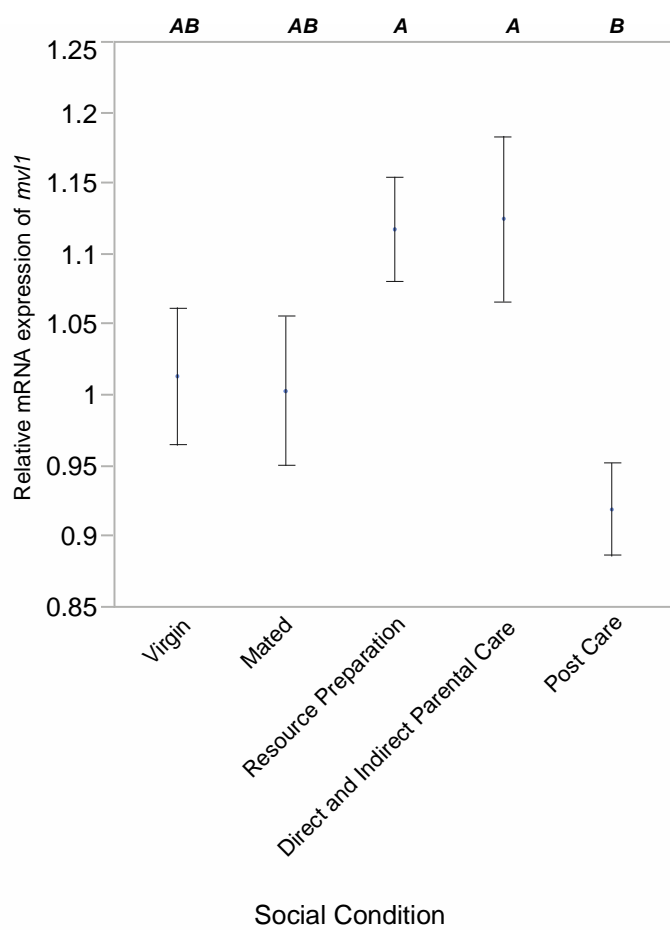




462 **Figure 2b.**



464 Figure 3a



466 Figure 3b

