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An ant–plant mutualism through the lens of cGMP-dependent kinase genes

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In plant–animal mutualisms, how an animal forages often determines how much benefit its plant partner receives. In many animals, foraging behaviour changes in response to *foraging* gene expression or activation of the cGMP-dependent protein kinase (PKG) that *foraging* encodes. Here, we show that this highly conserved molecular mechanism affects the outcome of a plant–animal mutualism. We studied the two PKG genes of *Allomerus octoarticulatus*, an Amazonian ant that defends the ant–plant *Cordia nodosa* against herbivores. Some ant colonies are better 'bodyguards' than others. Working in the field in Peru, we found that colonies fed with a PKG activator recruited more workers to attack herbivores than control colonies. This resulted in less herbivore damage. PKG gene expression in ant workers correlated with whether an ant colony discovered an herbivore and how much damage herbivores inflicted on leaves in a complex way; natural variation in expression levels of the two genes had significant interaction effects on ant behaviour and herbivory. Our results suggest a molecular basis for ant protection of plants in this mutualism.

1. Introduction

Cooperation is a trait or suite of traits in one individual that benefits con- or heterospecific individuals [1]. In animals, these traits are largely behavioural. Yet the rich literature on the evolution of cooperation has developed mostly in the absence of knowledge of the molecular mechanisms that modulate this animal behaviour. This is rapidly changing [2–4] as genetic and genomic tools are increasingly applied to social animals [5–7]. However, studies have focused mainly on interactions within conspecific social groups, while we still know almost nothing about the genes or molecular pathways that contribute to cooperative animal behaviours directed at heterospecific partners in mutualisms.

In the vast majority of plant–animal mutualisms, plants benefit from the foraging behaviour of their animal partners. For example, animals pollinate flowers as they forage for nectar or pollen, and disperse seeds as they forage for fruit. Similarly, ant 'bodyguards' protect plants from herbivores by foraging for insect prey on leaves and stems [8,9]. Thus, animal behaviour influences plant fitness, and feedback through this process may drive coevolution. Here, we propose that a family of genes that influence an animal's foraging behaviour may also affect the phenotype or fitness of its plant partner through mutualism.

A gene aptly named *foraging* (*for*), which encodes a cGMP-dependent protein kinase (PKG) [10], affects foraging behaviour in many animals, including ants [5,11–14]. The *for* gene is transcribed into mRNA (*for* gene expression) and translated into a protein kinase that remains inactive until it binds with cGMP (PKG activation), causing a conformational change that then allows the enzyme to phosphorylate numerous other proteins. In *Drosophila melanogaster*, two natural allelic variants show different foraging behaviours; individuals with a 'rover' allele

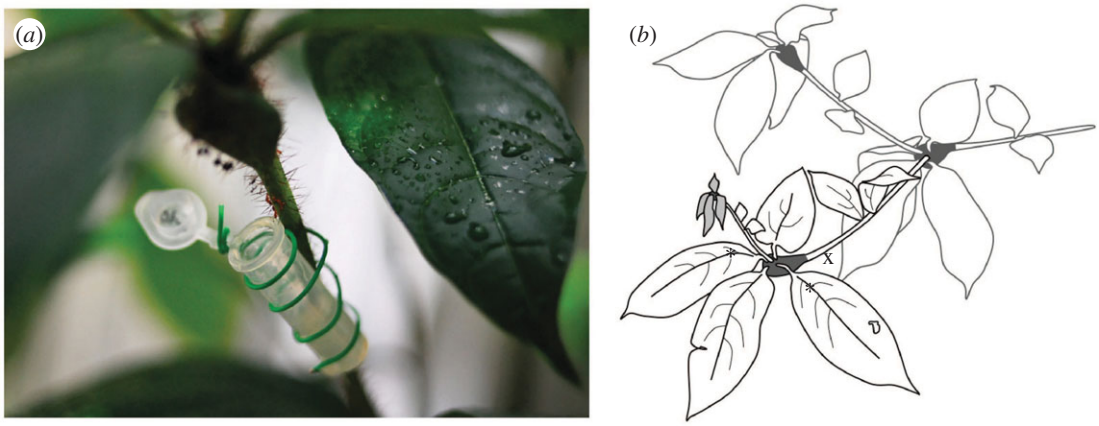

Figure 1. Experimental design. (*a*) Photograph of *A. octoarticulatus* workers foraging on a solution in a microcentrifuge tube on a *C. nodosa* branch at the Los Amigos Research Center in the Peruvian Amazon (photo credit: G.A. Miller). The workers live inside the domatium at the top of the branch in the photo. (*b*) Drawing of a *C. nodosa* branch showing three domatia (swollen stems coloured dark grey), location of microcentrifuge tube containing sucrose solution with or without 8-Br-cGMP depending on treatment (large X), positions of tethered grasshoppers in behavioural assays (*) and whorl of developing leaves on which herbivory was measured (light grey) (drawing credit: K.M. Turner).

move more, eat less, have lower triglyceride levels and higher *for* mRNA levels as larvae (in certain tissues) than individuals homozygous for the 'sitter' allele [10,15,16]. In honeybees, changes in *for* expression cause bees to transition from working inside the nest (e.g. caring for brood) to foraging outside the nest [5]. In two ant species (*Pogonomyrmex barbatus* and *Pheidole pallidula*), *for* expression has also been linked to worker task, age and daily rhythms [11,13,14] and to PKG enzyme activity [12]. Thus, *for* has been demonstrated to modulate foraging activities within ant and bee colonies. We investigated whether *for* may also contribute to the protective effect of ant bodyguards on plants, as an extended phenotype manifested through the effects of *for* on ant foraging behaviour.

The collective action of many individual ants foraging on a plant for insect prey is a highly effective form of plant defence [17]. This benefit has selected for plant traits like extrafloral nectaries, food bodies and ant domatia, and driven the evolution of ant–plant mutualisms [9]. Ant genes may evolve to provide effective protection to host plants because well-defended plants grow larger and provide more food and housing to resident ant colonies [8]. Thus, herbivore damage to an ant–plant can be considered an extended phenotype of the genes of its symbiotic ant colony, *sensu* Dawkins [18]. Working in the Peruvian Amazon, we studied *Allomerus octoarticulatus* (Formicidae: Myrmicinae) ant colonies that live in hollow, swollen stem domatia on the ant–plant *Cordia nodosa* (Boraginaceae; figure 1). Previous work on *A. octoarticulatus* has repeatedly shown that it significantly reduces damage to *C. nodosa* leaves [8,19,20]. Evidence suggests that *A. octoarticulatus* [8] and its congeners [21] decrease herbivore damage to plants because they search out and consume herbivorous insects on plant surfaces. Ness *et al.* [22] probably put it best: 'for ant-protected plants, the best defence is a hungry offense'.

We studied whether variation in the expression of genes from the PKG family or activation of the resulting protein kinases correlate with changes in *A. octoarticulatus* foraging behaviour that in turn affect herbivore damage to plants. Throughout, we use 'PKG expression' to refer to the level of mRNA expression of PKG genes and 'PKG activity' to refer to PKG proteins activated as the result of treatment with a synthetic cGMP analogue, 8-bromoguanosine 30,50-cyclic monophosphate (8-Br-cGMP). It is important to note that expression and activation of PKG genes and their products are independent mechanisms and not necessarily correlated [23]. To investigate whether PKGs play a role in ant defence of plants, first we sequenced the *A. octoarticulatus* genome and assembled putative PKG gene sequences. Second, we reconstructed the phylogeny of PKG genes in arthropods to identify likely orthologues of the two assembled *A. octoarticulatus* PKG sequences: the *A. octoarticulatus* orthologue of *for* (hereafter *Aofor*) and a second PKG in the *A. octoarticulatus* genome (hereafter *Aopkg*, see below). Next, at our field site in the Peruvian Amazon, we fed *A. octoarticulatus* workers with 8-Br-cGMP, a known PKG activator, and measured treatment effects on the plant-protective behaviour of ant colonies and herbivory on developing *C. nodosa* leaves. Finally, we measured treatment effects on *Aofor* and *Aopkg* mRNA levels and correlated PKG gene expression with ant behaviour and herbivory (in control colonies only).

2. Material and methods

(a) Study system and site

We studied the plant–protective behaviour of *A. octoarticulatus* colonies on *C. nodosa* trees at the Los Amigos Research Center (12°34′ S, 70°05′ W; elevation approximately 270 m) in Peru. This site is mostly primary tropical rainforest, with a mix of floodplain and *terra firme* habitats. Here, *A. octoarticulatus* appears to be an obligate associate of *C. nodosa*, with a single monogynous colony per plant [19,24]. *Cordia nodosa* produces domatia whether or not ants are present, growing one domatium per internode together with a whorl of four leaves (figure 1). When ants are present, each new domatium is quickly filled with brood and workers; the number of domatia on a *C. nodosa* tree is thus a good measure of *A. octoarticulatus* colony size [25]. The ants get food from the food bodies produced on the surfaces of young leaves as well as from the honeydew excreted by the scale insects (Hemiptera: Sternorrhyncha: Coccoidea) they tend inside domatia [26]. They also prey on insects they capture while actively patrolling leaves. This behaviour results in the death or deterrence of phytophagous insects that would have otherwise fed on *C. nodosa* tissues, lowering herbivore damage on leaves and promoting plant growth [8,20].

(b) Genome sequencing and cGMP-dependent protein kinase gene assembly

We performed shotgun sequencing of the *A. octoarticulatus* genome and we assembled de novo the sequences of putative PKG genes in *A. octoarticulatus* using the generated read database, taking a similar approach to [27] (SRA BioSample accession: SAMN07414042; see also Additional Methods in the electronic supplementary material). In order to check that the two sequences that we retrieved were plausibly encoding PKG, we translated the predicted coding DNA sequences and searched for functional domains using the InterProScan plugin v. 1.0.6 implemented in Geneious v. 6.0.5 [28].

We reconstructed a phylogenetic hypothesis for the various PKGs in arthropods to identify *D. melanogaster* orthologues of *A. octoarticulatus* PKG genes. Putative orthologues of PKGs in arthropods were downloaded using OrthoDB (Group EOG8PP0WX), and aligned with the two protein sequences inferred for *A. octoarticulatus* and the three protein sequences of PKG identified in *D. melanogaster*: isozyme 1 (*Pkg21D*), isozyme 2 (*foraging*) and *CG4839* (GenBank accession nos. Q03042, Q03043 and AAF52864, respectively) [29]. Phylogenetic reconstruction was performed by maximum-likelihood inference (ML) using RAxML software [30]. Details about PKG assembly and phylogenetic reconstruction can be found in the electronic supplementary material.

(c) cGMP-dependent protein kinase activator experiment

Following Ben-Shahar *et al*. [5] and Lucas & Sokolowski [12], we artificially increased PKG activity in *A. octoarticulatus* colonies at our field site using 8-Br-cGMP, a membrane-permeable derivative of endogenous cGMP. In January 2012, we studied 40 *A. octoarticulatus* colonies nesting in *C. nodosa* trees found along the trails at the Los Amigos Research Center and randomly assigned each one to a control or PKG activator treatment after stratifying by estimated standing herbivore damage to leaves. In both treatments, we attached a 2-ml microcentrifuge tube containing a 20% w/v sucrose solution to the stem below the whorl of focal leaves (figure 1); ant colonies in the control treatment received the sucrose solution with nothing added. In the PKG activator treatment, the solution was supplemented with 2.5 mM 8-Br-cGMP. We replaced the tubes twice over the course of the 14-day experiment. We regularly observed *A. octoarticulatus* workers feeding on the solutions in the tubes in both treatments.

To assess the effect of the activator treatment on ant behaviour, we measured whether ants discovered a grasshopper tethered to a leaf (grasshopper discovery) and how many ant 'bodyguards' attacked the grasshopper, as well as herbivory on the associated young leaves (figure 1). Specifically, we measured the number of ants attacking common *C. nodosa* herbivores, grasshoppers in the family Eumastacidae (cf. *Paramastax* spp.). We collected grasshoppers 1–3 days prior to the start of the assay and maintained them at ambient temperature. At the start of the assay, we placed one grasshopper on one of the four fully expanded leaves right below the focal whorl (figure 1). We tethered the grasshopper to this leaf by tying it to an insect pin with thread and pushing the pin through the leaf approximately 5 cm from the domatium. We counted the number of ants interacting with (i.e. stinging, biting or walking on) the grasshopper every minute for 5 min; we averaged the five counts for subsequent data analysis, which is a more conservative approach than modelling all the counts and including time and ant colony as (random) factors. These assays were conducted twice: once before imposing the activator and control treatments and once 14 days later. At the end of the second assay, we collected at least 10 patrolling workers and placed them in RNAlater solution (see more below). For each tree, we also counted the number of ant-inhabited domatia, which is an effective proxy for ant colony size [25]. The focal leaves were photographed once before the experiment and once at the end of the experiment using a digital camera. At the beginning of the experiment, these were all young leaves, and not yet fully expanded. In *C. nodosa* as in many tropical plants, most herbivory occurs during the first few weeks of a leaf's life, while it is expanding, making two weeks a reasonable window over which to measure damage to young leaves. We used the photographs to estimate herbivory by highlighting the contours of leaves and damaged areas using a graphics tablet, and counting the number of pixels in the drawn shapes in ImageJ v. 1.48. When tissue was missing from the edge of a leaf, its shape was visually extrapolated based on the global leaf shape. The leaves were grouped into three categories: less than 1% leaf area missing, between 1% and 10% leaf area missing, and over 10% leaf area missing. We binned the herbivory measurements into these three categories because statistical models fit the data poorly when the response variable was continuous; however, the results were qualitatively similar.

(d) Measurement of cGMP-dependent protein kinase gene expression

We measured the expression of the two PKG genes (*Aofor* and *Aopkg)* identified in *A. octoarticulatus* in both the PKG-activator and control colonies. Total RNA was extracted from 10 worker heads per colony (except in two colonies with fewer sampled workers), using the RNeasy Mini Kit (Qiagen). Briefly, we ground heads for 10 s in a solution of 350 µl RLT buffer and 10 µl β-mercaptoethanol using a VWR 200 Homogenizer. The lysate was further homogenized using QIAshredder spin columns (Qiagen) and total RNA was purified following the manufacturer's protocol. We stored RNA at −80°C before synthesizing cDNA using the iScript Advanced cDNA Synthesis Kit for qRT-PCR (Bio-Rad) following the enclosed protocol.

We designed intron-spanning primer pairs for amplifying cDNA from the PKG genes using Primer3 v. 0.4.0 as implemented in Geneious v. 6.0.5 [31], with an expected annealing temperature of 60°C. Primer quality was assessed by endpoint RT-PCR on pooled cDNA samples using a Multiplex PCR Kit (Qiagen). Amplification products were run on a 1% agarose electrophoresis gel and primer pairs with single products of the correct size were selected for qRT-PCR. Nucleic acid sequences of nine housekeeping genes were assembled using the Illumina read database, following the same approach as for PKG genes. Primer pairs for amplifying cDNA corresponding to these genes were designed as above (see electronic supplementary material, table S1 for gene sequence names, accession numbers and other details as well as primer sequences).

To quantify expression, *Aofor* and *Aopkg* primer efficiencies and specificity were assessed on a pooled cDNA dilution series. Relative expression levels were measured on a CFX384 Real-Time System (Bio-Rad), using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. Cycle threshold (C_t) was automatically determined using the accompanying CFX Manager Software (Bio-Rad) and *Aofor* and *Aopkg* C_t were normalized using 14-3-3ε, which had the best stability value across samples. Fold-change ($\Delta\Delta C_t$) was calculated relative to the lowest C_t measured.

(e) Statistical analyses

Statistical analyses were conducted using R v. 3.3.0, within the RStudio environment. R code and data are archived on Dryad [32]. We tested for a correlation between expression levels of the two PKG genes using the Spearman's rank coefficient (for non-normally distributed data) and we assessed the effect of the PKG activator and ant colony/plant size on PKG gene expression

using generalized linear models (GLMs) with the glm function and quasi-Poisson error distributions.

We assessed the effect of the PKG activator treatment on ant behaviour towards tethered grasshoppers. To account for two components of ant foraging, specifically discovery and dominance, we built two separate models. First, the effect of treatment on grasshopper discovery was assessed using a GLM with a binomial error distribution. Second, the effect of treatment on the number of ant bodyguards attacking a grasshopper was assessed using a GLM with a quasi-Poisson error distribution. We initially included ant colony/plant size, as well as pre-treatment counts of the number of ant bodyguards in models as covariates, but only the latter was retained in the final models that were selected based on BIC scores.

We examined the effect of the PKG activator treatment on herbivory during the experiment using cumulative link mixed models with the clmm function in the package *ordinal* [33]. Total leaf area and treatment were used as fixed effects (again, retained in the final models based on BIC scores), and plant ID as a random effect to account for having measured herbivory on four leaves per plant.

Finally, we assessed the effect of *Aofor* and *Aopkg* expression level on grasshopper discovery, number of ant bodyguards and herbivory during the experiment. We used the same three types of models as described above, but replaced PKG activator treatment with PKG gene expression. Again, we selected final models based on BIC scores, and initially included ant colony/plant size, initial number of bodyguards, and, in the herbivory model, leaf area, but retained only leaf area based on BIC scores. Because our analyses revealed that the PKG activator treatment feeds back to reduce PKG gene expression (see Results and figure 3*c*), only un-manipulated control colonies were used to analyse the relationship between PKG gene expression and ant behaviour or plant damage.

When appropriate, we assessed model fit through the visual evaluation of residual plots and by using the Shapiro–Wilk normality test, and tested for the autocorrelation and heteroskedasticity of the residuals by conducting the Durbin–Watson and Breusch–Pagan tests using the dwtest and the bptest functions, respectively, in the lmtest package [34]. Type III ANOVAs were used to test for the statistical significance of fixed effects.

3. Results

We assembled two genomic DNA sequences that are 26 029 and 4897 nucleotides long, encompassing *Aofor* and *Aopkg*, respectively (see electronic supplementary material, table S1 for GenBank accession numbers). Both sequences have the single protein kinase and two cGMP-binding domains characteristic of most PKG genes. We found that most arthropods had at least two PKG genes in their genomes, and there were two main clades (figure 2). Clade 1 included *D. melanogaster foraging* and its orthologues in arthropods, so we named the *A. octoarticulatus* sequence in this clade *Aofor*. Clade 2 included *D. melanogaster Pkg21D* (also known as *dg1*) and *D. melanogaster CG4839*, as well as the second *A. octoarticulatus* PKG sequence. Given the potential molecular function of the *A. octoarticulatus* gene in this clade, we named it *Aopkg*.

Over the two-week duration of our field experiment in the Peruvian Amazon, some ant colonies consistently attacked grasshoppers more than others, but ant bodyguard behaviour was modulated by treatment with the PKG activator. The number of ants attacking a grasshopper before the experiment started (i.e. the initial number of bodyguards) was a good predictor of whether the ants found the grasshopper at the end of the experiment (table 1). Grasshopper discovery was not influenced by the PKG activator treatment, although there was a marginally significant interaction between treatment and the initial number of bodyguards; the PKG activator tended to increase discovery of grasshoppers in colonies that initially had fewer bodyguards (table 1). Treatment significantly affected the number of bodyguards attacking grasshoppers at the end of the experiment; more ants attacked grasshoppers in PKG activator than control colonies, especially in colonies with few bodyguards initially (table 1, figure 3*a*). Treatment effects on ant behaviour were reflected in how much herbivores damaged leaves during the experiment; the PKG activator significantly reduced herbivory (table 1 and figure 3*b*).

We also measured PKG gene expression in workers at the end of the experiment, and found that measurements of *Aofor* and *Aopkg* expression were not correlated, not in un-manipulated, control colonies (Spearman's $\rho = -0.379$, p-value $= 0.110$), nor in all colonies in the field experiment (i.e. both control colonies and colonies treated with 8-Br-cGMP, Spearman's $\rho = -0.014$, p-value $= 0.935$). There was a significant effect of the interaction between treatment and plant size (i.e. number of domatia) on *Aofor* expression (table 1 and figure 3*c*). Specifically, ant colonies in larger plants expressed more *Aofor* mRNA, but only in the control treatment, and in large plants, PKG activation decreased *Aofor* expression levels relative to controls (figure 3*c*). By contrast, neither treatment nor any other tested variables significantly influenced *Aopkg* expression level (table 1 and figure 3*d*). Because treatment with the PKG activator decreased *Aofor* expression, suggesting that enzyme activation fed back to suppress gene expression, we modelled the relationship between PKG gene expression and ant behaviour/herbivore damage *in un-manipulated control colonies only*.

In the control colonies, PKG gene expression was significantly associated with ant behaviour and herbivore damage to plants. However, the influence of *Aofor* expression depended on *Aopkg* expression and vice versa, and PKG gene expression sometimes had different effects than PKG enzyme activation on the measured phenotypes. The interaction between *Aofor* and *Aopkg* RNA expression levels significantly explained the probability of ants finding the grasshopper: as RNA expression of either PKG gene increased, grasshopper discovery decreased, but only at low expression levels of the other PKG gene (table 2 and figure 4*a*,*b*). RNA expression of either PKG gene did not affect grasshopper discovery when the expression levels of the other PKG gene were high. Although the number of ant bodyguards was not influenced by the expression of either PKG gene (table 2), the interaction between *Aofor* and *Aopkg* expression levels had a highly significant effect on herbivory. Higher *Aopkg* expression was associated with more herbivory, but only at low levels of *Aofor* expression, whereas more *Aofor* expression was associated with less herbivory, but only at high levels of *Aopkg* expression (table 2 and figure 4*c*,*d*). Note that with the significant interaction between *Aofor* and *Aopkg* expression on herbivory, the significant main effect of either *Aofor* or *Aopkg* expression on herbivory holds true only when expression of the other PKG gene is zero.

4. Discussion

Our results suggest that the outcome of the *A. octoarticulatus*–*C. nodosa* mutualism is sensitive to PKG activity or gene expression in the ant partner. We predicted that the *foraging*

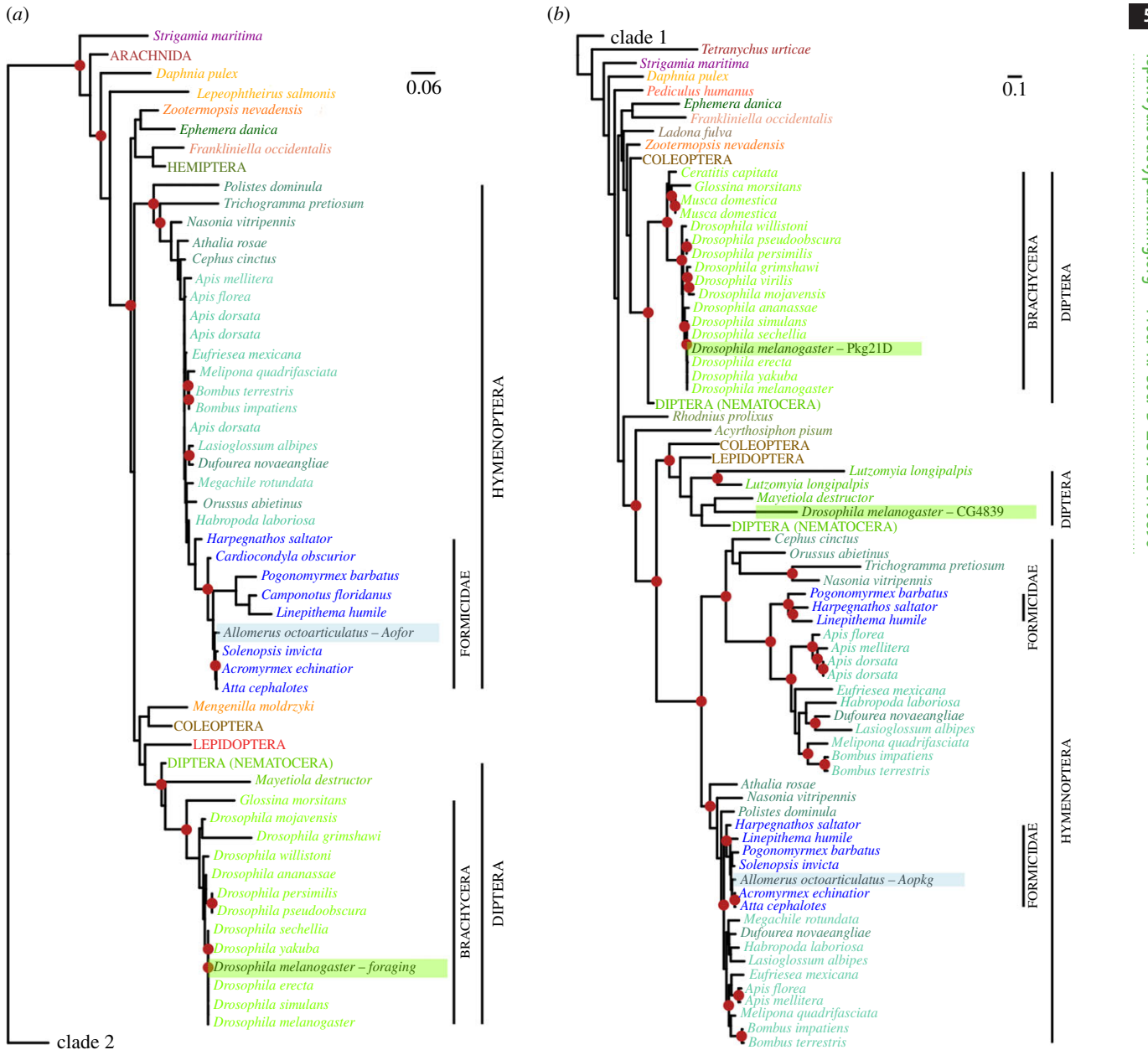

Figure 2. Maximum-likelihood phylogenetic tree for 196 arthropod PKG sequences. Panels (*a*) and (*b*) are clade 1 and clade 2, respectively. Branch lengths are drawn proportional to the number of nucleotide substitutions per site. Red dots account for bootstrap support values (using RAxML) above 70%. Tips are coloured by taxonomic group. Collapsed monophyletic clades are labelled in uppercase (see electronic supplementary material, figure S1 for a tree without collapsed nodes).

gene, known to modulate foraging behaviour in many animals, would influence foraging and, by extension, plant-protective behaviour in *A. octoarticulatus* ant colonies. We found that treatment with a PKG activator increased ant defence of plants and that RNA expression of both of the ant's PKG genes, *Aofor* and *Aopkg*, correlated with herbivory on *C. nodosa* leaves under natural conditions. Unexpectedly, there was a strong statistical interaction between *Aofor* and *Aopkg* RNA expression on herbivory, with increased *Aofor* expression decreasing herbivory only when *Aopkg* expression was high, and increased *Aopkg* expression increasing herbivory when *Aofor* expression was low. In fact, when either PKG gene was highly expressed (a condition simulated, perhaps, by the PKG activation treatment), increasing expression of the other PKG gene decreased herbivory and the results of the PKG activation treatment and the PKG gene expression analyses were in the same direction. Thus, although plant defence by ants is a complex trait and likely influenced by many sources of genetic and environmental variation [35], our results suggest that both PKG genes in *A. octoarticulatus* contribute to the phenotype. In this regard, herbivory on *C. nodosa* may be considered an extended phenotype of genes modulating *A. octoarticulatus* foraging behaviour.

The phylogenetic reconstruction showed that having one copy of *foraging* and a second copy of another PKG gene is a shared characteristic of insects. All taxa have one copy orthologous to *foraging* in *D. melanogaster*, highlighting the physiological importance and conserved function of this gene. The second PKG was orthologous to *D. melanogaster Pkg21D* (also called *dg1*) in the least recently derived taxa, while it was closer to *D. melanogaster CG4839* in lepidopterans and hymenopterans. 'Intermediate' taxa such as dipterans and coleopterans had three PKG genes. This might indicate a duplication of the ancestral gene that led to *Pkg21D* and *CG4839*, followed by a loss of the original copy over the course of insect evolution. Although *dg1* has a well-studied role in

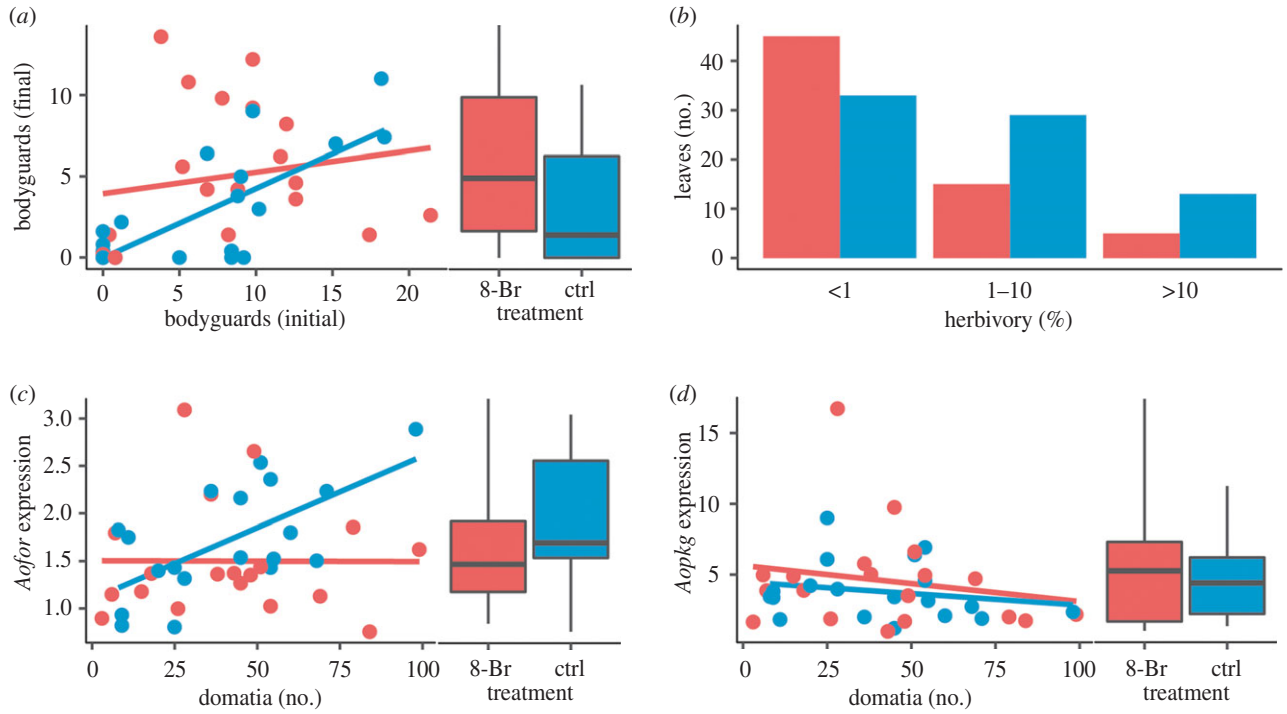

Figure 3. PKG activator effects on ant behaviour, herbivory, and PKG gene expression. (*a*) Number of *A. octoarticulatus* ants attacking grasshoppers in behavioural assays, and (*b*) number of leaves per damage category in the PKG activator and control treatments. (*c*,*d*) Expression levels in fold change of (*c*) *Aofor* or (*d*) *Aopkg* in heads of *A. octoarticulatus* workers in the PKG activator and control treatments. Lines show linear models (for visualization purposes only). In all panels, light red points, lines or bars are PKG-activator ant colonies (8-Br-cGMP plus sucrose solution) and dark blue points, lines or bars are control ant colonies (sucrose solution only). Boxplots show the median (line), first and third quartiles (box edges), 5th and 95th percentiles (whiskers) and outliers (dots). See table 1 for statistical significance in GLMs.

diuresis, but no known role in foraging-related traits [36], very little is known about *CG4839*, apart from its expression patterns across different fruit fly tissues [37–40], making it difficult to speculate about its function.

In our field experiment, ant colonies treated with 8-Br-cGMP were more aggressive towards grasshoppers (figure 3*a*) and their host plants suffered less herbivory (figure 3*b*), suggesting cascading effects of the treatment on ant behaviour and plant damage. PKG activity monitoring was not possible in our field study in Peru as it requires brain dissections on dry ice and storage at −80°C, which were not feasible in the Amazonian rainforest. However, 8-Br-cGMP has reproduced the effects of endogenous cGMP on insect physiology and behaviour in many studies [5,12,41–44]. For example, in honeybees, treatment with 8-Br-cGMP induced precocious foraging and high sucrose responsiveness in young workers through the activation of PKGs [5,45]. Similarly, the ant *P. pallidula* became more aggressive towards invaders in response to PKG activation by 8-Br-cGMP treatment [12]. Thus, the effect of 8-Br-cGMP on *A. octoarticulatus* foraging behaviour mirrored the effects of increasing cGMP in other insects and was likely driven by changes in PKG activity.

The 8-Br-cGMP treatment decreased *Aofor* expression in large plants/ant colonies (figure 3*c* and table 1). One possibility is that 8-Br-cGMP increased PKG activity in treated ants as expected, and in turn triggered the suppression of *Aofor* expression via negative feedback control, as described for PKG-I in mammals [46]. Although we might have predicted a similar effect of 8-Br-cGMP treatment on *Aopkg* expression, given the gene's two cGMP binding domains, activation of this enzyme, if it occurred, did not appear to feed back to affect *Aopkg* expression, perhaps because of lower binding affinity. It is also possible that the *Aopkg* protein was not activated by 8-Br-cGMP. While 8-Br-cGMP has been repeatedly used to activate proteins encoded by PKGs in other hymenopterans, with studies directly confirming greater PKG enzyme activity in response to treatment [5,12], it is not known whether 8-Br-cGMP activates all PKGs equally.

Treatment with 8-Br-cGMP decreased herbivory (figure 3*b*), as did increased *Aofor* expression when *Aopkg* expression was high (figure 4*c*). However, greater *Aopkg* expression was positively correlated with herbivory on *C. nodosa* leaves at low *Aofor* mRNA levels (figure 4*d*), meaning that we might have expected *Aopkg* activation to increase herbivory. Again, one possible explanation is differential activation of *Aofor* and *Aopkg* by 8-Br-cGMP. There is ample scope for differences in cGMP binding affinity between the predicted *Aofor* and *Aopkg* proteins as their first and second cGMP-binding domains share only 36% and 44% amino acid sequence identity, respectively (results not shown). If *Aofor* protein binds more readily to cGMP than *Aopkg* protein, this could explain why the association between *Aofor*, but not *Aopkg*, expression and herbivory was in the same direction as the effects of treatment with 8-Br-cGMP on herbivory.

Interestingly, our qRT-PCR analyses revealed significant interactions between *Aofor* and *Aopkg* expression on both the likelihood that ants discovered the tethered grasshopper and on herbivory (table 2 and figure 4). In other words, the relationship between *Aofor* expression and these traits depended on *Aopkg* expression, and vice versa (figure 4). Because the physiological function of *Aopkg* and its orthologues in other arthropods has not been studied, we are unable to say what kind of interactions occurs between the two PKGs at a molecular level. Complex behaviours like foraging may often depend

Table 1. Statistical model results for PKG activator effects on ant behaviour, herbivory and PKG gene expression. *Initial bodyguards*, mean number of ants interacting with the grasshopper at the beginning of the experiment; *treatment*, treatment with PKG activator as the baseline; *leaf area*, total leaf area; *domatia*, total number of domatia on the plant; *Aofor*, *Aofor* fold-change; *Aopkg*, *Aopkg* fold-change. Statistical significance is indicated by asterisks.

	estimate	χ^2	*p*-value
grasshopper discovery			
initial bodyguards	0.169	9.126	0.003**
treatment	0.616	0.247	0.621
initial bodyguards × treatment	−0.139	3.296	0.069
final bodyguards (number of ants interacting with the grasshopper)			
initial bodyguards	0.026	0.723	0.395
treatment	−1.614	7.904	0.005**
initial bodyguards × treatment	0.112	5.281	0.022*
herbivory			
leaf area	0.511	0.000	1.000
treatment	1.633	3.940	0.047*
leaf area × treatment	−0.804	2.133	0.144
Aofor expression			
domatia	0.000	0.000	0.984
treatment	−0.238	1.161	0.281
domatia × treatment	0.009	3.877	0.049*
Aopkg expression			
domatia	−0.003	0.412	0.521
treatment	0.022	0.004	0.949
domatia × treatment	−0.001	0.029	0.865

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 2. Statistical model results for PKG gene expression effects on ant behaviour and herbivory. *Aofor*, *Aofor* fold-change; *Aopkg*, *Aopkg* fold-change; *leaf area*, total leaf area. Statistical significance is indicated by asterisks.

	estimate	χ^2	*p*-value
grasshopper discovery			
Aofor	−6.645	5.842	0.016*
Aopkg	−2.424	4.926	0.026*
Aofor × *Aopkg*	1.232	4.229	0.040*
final bodyguards (number of ants interacting with the grasshopper)			
Aofor	−0.201	0.019	0.890
Aopkg	0.036	0.003	0.957
Aofor × *Aopkg*	−0.197	0.233	0.629
herbivory			
leaf area	−1.308	0.597	0.440
Aofor	8.348	13.242	<0.001***
Aopkg	4.716	16.637	<0.001***
leaf area × *Aofor*	0.246	0.010	0.919
leaf area × *Aopkg*	−0.402	0.091	0.762
Aofor × *Aopkg*	−2.305	14.052	<0.001***
leaf area × *Aofor* × *Aopkg*	0.360	0.324	0.570

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

on the interaction between *for* and other genes; for example, epistasis occurs between *Amfor* (the honeybee *foraging* orthologue) and other QTLs important for foraging behaviour in honeybees [47].

The positive correlation between the two behavioural assays performed two weeks apart (figure 3*a*) indicates that ant colonies behaved consistently through time; some colonies are naturally better protectors than others. This could be indicative of genetic differences in the colonies as in *D. melanogaster* [48,49], or because of environmental or ontogenetic factors such as colony age, size, diet, disease, etc. The genome sequencing done in the present study does not allow us to assess genetic variation in these PKG genes but this is an important future research direction. Differences in transcriptional or post-transcriptional regulation could also explain variation in PKG expression levels, resulting in consistent behavioural differences among colonies [50,51]. Several transcripts, with potentially different functions, have been isolated for *foraging* in *D. melanogaster* [16,39,40,52,53]. However, only one has been identified for *Pbfor* in the ant *P. barbatus* [11], and we found only one transcript for each of the two PKGs identified in the *Solenopsis invicta* EST database [54] (results not shown). Of course, environmental or ontogenetic conditions that have consequences for *Aofor* and *Aopkg* gene expression levels might account for some or all of the variation in the ant behaviours we observed [14].

Previous research has shown that an insect's food or social environment can influence *foraging* expression. In ants, wasps and some *Bombus* species, food deprivation decreases *foraging* expression [12,55], and foraging activities are negatively correlated with *foraging* expression and enzymatic activity [11,13,56,57]. Here, we found a positive correlation between plant/ant colony size and *Aofor* expression (figure 3*c*). This could reflect changes in task allocation within colonies as they grow [58]; there is some suggestion that *A. octoarticulatus* workers engage in specific tasks like nursing or foraging [59], and they might express different amounts of *Aofor* mRNA, as in some other Hymenoptera [5,11]. The positive correlation between ant colony/plant size and *Aofor* expression could also be driven by more intense social interactions in larger ant colonies, much as gregarious locusts express more *foraging* mRNA than solitary locusts [55].

In ant–plants, the amount of damage caused by herbivores can be considered an extended phenotype of the ant colony living inside the plant. We might predict that gene expression should explain less of the variation in an extended phenotype than in other traits, because we expect the correlation between gene expression and any measured trait to weaken the further removed the phenotypic trait is from the gene. Yet the underlying *Aofor* and *Aopkg* mRNA levels were more strongly correlated with herbivory than with ant 'bodyguard' behaviour (table 2). This could be because herbivory accumulates gradually on leaves and thus may integrate the effects of PKG gene expression on plant protection by ants across longer time periods. In contrast, we measured the number of *A. octoarticulatus* workers interacting

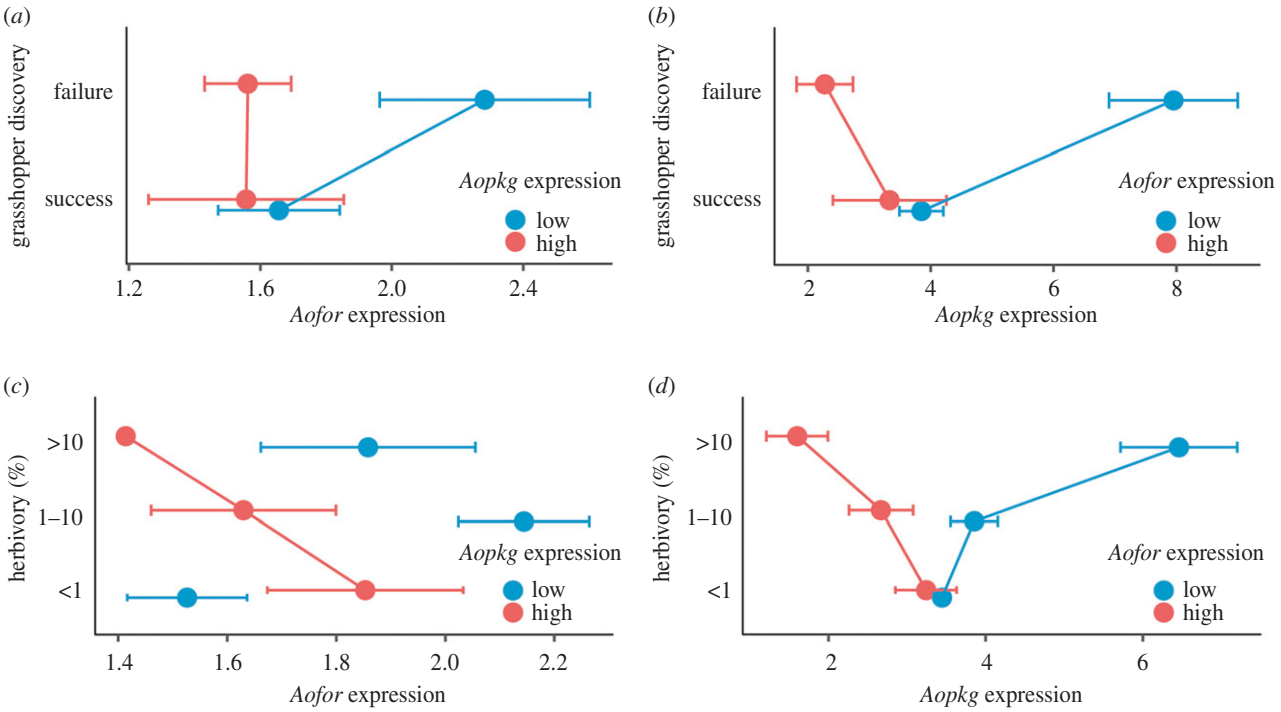

Figure 4. Interactive effects of *Aofor* and *Aopkg* expression on ant behaviour and herbivory. (*a*,*b*) Grasshopper discovery (success/failure) as a function of (*a*) *Aofor* and (*b*) *Aopkg* expression level in fold change. (*c*,*d*) Herbivory on *C. nodosa* leaves as a function of (*c*) *Aopkg* or (*d*) *Aofor* expression in fold change. In all panels, points are means and whiskers are standard errors, light red denotes high (above the sample median) expression levels and dark blue denotes low (below the sample median) expression levels. See table 2 for statistical significance in GLMs.

with a tethered grasshopper over only 5 min once before and once after the PKG activation experiment. On the other hand, these observations were sufficient to observe significant effects of PKG activation on ant behaviour.

Our work suggests that a molecular mechanism influences the quality of mutualistic services that an animal provides. It also adds to the small but growing number of studies linking genes or molecules to complex animal behaviours and their ecological consequences under field conditions [60,61]. We studied naturally occurring ant colonies and plants in a hyper-diverse tropical rainforest in Peru, where they experienced a wide range of abiotic and biotic conditions. Despite strong environmental variation, our results suggest that the cGMP-PKG signalling pathway modulates the plant-protective behaviour of *A. octoarticulatus*, with an extended phenotype in the ants' host plants. The cGMP-PKG signalling pathway may modulate mutualist quality and have extended phenotypic effects in a wide array of plant–animal mutualisms because the *foraging* gene influences food-related behaviours in many insects, and plants benefit from animal foraging in pollination, seed dispersal and ant–plant protection mutualisms.

Ethics. We obtained permits from the Dirección General Forestal y de Fauna Silvestre of the Ministerio de Agricultura in Peru for fieldwork and sample collection (no. 0299-2011-AG-DGFFS-DGEFFS) and for molecular analyses of samples (no. 0046-2014-MINAGRI-DGFFS-DGEFFS).

Data accessibility. The read database is archived in SRA (BioSample accession no.: SAMN07414042), assembled DNA sequences are archived in GenBank (accession no.: KX809572-82, see electronic supplementary material, table S1 for details), and R code and data are archived in Dryad (http://dx.doi.org/10.5061/dryad.c6bf5) [32].

Authors' contributions. P.-J.G.M. performed molecular biology and statistical analyses. K.M.T. conducted field experiments. M.D. performed leaf measurements. I.A., A.M.A. and M.B.S. assisted in the molecular biology analyses design. P.-J.G.M. and M.E.F. wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

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References

1. Barker JL, Bronstein JL, Friesen ML, Jones EI, Reeve HK, Zink AG, Frederickson ME. 2017 Synthesizing perspectives on the evolution of cooperation within and between species. *Evolution* **71**, 814–825. (doi:10.1111/evo.13174)
2. Fitzpatrick MJ, Benshahar Y, Smid H, Vet L, Robinson G, Sokolowski M. 2005 Candidate genes for behavioural ecology. *Trends Ecol. Evol.* **20**, 96–104. (doi:10.1016/j.tree.2004.11.017)
3. Sokolowski MB. 2010 Social interactions in 'simple' model systems. *Neuron* **65**, 780–794. (doi:10.1016/j.neuron.2010.03.007)
4. Taborsky M, Taborsky B. 2015 Evolution of genetic and physiological mechanisms of cooperative behaviour. *Curr. Opin. Behav. Sci.* **6**, 132–138. (doi:10.1016/j.cobeha.2015.11.001)
5. Ben-Shahar Y, Robichon A, Sokolowski MB, Robinson GE. 2002 Influence of gene action across different time scales on behavior. *Science* **296**, 741–744. (doi:10.1126/science.1069911)
6. Wang J, Wurm Y, Nipitwattanaphon M, Riba-Grognuz O, Huang Y-C, Shoemaker DW, Keller L. 2013 A Y-like social chromosome causes alternative colony organization in fire ants. *Nature* **493**, 664–668. (doi:10.1038/nature11832)
7. Kapheim KM *et al.* 2015 Genomic signatures of evolutionary transitions from solitary to group

living. *Science* **348**, 1139–1143. (doi:10.1126/science.aaa4788)

8. Frederickson ME, Ravenscraft A, Miller GA, Arcila Hernández LM, Booth G, Pierce NE. 2012 The direct and ecological costs of an ant–plant symbiosis. *Am. Nat.* **179**, 768–778. (doi:10.1086/665654)
9. Mayer VE, Frederickson ME, McKey D, Blatrix R. 2014 Current issues in the evolutionary ecology of ant–plant symbioses. *New Phytol.* **202**, 749–764. (doi:10.1111/nph.12690)
10. Osborne KA, Robichon A, Burgess E, Butland S, Shaw RA, Coulthard A, Pereira HS, Greenspan RJ, Sokolowski MB. 1997 Natural behavior polymorphism due to a cGMP-dependent protein kinase of *Drosophila*. *Science* **277**, 834–836. (doi:10.1126/science.277.5327.834)
11. Ingram KK, Oefner P, Gordon DM. 2005 Task-specific expression of the *foraging* gene in harvester ants. *Mol. Ecol.* **14**, 813–818. (doi:10.1111/j.1365-294X.2005.02450.x)
12. Lucas C, Sokolowski MB. 2009 Molecular basis for changes in behavioral state in ant social behaviors. *Proc. Natl. Acad. Sci. USA* **106**, 6351–6356. (doi:10.1073/pnas.0809463106)
13. Ingram KK, Kleeman L, Peteru S. 2011 Differential regulation of the foraging gene associated with task behaviors in harvester ants. *BMC Ecol.* **11**, 19. (doi:10.1186/1472-6785-11-19)
14. Ingram KK, Gordon DM, Friedman DA, Greene M, Kahler J, Peteru S. 2016 Context-dependent expression of the *foraging* gene in field colonies of ants: the interacting roles of age, environment and task. *Proc. R. Soc. B* **283**, 20160841. (doi:10.1098/rspb.2016.0841)
15. Sokolowski MB. 1980 Foraging strategies of *Drosophila melanogaster*: a chromosomal analysis. *Behav. Genet.* **10**, 291–302. (doi:10.1007/BF01067774)
16. Allen AM, Anreiter I, Neville MC, Sokolowski MB. 2017 Feeding-related traits are affected by dosage of the *foraging* gene in *Drosophila melanogaster*. *Genetics* **205**, 761–773. (doi:10.1534/genetics.116.197939)
17. Chamberlain SA, Holland JN. 2009 Quantitative synthesis of context dependency in ant-plant protection mutualisms. *Ecology* **90**, 2384–2392. (doi:10.1890/08-1490.1)
18. Dawkins R. 2004 Extended phenotype—but not too extended. A reply to Laland, Turner and Jablonka. *Biol Philos.* **19**, 377–396. (doi:10.1023/B:BIPH.0000036180.14904.96)
19. Yu DW, Pierce NE. 1998 A castration parasite of an ant–plant mutualism. *Proc. R. Soc. Lond. B* **265**, 375–382. (doi:10.1098/rspb.1998.0305)
20. Frederickson ME. 2005 Ant species confer different partners benefits on two neotropical myrmecophytes. *Oecologia* **143**, 387–395. (doi:10.1007/s00442-004-1817-7)
21. Dejean A, Solano PJ, Ayroles J, Corbara B, Orivel J. 2005 Arboreal ants build traps to capture prey. *Nature* **434**, 973. (doi:10.1038/434973a)
22. Ness JH, Morris WF, Bronstein JL. 2009 For ant-protected plants, the best defense is a hungry offense. *Ecology* **90**, 2823–2831. (doi:10.1890/08-1580.1)
23. Schwanhäusser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Chen W, Selbach M. 2011 Global quantification of mammalian gene expression control. *Nature* **473**, 337–342. (doi:10.1038/nature10098)
24. Frederickson ME, Gordon DM. 2009 The intertwined population biology of two Amazonian myrmecophytes and their symbiotic ants. *Ecology* **90**, 1595–1607. (doi:10.1890/08-0010.1)
25. Frederickson ME. 2006 The reproductive phenology of an Amazonian ant species reflects the seasonal availability of its nest sites. *Oecologia* **149**, 418–427. (doi:10.1007/s00442-006-0460-x)
26. Solano P-J, Belin-Depoux M, Dejean A. 2005 Formation and structure of food bodies in *Cordia nodosa* (Boraginaceae). *C. R. Biol.* **328**, 642–647. (doi:10.1016/j.crvi.2005.05.004)
27. Malé P-JG *et al.* 2014 Genome skimming by shotgun sequencing helps resolve the phylogeny of a pantropical tree family. *Mol. Ecol. Resour.* **14**, 966–975. (doi:10.1111/1755-0998.12246)
28. Zdobnov EM, Apweiler R. 2001 InterProScan—an integration platform for the signature-recognition methods in InterPro. *Bioinformatics* **17**, 847–848. (doi:10.1093/bioinformatics/17.9.847)
29. dos Santos G, Schroeder AJ, Goodman JL, Strelets VB, Crosby MA, Thurmond J, Emmert DB, Gelbart WM. 2014 FlyBase: introduction of the *Drosophila melanogaster* release 6 reference genome assembly and large-scale migration of genome annotations. *Nucleic Acids Res.* **43**, D690–D697. (doi:10.1093/nar/gku1099)
30. Stamatakis A. 2006 RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**, 2688–2690. (doi:10.1093/bioinformatics/btl446)
31. Untergrasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. 2012 Primer3—new capabilities and interfaces. *Nucleic Acids Res.* **40**, e115. (doi:10.1093/nar/gks596)
32. Malé P-JG, Turner KM, Doha M, Anreiter I, Allen AM, Sokolowski MB, Frederickson ME. 2017 Data from: An ant–plant mutualism through the lens of cGMP-dependent kinase genes. Dryad Digital Repository. (http://dx.doi.org/10.5061/dryad.c6bf5)
33. Christensen RHB. 2015 *ordinal*: Regression models for ordinal data. R package version 6-28.
34. Zeileis A, Hothorn T. 2002 Diagnostic checking in regression relationships. *R News* **2**, 7–10.
35. Pringle EG, Akçay E, Raab TK, Dirzo R, Gordon DM. 2013 Water stress strengthens mutualism among ants, trees, and scale insects. *PLoS Biol.* **11**, e1001705. (doi:10.1371/journal.pbio.1001705)
36. MacPherson MR, Lohmann SM, Davies S-A. 2004 Analysis of *Drosophila* cGMP-dependent protein kinases and assessment of their *in vivo* roles by targeted expression in a renal transporting epithelium. *J. Biol. Chem.* **279**, 40 026–40 034. (doi:10.1074/jbc.M405619200)
37. Chintapalli VR, Wang J, Dow JAT. 2007 Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. *Nat. Genet.* **39**, 715–720. (doi:10.1038/ng2049)
38. Graveley BR *et al.* 2011 The developmental transcriptome of *Drosophila melanogaster*. *Nature* **471**, 473–479. (doi:10.1038/nature09715)
39. Brown JB *et al.* 2014 Diversity and dynamics of the *Drosophila* transcriptome. *Nature* **512**, 393–399. (doi:10.1038/nature12962)
40. Attrill H, Falls K, Goodman JL, Millburn GH, Antonazzo G, Rey AJ, Marygold SJ. 2016 FlyBase: establishing a Gene Group resource for *Drosophila melanogaster*. *Nucleic Acids Res.* **44**, D786–D792. (doi:10.1093/nar/gkv1046)
41. Morton DB, Truman JW. 1985 Steroid regulation of the peptide-mediated increase in cyclic GMP in the nervous system of the hawkmoth, *Manduca sexta*. *J. Comp. Physiol. A* **157**, 423–432. (doi:10.1007/BF00615142)
42. Baker JD, McNabb SL, Truman JW. 1999 The hormonal coordination of behavior and physiology at adult ecdysis in *Drosophila melanogaster*. *J. Exp. Biol.* **202**, 3037–3048.
43. Ben-Shahar Y, Leung H-T, Pak WL, Sokolowski MB, Robinson GE. 2003 cGMP-dependent changes in phototaxis: a possible role for the foraging gene in honey bee division of labor. *J. Exp. Biol.* **206**, 2507–2515. (doi:10.1242/jeb.00442)
44. Müller U, Hildebrandt H. 2006 The nitric oxide/cGMP system in the antennal lobe of *Apis mellifera* is implicated in integrative processing of chemosensory stimuli. *Eur. J. Neurosci.* **7**, 2240–2248. (doi:10.1111/j.1460-9568.1995.tb00645.x)
45. Thamm M, Scheiner R. 2014 PKG in honey bees: spatial expression, *Amfor* gene expression, sucrose responsiveness, and division of labor. *J. Comp. Neurol.* **522**, 1786–1799. (doi:10.1002/cne.23500)
46. Sellak H, Choi C-S, Dey NB, Lincoln TM. 2012 Transcriptional and post-transcriptional regulation of cGMP-dependent protein kinase (PKG-I): pathophysiological significance. *Cardiovas. Res.* **97**, 200–207. (doi:10.1093/cvr/cvs327)
47. Rüppell O, Pankiw T, Page Jr RE. 2004 Pleiotropy, epistasis and new QTL: the genetic architecture of honey bee foraging behavior. *J. Hered.* **95**, 481–491. (doi:10.1093/jhered/esh072)
48. de Belle JS, Sokolowski MB. 1987 Heredity of rover/sitter: alternative foraging strategies of *Drosophila melanogaster* larvae. *Heredity* **59**, 73–83. (doi:10.1038/hdy.1987.98)
49. de Belle JS, Sokolowski MB, Hilliker AJ. 1993 Genetic analysis of the *foraging* microregion of *Drosophila melanogaster*. *Genome* **36**, 94–101. (doi:10.1139/g93-013)
50. Wray GA. 2007 The evolutionary significance of *cis*-regulatory mutations. *Nat. Rev. Genet.* **8**, 208–216. (doi:10.1038/nrg2063)
51. Yan H, Simola DF, Bonasio R, Liebig J, Berger SL, Reinberg D. 2014 Eusocial insects as emerging models for behavioural epigenetics. *Nat. Rev. Genet.* **15**, 677–688. (doi:10.1038/nrg3787)

52. Kalderon D, Rubin GM. 1989 cGMP-dependent protein kinases genes in *Drosophila*. *J. Biol. Chem.* **264**, 10 738–10 748.
53. Stapleton M *et al.* 2002 The *Drosophila* gene collection: identification of putative full-length cDNAs for 70% of *D. melanogaster* genes. *Genome Res.* **12**, 1294–1300. (doi:10.1101/gr.269102)
54. Wang J, Jemielity S, Uva P, Wurm Y, Gräff J, Keller L. 2007 An annotated cDNA library and microarray for large-scale gene-expression studies in the ant *Solenopsis invicta*. *Genome Biol.* **8**, R9. (doi:10.1186/gb-2007-8-1-r9)
55. Tobback J, Verlinden H, Vuerinckx K, Vleugels R, Vanden Broeck J, Huybrechts R. 2013 Developmental- and food-dependent foraging transcript levels in the desert locust. *Insect Sci.* **20**, 679–688. (doi:10.1111/1744-7917.12012)
56. Tobback J, Heylen K, Arckens L, Tobback J, Billen J, Gobin B, Huybrechts R. 2008 Cloning and expression of PKG, a candidate foraging regulating gene in *Vespula vulgaris*. *Anim. Biol.* **58**, 341–351. (doi:10.1163/157075608X383665)
57. Kodaira Y, Ohtsuki H, Yokoyama J, Kawata M. 2009 Size-dependent foraging gene expression and behavioral caste differentiation in *Bombus ignitus*. *BMC Res. Notes.* **2**, 184. (doi:10.1186/1756-0500-2-184)
58. Thomas ML, Elgar MA. 2003 Colony size affects division of labour in the ponerine ant *Rhytidoponera metallica*. *Naturwissenschaften* **90**, 88–92.
59. Edwards DP, Arauco R, Hassall M, Sutherland WJ, Chamberlain K, Wadhams LJ, Yu DW. 2007 Protection in an ant–plant mutualism: an adaptation or a sensory trap? *Anim. Behav.* **74**, 377–385. (doi:10.1016/j.anbehav.2006.07.022)
60. Weber JN, Peterson BK, Hoekstra HE. 2013 Discrete genetic modules are responsible for complex burrow evolution in *Peromyscus* mice. *Nature* **493**, 402–405. (doi:10.1038/nature11816)
61. Edelsparre AH, Vesterberg A, Lim JH, Anwari M, Fitzpatrick MJ. 2014 Alleles underlying larval foraging behaviour influence adult dispersal in nature. *Ecol. Lett.* **17**, 333–339. (doi:10.1111/ele.12234)