PROCEEDINGS B

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Cite this article: Bell EF, Wyatt CDR, Taylor D, Radford AN, Sumner S. 2026 Limits to behavioural plasticity in tropical paper wasps. *Proc. R. Soc. B* **293**: 20260252.
https://doi.org/10.1098/rspb.2026.0252

Received: 30 January 2026
Accepted: 11 March 2026

Subject Category:
Behaviour

Subject Areas:
behaviour, genomics

Keywords:
caste, paper wasps, *Polistes canadensis*, gene expression, sociality

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.8435155.

THE ROYAL SOCIETY
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Limits to behavioural plasticity in tropical paper wasps

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Cooperative breeders are defined by a division of reproductive labour among group members that can respond flexibly to changing conditions via phenotypic plasticity. But such plasticity can be costly and is likely to be influenced by previous experience. *Polistes* paper wasps live in small societies where non-reproductive (worker) and reproductive (queen) individuals can switch roles throughout adulthood. Such plasticity in reproductive roles positions them as important models for social evolution. However, the limits of their individual-level plasticity have not been fully tested. We experimentally forced queens and workers of *Polistes canadensis* to nest alone, requiring them to express reproductive and non-reproductive characteristics simultaneously. At the behavioural level, although all isolated wasps laid eggs and foraged, ex-queens were less good than ex-workers at brood rearing. We attribute this to subtle differences in neuroplasticity. While brain transcription of both ex-queens and ex-workers changed in response to the manipulation, converging on a state intermediate to that of control queens and workers, ex-queens did not upregulate some key molecular processes required for expression of an effective worker phenotype. Our findings demonstrate that both *Polistes* queens and workers can exhibit behavioural, physiological and molecular plasticity, but reveal how previous life history can impose limits to that plasticity.

1. Introduction

Social groups typically exhibit a division of reproductive labour, with one or a few breeders and several or many non-reproductive helpers that cooperatively rear related offspring [1,2]. These different individual strategies are alternative phenotypic expressions of the same genome: helpers and breeders are phenotypes that are conditionally expressed in response to the environment [3]. In the simplest societies—primitively eusocial bees and wasps, as well as cooperatively breeding mammals and birds—individuals exhibit complete phenotypic plasticity (full 'totipotency') [4,5]. If conditions change—e.g. through altered group composition, external opportunities or constraints—the balance between individuals that breed and those that help can change within the lifetime of the group—and even within that of an individual. Thus, plasticity can be an essential component of group living, ensuring that individuals maximize fitness and adapt to unpredictable environments.

In simple social groups, individuals typically express plasticity in their roles, switching from being non-reproductive to reproductive and the reverse. If a reproductive individual in a group dies or disappears, then a non-reproductive groupmate often takes their place in both invertebrate and vertebrate species [6,7]. Similarly, non-reproductive individuals may choose to leave the group to start another group (either alone or with others), or they may move to join another existing group where they become the reproductive [7,8]. However, there can also be a switch from a reproductive to a non-reproductive role. In cooperatively breeding birds and mammals, a former dominant breeding individual may be evicted or usurped and take up a non-breeding helping role either in the same or a different group [7]. In *Polistes* paper wasps, a group of hopeful reproductives will compete over a short period to be the reproductive (queen); those that fail may regress their ovaries and adopt a subordinate, non-reproductive role (worker) [9], and reversals of the dominant reproductive are apparent from brood maternity analyses [10,11]. Reproductives at any colony stage may need to switch to worker behaviour if the nest is destroyed by parasites or predators [9,10,12,13] or their worker force is lost; the mortality rate for workers is high, especially in the tropics [14,15]. Plasticity thus affords individuals autonomy over their reproductive strategy, but retaining such plasticity may be costly, especially if specialization in a specific role is beneficial for inclusive fitness [16,17]. There may also be constraints on plasticity such that not all individuals express the optimum phenotype in response to a heterogeneous set of environmental conditions [16,18]. Determining what constrains plasticity, how such limits are imposed and why they evolve is a key part of the puzzle in understanding sociality.

Individual-level plasticity in simple societies is known to be influenced by age, order of arrival/emergence as adults, size and previous life experience. For example, behavioural experiments with paper wasps (*Polistes*) and hover wasps (Stenogastrinae) show that age strongly determines who succeeds the queen if she dies, with the oldest worker typically taking on the role, often with little conflict (reviewed in [19]). Such plasticity is also reflected at the molecular level through changes in brain transcription [20,21]. Similarly, age and/or size determine which non-reproductive succeeds the breeder in primitively social halictid and carpenter bees [22,23]. However, most research focuses on the switch from a non-reproductive to reproductive role; few studies have explored the converse switch, from reproductive to non-reproductive. Exceptions include work on post-reproductive females of the subsocial bee *Ceratina calcarata*, which showed reduced brain size in the mushroom body calyces and antennal lobes following a switch from reproductive to a non-reproductive role [24]. Similarly, gamergates in queenless ant societies that are forced to switch back to being non-reproductive workers show plasticity in brain size, reverting to a larger brain volume [25]. Thus, individuals clearly have the capacity for reproductive plasticity, but it is unknown to what extent reproductives and non-reproductives are equally plastic. There is good reason to hypothesize that reproductives should express less phenotypic plasticity than non-reproductives, given the costs of plasticity, the benefits of being a breeder and the role of caste specialization (canalization) in social evolution. Testing whether previous life experience (as a reproductive or non-reproductive) affects plasticity matters if we want a comprehensive understanding of alternative strategies in group-living animals: this is because stochastic environmental, social and disease-related events alter the conditions and opportunities for *all* group members, not just the non-reproductives. Moreover, losses of individual-level plasticity are signatures of caste evolution in more complex societies where workers have lost the ability to mate and/or lay eggs and where queens have lost the ability to forage and rear brood [3,5,17]. Thus, determining whether there are signs of this in the simplest societies may provide insights into the evolution of complex sociality [5].

In this study, we experimentally explore the limits of individual plasticity in behaviour and reproduction exhibited by queens and workers of the neotropical paper wasp, *Polistes canadensis*, and examine the molecular basis of such plasticity using brain transcriptomics. *P. canadensis* live in small groups of females (typically 10–100 individuals); colonies are typically founded by groups of females (mean 7.5 females; $n = 576$ colonies [13]). Established colonies have a single long-lived breeder at any one time [26]. Despite this, females can exploit reproductive opportunities throughout their life because there are no seasonal limits to breeding cycles [27–29], and neither age nor size plays a role in determining who becomes the new breeder when the queen dies. Moreover, reproductive investment for any individual is not limited to a single group, as a large proportion of females (30–50%) spend time on neighbouring nests where they help to raise distantly related brood [8,14]. Mechanisms of plasticity among group members are reflected in the brain through gene expression [30–32]. A genome sequence for *P. canadensis* [31], along with a body of comparative transcriptome data for *Polistes* (including *P. canadensis*), makes this species a highly tractable model for exploring limits and mechanisms of behavioural plasticity [30,33].

Using *P. canadensis*, we tested the null hypothesis that previous life experience as a reproductive versus a non-reproductive imposes no specific limits on behavioural plasticity, while accounting for other variables such as size and age. To do this, we forced queens and workers to exhibit the characteristics of their counterpart phenotype by becoming a lone female. Single female nests occur naturally during the foundress period: Pickering [13] reported that 17–28% of foundress colonies had a single female, depending on the time of year (data extracted from figure 10-5 in [13]; $n = 576$ colonies [13]). If the null hypothesis is true, we expect to find no differences in the behaviour, reproduction and/or underlying molecular regulatory machinery of isolated individuals, whether they were previously a queen or a worker. Alternatively, if there are limits to plasticity regarding previous reproductive life, we expected ex-queens to be less successful in accommodating the full repertoire of behaviours required (e.g. foraging, brood care) compared with workers, and there to be signs of the mechanisms responsible for limiting plasticity in the underlying molecular regulatory machinery.

2. Methods

(a) Overview

Although it is experimentally simple to induce a *Polistes* worker to become a queen in the presence of her nestmates by removing the existing queen [6,19,29,34], it is almost impossible to induce a *Polistes* queen to become a worker in the presence of her own workers [12]. To overcome this problem, we tested the behavioural plasticity of focal queens and workers by removing all their nestmates, thereby leaving one isolated wasp (an 'ex-queen' or 'ex-worker') on her nest; this compels her to perform simultaneously all reproductive *and* non-reproductive roles required to realize fitness (figure 1). Wasps removed at manipulation were preserved to obtain comparative data on the reproductive physiology and brain gene expression profiles of 'typical queens' and 'typical workers' on unmanipulated nests. Following manipulation, we observed the focal wasps for a maximum of two weeks to compare levels of foraging activity. Occurrences of egg laying and successful rearing of brood to the next developmental stage were determined from detailed mapping of nest cartons over the same period. At the end of the experiment, we collected the focal females, dissected them to determine whether their ovaries were developed and if they were mated and extracted RNA from their brains for gene expression analysis.

(b) Experimental set-up

We conducted the experiment during May–July 2012 and May–July 2013 within the Province of Colón in the Republic of Panamá at two sites: Punta Galeta (9°24′08.49″N, 79°52′19.46″W) and Fort Sherman (9°21′42.62″N, 79°56′58.34″W). We used a combination of pre-worker emergence nests (*n* = 30) and post-worker emergence nests (*n* = 35) to sample ex-queens and ex-workers across a spectrum of ages and levels of relatedness [26]. Ex-queens on all types of nests are likely to be in excess of 50 days old [27]. Ex-workers on pre-worker emergence nests were co-foundresses of their colony; co-foundresses are typically sisters, and so pre-emergence ex-workers are also expected to be in excess of 50 days old [26]. Ex-workers on post-emergence nests were first-generation individuals, offspring of the existing queen and approximately 15 days old. This age is considered the point at which workers can develop their ovaries and lay eggs should the opportunity arise [27]. Thus, if age influences plasticity, we would expect ex-workers on post-emergence nests to differ from ex-workers on pre-emergence nests. As a tropical species, the effects of seasonality are limited in *P. canadensis* compared with their temperate counterparts. Colonies exist at all stages of development at any one time, such that males are always present in the population; this means that a female has the potential to become a queen irrespective of when she emerges [27–29].

Pre-manipulation, we individually marked females on all nests using 'POSCA Pens' or 'queen bee tags' and used hand-held callipers to obtain a measurement of their wings (in cm) as a proxy for wasp size. We monitored nests daily with multiple censuses to identify the queen and to quantify foraging effort by workers. Nests were visited approximately every 45 min between 10.00 and 17.00 on sunny days for a minimum of 4 days to ascertain which individuals were present (electronic supplementary material, dataset S1: table S1.1). Wasps need to leave the nest to forage and collect nesting material, so the relative time spent away from nests provides a good indication of foraging rate [27] (figure 1). We estimated foraging effort as a proportion of censuses in this period when a female was not present on a nest. Workers targeted for the study were wasps that spent approximately 40% of their time off the nest [32]. We identified queens as the one wasp per nest that was present >90% of the time and confirmed this through the observation of egg laying. Following phenotype identification, we randomly assigned each nest to one of the two experimental treatments: ex-queens (*n* = 33) or ex-workers (*n* = 32), with an equal representation of colony stages in each treatment (figure 1).

Once assigned, we removed all individuals except the focal female from each nest and stored their bodies in 70% ethanol and heads in RNAlater. We drew a full brood map of each nest on the day of manipulation (Day 0) and on days 4, 7, 10 and 14 after manipulation (electronic supplementary material, dataset S1: table S1.2). For all brood maps, we counted the total number of cells to give the overall nest size and checked each cell for the presence of brood parasites (evident from the pupal cap), which may influence the behaviour of focal wasps. Following manipulation, we collected observational data for up to two weeks. Some focal wasps disappeared before the end of the planned two-week manipulation period (ex-queens: *n* = 22, ex-workers: *n* = 16, range of days remaining post-manipulation 4–14 days); in these nests, data collection stopped when the individual had not been seen for 48 h (electronic supplementary material, dataset S1: table S1.1). Any emerging wasps or joining females were removed to ensure that focal females remained alone. At the end of the post-manipulation period, we collected the focal female, stored her body in 70% ethanol and her head in RNAlater; this constituted the end of the experimental trial (figure 1).

(c) Foraging behaviour

Post-manipulation, we visited nests daily every 45 min between 10.00 and 17.00 to determine when the focal individual was present. From this, we calculated the proportion of nest censuses when the focal individual was absent (presumed foraging; electronic supplementary material, dataset S1: table S1.1). We used a binomial generalized linear mixed model (GLMM) in R v. 3.0.2 [35] to compare ex-queens and ex-workers in their foraging time. The GLMM included year, individual female identity and experimental day as random effects, with wasp phenotype (ex-queen or ex-worker), wasp size, nest size, nest stage (pre-emergence, post-emergence) and presence of potential parasites as fixed effects. A full model summary is provided in the electronic supplementary material, table S1. We used Wilcoxon signed-rank tests to compare the proportion of time that focal wasps spent on the nest pre- and post-manipulation, with separate tests for ex-queens and ex-workers and using the mean value for a given wasp from the different observation days.

(d) Egg-laying and ovary development

To compare the differences in egg-laying capabilities of ex-queens and ex-workers, we estimated whether each focal female had laid eggs in the nest carton post-manipulation. Eggs have a fast development time (approx. 4 days) to the next brood stage, so if more than 4 days had elapsed and a cell still had an egg in it then this was recorded as a 'new' egg. Likewise, the appearance of an egg following a different brood stage indicated a 'new' egg. Evidence of egg laying was determined as a binary count per

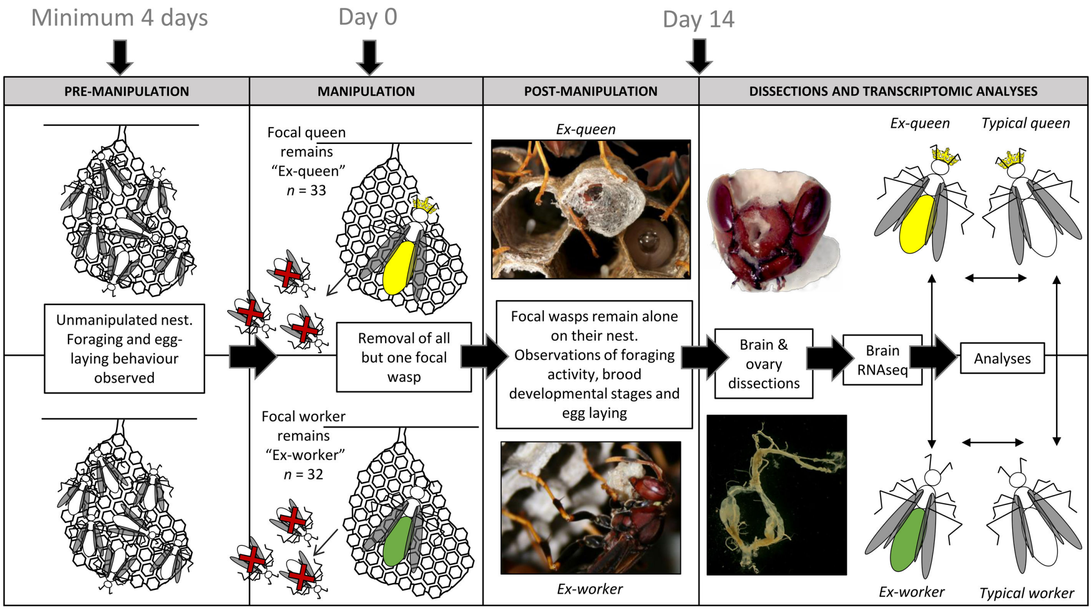


Figure 1. Schematic showing the methodological approach of the study. Foraging and egg-laying behaviour were observed on unmanipulated nests for a minimum of 4 days prior to manipulation. All but one focal wasp—either an 'ex-queen' ($n = 33$ total: $n = 15$ pre-emergence and $n = 18$ post-emergence) or an 'ex-worker' ($n = 32$ total: $n = 15$ pre-emergence and $n = 17$ post-emergence)—were removed from the nest on the day of manipulation (Day 0). Post-manipulation, the focal wasp remained alone on the nest (any emerging wasps or joiners were removed) and observation of foraging activity, egg laying and brood developmental stages was made. After 14 days, all focal wasps remaining were collected, heads were stored in RNAlater and bodies were stored in 70% ethanol. Measures of reproductive physiology were taken to determine the egg-laying status of females, and brain tissue was used for transcriptomic analyses of gene expression. Comparisons of ovarian development, foraging rates and brood development were made between ex-workers and ex-queens and typical counterparts (i.e. 'typical-workers' and 'typical-queens' from pre-manipulation nests). Brain transcriptomes were compared between ex-queens and ex-workers and between typical-queens and typical-workers.

cell (0 for no evidence of egg laying and 1 for evidence of egg laying) within nests across the manipulation period. An estimate of egg-laying evidence per focal female was calculated by log-transforming the data to ensure comparability between nests of different sizes and then comparing between phenotypes using a Mann–Whitney–Wilcoxon test (electronic supplementary material, dataset S1: table S1.2).

We dissected focal wasps to count the total number of oocytes in one ovariole as an indicator of the potential fecundity of females post-manipulation [28] (electronic supplementary material, dataset S1: table S1.3). We also dissected a subset of females collected from the same nests on manipulation day to create an ovarian profile for 'typical' queens ($n = 21$) and workers ($n = 41$) with which ex-queens and ex-workers could be compared. We used separate Mann–Whitney–Wilcoxon tests to compare the number of oocytes on an ovariole for typical versus ex-queens and for typical versus ex-workers.

(e) Brood survival

The unenveloped nest combs of *Polistes* wasps enable tracking of brood development and survival within individual nest cells over time. The brood map drawn on Day 0 acted as a proxy for the number of cells and brood within the nest, prior to manipulation (electronic supplementary material, dataset S1: table S1.2). If focal wasps disappeared before the end of the two-week manipulation period, the final brood map was drawn when the focal female had not been observed for 48 h. The appearance of a newly laid egg and the development of brood within cells were assessed visually; brood were scored as small larvae, medium larvae or large larvae (electronic supplementary material, figure S1). The disappearance of larvae could be because they died or were cannibalized to feed other brood: we consider either case to indicate lack of brood care. We analysed differences in the survival of small, medium and large larvae between nests of ex-queens and ex-workers using separate log-rank Mantel–Cox tests for each larval size, whereby survival means progression to the next developmental stage (electronic supplementary material, dataset S1: tables S1.4-S1.6).

(f) Transcriptome sequencing and gene expression analyses

We sequenced individual brain RNA samples from 14 focal wasps (ex-queens: $n = 7$, ex-workers: $n = 7$). We also sequenced RNA from 11 typical queens and 15 typical workers. This larger sample size of typical phenotypes included individual-level sequencing ($n = 5$ queens; $n = 6$ workers) and pools ($n = 3$ pools of queens; $n = 3$ pools of workers), where each pool comprised three individuals (see electronic supplementary material, dataset S2: table S2.1). Typical phenotypes also included individuals from foundress colonies for comparison (for details see electronic supplementary material, dataset S2: table S2.1). We extracted total RNA from single brains using the Qiagen RNeasy® column kit according to the manufacturer's instructions. The yield and quality of RNA were initially assessed using the A280/A260 ratio (NanoDrop 2000). Further quality assessments on the concentration and quality of extracted RNA were made using an Agilent 2100 bioanalyzer (Agilent Technologies). Samples that passed quality control were then submitted for sequencing. We constructed RNA sequencing libraries at the University of Bristol Genomics facility using the Illumina TruSeq stranded RNA LT kit RiboZero. A total of 30 libraries were constructed and five samples were multiplexed per lane. Each lane yielded up to 250M reads, and samples were randomly distributed across five lanes to give approximately 50M reads per sample. The total number of reads achieved per sample ranged from 23.8 to 68.5M reads. We pre-processed the reads using Trimmomatic (v. 3, https://trimmomatic.com/) to remove Illumina adaptors and low-quality reads. After this, samples had between 16.0 and 54.6M reads per sample. Reads were deposited at GEO (GSE169493, https://www.omicsdi.org/dataset/geo/GSE169493). All bioinformatics analyses were performed on the computer cluster at University College London (Myriad).

We estimated gene expression using a default Nextflow [36] (v. 20.04.1) pipeline from nf-core [37] ('rna-seq' pipeline (v. 1.4.2)) [36] using STAR [38] (v. 2.6.1d) to map reads to the publicly available *P. canadensis* genome (GCF_001313835.1). Quality trimming was performed with TrimGalore (v. 0.6.4, https://github.com/FelixKrueger/TrimGalore), and raw read counts were calculated using featureCounts [39] (v. 1.6.4; electronic supplementary material, dataset S2: table S2.3). Prior to running the Nextflow pipeline, we pre-filtered the raw reads by mapping to the *P. canadensis* genome (GCF_001313835.1) using Tophat2 [40]. R (v. 3.6.2) [35] was used for the calculation of differentially expressed genes (DEGs) using the DESeq2 package (v. 1.26.0, https://bioconductor.org/packages/release/bioc/html/DESeq2.html; electronic supplementary material, dataset S2: tables S2.5–S2.8), along with the categorization of queen/worker, ex-queen/ex-worker (electronic supplementary material, dataset S2: table S2.2). We calculated normalized gene expression using DESeq2 (counts(dds, normalized = TRUE)), with further scaling to values 0–1 shown in the electronic supplementary material, dataset S2: table S2.9.

We compared transcription across 55 brain RNA-seq samples from ex-queens, ex-workers, typical queens and typical workers (electronic supplementary material, dataset S2: table S2.1). However, the foundress samples differed significantly from the pre- and post-emergence nests and so were removed from the remaining analyses (see electronic supplementary material for results and discussion of these data). The remaining samples included 37 individuals sequenced as 28 samples (ex-queens: $n = 7$, ex-workers: $n = 7$, typical queens: $n = 8$ individuals as 5 samples and typical workers: $n = 15$ individuals as 9 samples).

We performed principal component analysis (PCA) using 'prcomp' in R (v. 3.6.2) [35] with default settings. Gene ontology (GO) assignment of genes was generated using 'excon' v. 1.0.1 [26] and GO enrichment was performed using a custom wrapper (https://github.com/chriswyatt1/chopgo [41]) to run TopGO (v. 2.48.0, https://bioconductor.org/packages/release/bioc/html/topGO.html) with multiple statistical tests performed (two-sided tests, using either classic fisher, holm, Hochberg, Bonferroni, FDR (false discovery rate) and none (no FDR)) (electronic supplementary material, dataset S2: tables S2.10–S2.11). Additionally, we explored putative key genes, expression of which would be expected to change in focal wasps for effective worker-like and queen-like behaviour. To do this, we compared the residuals of scaled gene expression in ex-queens and ex-workers for the most highly differentially expressed worker-biased and queen-biased genes found among typical phenotypes. This enabled us to explore the extent to which focal wasps have upregulated or downregulated critical worker-biased and queen-biased genes (electronic supplementary material, dataset S2: table S2.9).

3. Results and discussion

(a) Ex-queens and ex-workers exhibit plasticity in foraging and egg laying but differ in brood-rearing success

All focal wasps ($n = 65$) changed phenotype in response to our experimental manipulation. After controlling for the negative effect of parasite presence on time spent off the nest (electronic supplementary material, table S1), ex-queens and ex-workers spent similar amounts of time probably foraging (GLMM: $Z = -1.599$, $p = 0.110$). Compared with pre-manipulation, ex-queens showed an increase in the proportion of time that they spent foraging (Wilcoxon signed-rank test: $W = 258$, $n = 33$, $p = 0.002$; figure 2a). By contrast, ex-workers decreased the proportion of time that they spent foraging compared with pre-manipulation ($W = -488$, $n = 32$, $p < 0.001$; figure 2a). Some ex-queens and ex-workers were not recorded foraging during the censuses; this is likely to be a result of limited sampling rather than any reflection on their foraging behaviour (see electronic supplementary material) as it does not preclude the likelihood that they foraged outside of the census periods. All focal wasps laid eggs: there was no significant difference between ex-queens and ex-workers in the log-estimated numbers of eggs laid (Mann–Whitney test: $U = 162$, $n_{\text{ex-queens}} = 18$, $n_{\text{ex-workers}} = 20$, $p = 0.606$; figure 2b). There was no significant difference between typical queens (i.e. those from pre-manipulated nests) and ex-queens in the number of oocytes in any single ovariole in their reproductive tracts ($W = 221$, $n_{\text{typical}} = 22$, $n_{\text{ex}} = 21$, $p = 0.841$). However, ex-workers had a greater number of oocytes in any single ovariole than did typical workers on pre-manipulated nests ($W = 132$, $n_{\text{typical}} = 41$, $n_{\text{ex}} = 20$, $p < 0.001$; figure 2c). Thus, both ex-queens and ex-workers exhibited behavioural and physiological plasticity: ex-queens could forage to provide for their brood, while ex-workers could realize direct fitness opportunities by producing offspring. These results are in line with our null hypothesis that both phenotypes express the required plasticity in foraging and reproductive behaviours for their new circumstances: as sole occupants of their natal nest, focal wasps invested similar foraging effort and had equal egg-laying ability, irrespective of their previous role.

Despite this behavioural and physiological plasticity, not all females were equally successful at rearing brood. The survivorship (to the next developmental stage) of brood raised by ex-queens was significantly lower than brood raised by ex-workers for both small larvae (Mantel–Cox: $X^2 = 12.39$, $d.f. = 1$, $p < 0.001$; figure 2d) and large larvae ($X^2 = 23.41$, $d.f. = 1$, $p < 0.001$; figure 2e) but not significantly different for medium larvae ($X^2 = 0.003$, $d.f. = 1$, $p = 0.959$). Removing all wasps except one from a nest inevitably results in a large proportion of the brood disappearing because a single female cannot compensate for the loss of a whole colony's workforce. We therefore expected females to lose brood. Brood cannibalism is commonly observed in social

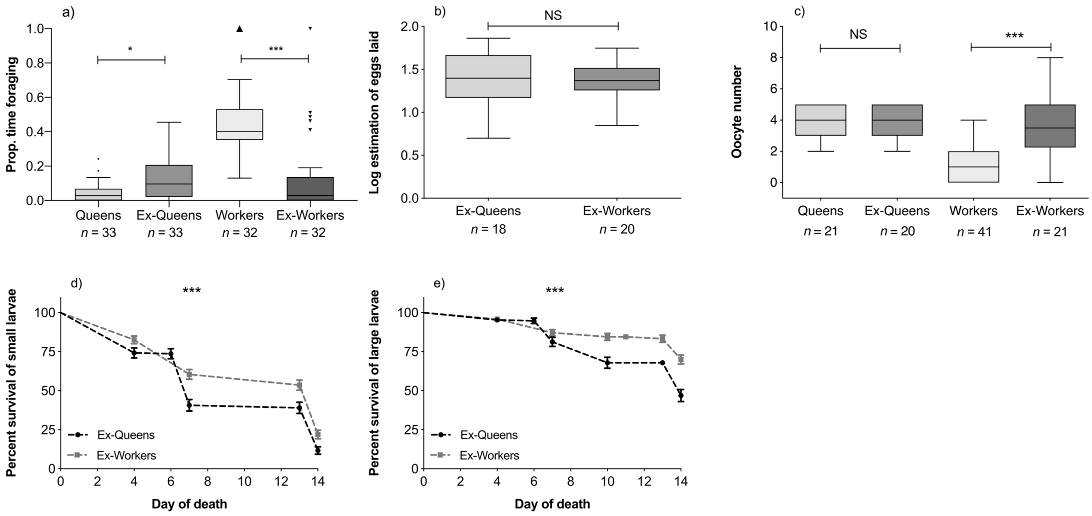


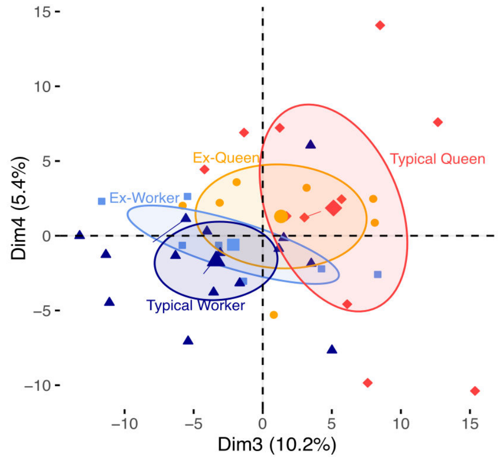
Figure 2. Comparisons of phenotypic traits for unmanipulated (queens and workers) and manipulated (ex-queens and ex-workers) individuals. (a) Both ex-queens and ex-workers showed a significant change in foraging activity after manipulation, such that the foraging efforts of ex-queens and ex-workers were not significantly different. (b) Ex-queens and ex-workers invested similarly in egg laying. (c) Ex-queens did not differ from unmanipulated (typical) queens in their oocyte number, whereas ex-workers had a larger number of oocytes than typical workers. (d) Survivorship of small larvae was significantly lower in nests with ex-queens ($n = 126$) than in nests with ex-workers ($n = 153$). (e) Survivorship of large larvae was significantly lower in nests with ex-queens ($n = 147$) than in nests with ex-workers ($n = 176$). For (a–c), sample sizes (n, number of wasps) are given on the plots. Boxplots show the median and extend to the 25th and 75th percentiles, and whiskers are shown with a maximum of 1.5x interquartile range. Significance values for pairwise comparisons: NS = non-significant, $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$. For (d) and (e), means and s.e. are reported.

insects, where typically smaller larvae that have received less investment will be sacrificed and fed to larger larvae; larger larvae have already received significant investment and will soon provide new nestmates that are likely to adopt worker roles. This pattern is evident for brood reared by both ex-queens and ex-workers: around 25% of small brood survived the full two-week experimental period, compared with 50–75% of large larvae. At the start of the experiment (Day 0), ex-queen and ex-worker nests had similar representations of each brood stage (t-tests: all t-statistics < 1.94; all $p > 0.1$). Survivorship of brood reared on ex-queen and ex-worker nests only started to diverge one week after the manipulation, with a steep drop-off in brood survival for larvae in the nests of ex-queens compared with ex-workers (figure 2); seven days may represent the time for which larvae can survive with little feeding or care.

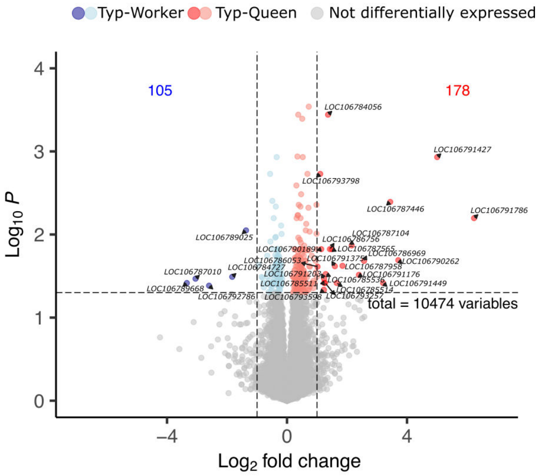
We interpret these findings as evidence that previous social status—as a reproductive—affects the ability of a wasp to rear brood successfully. Other possible explanations for the difference in brood-rearing abilities were controlled for by our experimental design. For example, fitness payoffs are unlikely to explain any differences in brood-rearing effort or motivation: ex-queens are likely to be raising offspring (relatedness = 0.5), while ex-workers are either raising full sisters ($r = 0.75$, in the post-emergence colonies) or nieces ($r = 0.375$, in the pre-emergence colonies) [26]. Likewise, age cannot explain these results since our experimental design using a combination of nest stages ensured that both ex-workers and ex-queens were from a range of overlapping ages. Using a mix of nest stages also provided a direct test of any age effect by including young ex-workers (from post-emergence nests, *ca* 15 days old) and old ex-workers (on pre-emergence nests, at least 60 days old; see §2): nest stage (and wasp size) had no significant effects in our models (electronic supplementary material, table S1).

(b) Neuro-transcription of ex-queens and ex-workers is intermediate to previous caste-specific social profiles but may explain their differences in behaviour

We found some evidence for global patterns of brain gene expression that reflect the observed shifts in behaviour after manipulation. Using PCA, typical queens and workers were shown to have some differences in transcriptomic patterns, where principal components (PCs) 3 and 4 appear to represent caste differences with little overlap in distributions (figure 3a), as expected for *Polistes* [30,31,33]. PCs 1 and 2 do not appear related to caste (see electronic supplementary material, figure S2). Between typical queens and workers, we identified 283 DEGs, which were enriched for ribosomal and translation gene expression terms (adjusted p-value < 0.05; figure 3b). Of these, 178 were queen-biased (i.e. upregulated in queens relative to workers) and 105 were worker-biased (electronic supplementary material, dataset S2: table S2.5). By contrast, ex-queens and ex-workers were transcriptomically similar, with PCA distributions that overlapped; their distributions also overlapped with those of typical queens and workers in PCs 3 and 4 (figure 3a). Only two genes (*pyruvate-* and *glucose-dehydrogenases*) were significantly differentially expressed between ex-queens and ex-workers (adjusted p-value <0.05; $\log_2$ fold change (FC) 0.48 and 0.50, respectively; electronic supplementary material, dataset S2: table S2.6; figure 3*c*). These results indicate that brain transcription in ex-queens and ex-workers occupies a state intermediate to that of typical queens and workers, reflecting the behavioural shifts of manipulated wasps from caste specialists (as typical social phenotypes) to generalists (as solitary females). However, ex-queens differed from typical queens (78 DEGs; electronic supplementary material, dataset S2: table S2.7) more than ex-workers did from typical workers (18 DEGs; electronic supplementary material, dataset S2: table S2.8; all DEGs after p-value a) Principal component 3 and 4



b) Typical Worker versus Typical Queen



c) Ex-Worker versus Ex-Queen

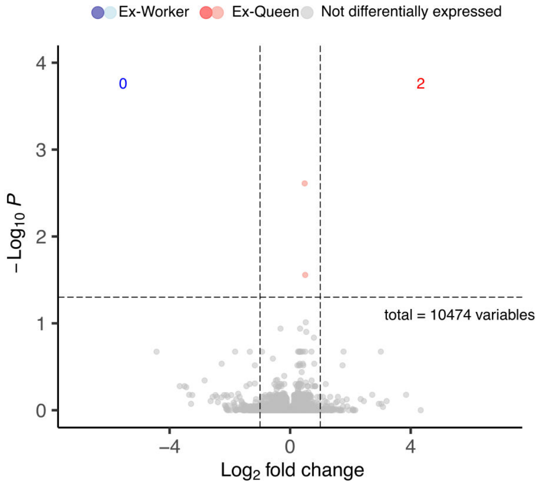


Figure 3. Brain gene expression comparisons of typical queens and typical workers and ex-queens and ex-workers**.** (a) PCA of gene expression in the four different phenotypes in the third (Dim 3) and fourth (Dim 4) PCs, with ellipsoids to show the general location of points: queens (red diamonds), ex-queens (orange circles), workers (blue triangles) and ex-workers (light blue squares). Each axis labels the variation represented as a percentage. PCs 1 and 2 are found in the electronic supplementary material, figure S2. (b) Volcano plot showing the DEGs in red (queen-upregulated) and blue (worker-upregulated), with genes not changing significantly in expression in grey, below 0.05 adjusted *p*-value (on *y* axis; denoted with dashed line). The *x*-axis shows the $\log_2$ FCs between the two castes (queen and worker). The vertical dotted lines denote the 1 and −1 $\log_2$ FC limits, with points greater than this in darker colours and with gene labels (LOC ids). (c) Shows the same plot as in (b), but with ex-queens (red) against ex-workers (blue), showing only two DEGs.

adjustments). This perhaps reflects the larger repertoire of new behaviours that ex-queens needed to accommodate (foraging, brood rearing, nest maintenance) compared to ex-workers (egg laying). Thus, at the global level, queens and workers are both able to express plasticity in neurotranscription, but the changes in gene regulation required by queens are larger than those required by workers when having to adopt additional roles.

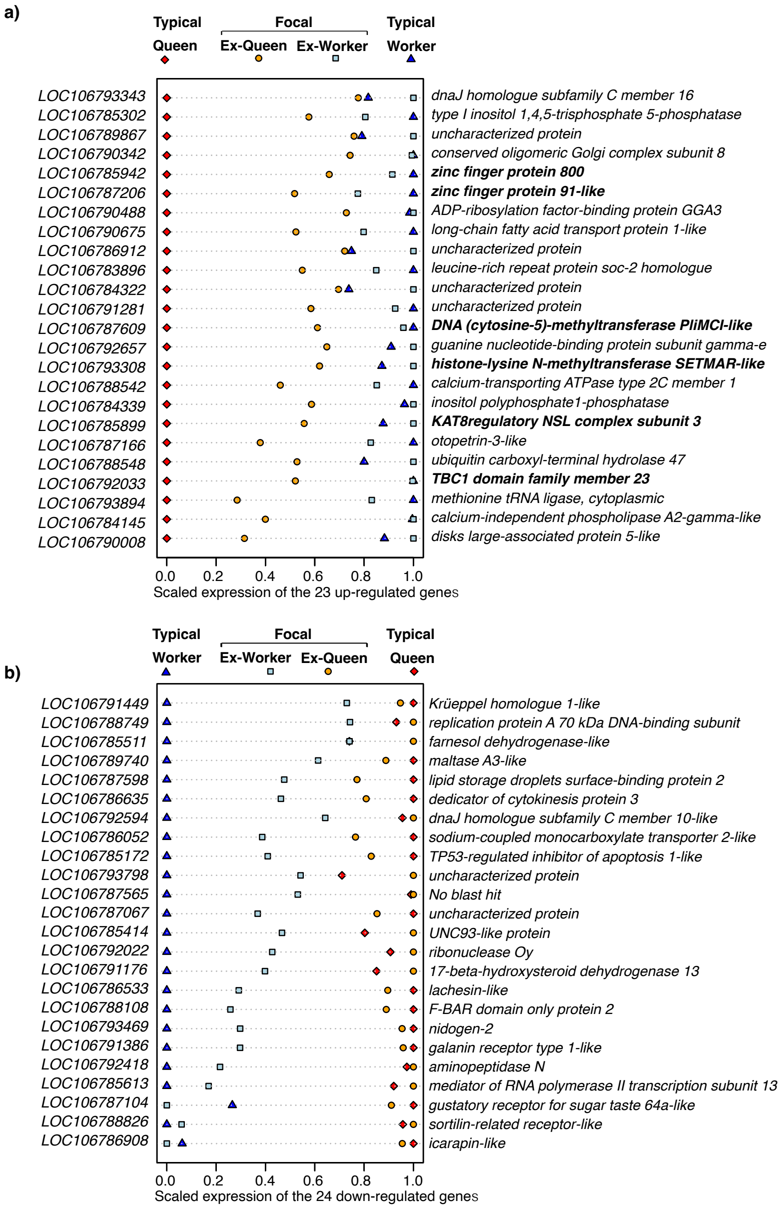


Figure 4. Residuals of scaled expression for critical caste-biased genes in ex-queens and ex-workers provide candidates for limiting ex-queen plasticity. (a) Critical upregulation genes are typical worker-biased genes that ex-workers do not downregulate (and thus are likely important for brood care and foraging) but that ex-queens fail to upregulate; figure shows scaled relative gene expression of these 23 genes (<0.05 adjusted p-value DESeq2; ≥0.2 scaled separation between the ex-queen average and ex-worker average: e.g. for a hypothetical gene, an ex-worker average at 0.8 and ex-queen average at 1, would have a difference of 0.2). (b) Critical downregulation genes are typical queen-biased genes that ex-workers do not upregulate (and thus are not essential for reproduction) but that ex-queens fail to downregulate. The figure shows scaled expression of these 24 genes (≥0.2 scaled expression difference; see main text for further details). In both plots, LOC identifiers correspond to individual genes/transcripts in the *P. canadensis* genome. Points on the graphs indicate the scaled averages of gene expression for each LOC; expression levels for the four phenotypes were scaled by setting queen expression to 0 and worker expression to 1 (and vice versa for panel b), with the corresponding expression in ex-queens and ex-workers. Blast hits are shown to the right of each gene line. Sample sizes: queen ($n = 5$), worker ($n = 9$), ex-queen ($n = 7$) and ex-worker ($n = 7$). Each point is an average of all individuals/samples in each of the phenotypes sub-categories. Bold formatted blast hits highlight genes of special interest mentioned within the main text.

Gene transcription in brain tissue reflects differences in phenotypes, including behavioural variants [20,42]; moreover, the effects of previous life experience on future reproductive strategies and behaviour can manifest as differences in gene expression [43]. We found some evidence of this and identified a small number of candidate genes that may explain the contrasting brood-rearing abilities in manipulated wasps. To explore the putative genes involved, we compared the residuals of scaled gene expression in ex-queens and ex-workers for genes most indicative of typical castes in social nests (i.e. the caste-biased genes identified in figure 3b). We were first interested in genes that we call 'critical upregulated genes'—genes that are putatively required to be *upregulated* for effective performance of worker-like behaviours (e.g. foraging and brood care), even in solitary females. We defined these as genes that were both: (i) worker-biased among typical castes—of which there were 105 (§3b; figure

3b); and (ii) that remained highly expressed in ex-workers (defined as >0.75 scaled expression)—of which there were 55 (in figure 4a). Of these 55 putatively critical genes, ex-queens did not upregulate 23 of them (electronic supplementary material, dataset S2: table S2.9; figure 4a). At least six of these 23 are putative candidates for involvement in regulatory processes such as transcription (e.g. *zinc finger proteins 91* (−0.20 $\log_2$FC) and 800 (−0.41 $\log_2$FC)), methylation and epigenetic modification (e.g. *histone-lysine N-methyltransferase* (−0.94 $\log_2$FC), TBC1 domain family member 23 (−0.19 $\log_2$FC)) and are known to be important in the regulation of social behaviour [44,45]. *Histone-lysine N-methyltransferase* may be involved in oogenesis [46] and is linked to the action of the key insect reproductive regulator, juvenile hormone [47]. Three genes were identified to have links with caste differentiation: *DNAJ homologue subfamily C* (a member of the heat shock protein family) (2 copies, LOC106793343 −0.32 $\log_2$FC and LOC106792004 + 0.20 $\log_2$FC), *otopetrin-3* (−0.51 $\log_2$FC) and *methionine tRNA ligase* (−0.20 $\log_2$FC). These genes are commonly caste-biased in social insects [48,49] and are potentially vital for the regulation of worker-like behaviours. A further two genes (*calcium-transporting ATPase type 2C* (−0.30 $\log_2$FC) and *KAT8 regulatory NSL complex subunit 3* (−0.18 $\log_2$FC)) have putative links with diapause pathways in insects and arthropods, and hence could have implications for caste evolution [47,50,51]. Together, the lack of upregulated genes associated with key regulatory processes, caste differentiation and diapause could explain why ex-queens performed less well than ex-workers in rearing brood.

Next, we considered genes putatively required to be *downregulated* by queens for effective performance as a solitary female: we refer to these as ‘critical downregulated genes’. We defined these as genes that were both: (i) queen-biased among typical castes (figure 3b), and that (ii) remained highly expressed in ex-queens, but that ex-workers did not upregulate (meaning that ex-workers putatively did not need to upregulate these genes to be an effective solitary female). Downregulation of queen-biased genes may be important for regulating specific pathways required for effective worker behaviours. Importantly, these genes are not likely to be critical for effective reproduction; otherwise, ex-workers would have upregulated them. Of the 178 queen-biased genes (electronic supplementary material, dataset S2: table S2.5; figure 3b), 27 remained highly expressed in ex-queens, and of these, 24 were not upregulated by ex-workers (electronic supplementary material, dataset S2: table S2.9; figure 4b). These 24 putatively ‘critical downregulated genes’ included key caste regulatory genes such as *Krüppel homologue 1-type* (3.20 $\log_2$FC), which is functionally critical in facilitating caste-appropriate behaviours in the queenless ant societies of *Harpegnathos saltator* [52]. But, interestingly, the genes with the greatest residual differences between expression in ex-queens and ex-workers were associated with sensory perception, cognition and neuronal function. For example, ex-queens did not downregulate *galanin*, which is involved in learning [48] as well as the regulation of juvenile hormone [49]; and *nidogen-2* (0.49 $\log_2$FC) and *sortilin* (0.28 $\log_2$FC), which are associated with nervous system patterning [53]. The lack of downregulated gustatory receptors may also indicate some deficiencies in the ability of ex-queens to forage and/or detect communication with the brood.

These results hint that there may be molecular mechanisms in the brain that are limiting plasticity in ex-queens. The lack of upregulated genes putatively associated with epigenetic processes and transcription, caste differentiation and diapause, and the lack of downregulated genes putatively involved in sensory perception, response to chemical cues and cognition may explain the difference in brood survival on nests with ex-queens and ex-workers. Although our analyses are limited to correlational patterns and thus do not demonstrate causation, these genes are prime candidates for future gene-manipulation work. That work could narrow down the functional effects of these genes in limiting caste plasticity and help to explain why ex-queens were less effective at brood rearing [54]. There may be additional mechanisms, such as differences in fat body content, metabolism and wing muscle efficiency, that limit the plasticity in former queens more than in former workers and that warrant further investigation.

4. Conclusion

For a cooperatively breeding social animal, behavioural plasticity is key because it affords the opportunity to exploit alternative reproductive opportunities throughout life. An extensive literature on vertebrates and invertebrates demonstrates such plasticity and its importance for non-reproductive group members in achieving reproductive status. Few studies, however, examine the plasticity of reproductive group members and their ability to behave like a helper after having experienced life as an established breeder, despite evidence that this does happen in nature [7,9,10,12,13]. The implicit assumption, therefore, was that plasticity is unlimited for all group members, irrespective of their previous life experience. Our study tested this null hypothesis in the cooperatively breeding wasp *P. canadensis* and found some support for it in terms of foraging behaviour, reproductive capacity and global neurotranscription. However, we also found evidence of limits to plasticity in ex-queens, which were less able to rear brood successfully compared with ex-workers; we also identified some of the putative molecular mechanisms that might be responsible for this difference in plasticity. Although less common than the switch from worker to queen, switches from queen to worker do occur naturally [6,9,10,12,13]. However, if there are costs to retaining plasticity, and if becoming a lone female after having been an established queen is rare in nature, there may have been little selection for retaining full plasticity. The sub-optimal ability of ex-queens to perform worker tasks may reflect this.

Detecting signs that plasticity is limited among queens of *Polistes* has broader significance to our understanding of the proximate and ultimate mechanisms of social evolution. The loss of phenotypic plasticity is a defining feature in the evolution of specialized queen and worker castes in social insects, with full caste plasticity limited to the developmental stage in the more complex, superorganismal societies [17,55]. Simple cooperative groups probably represent the ancestral state from which more complex social species evolved [5,55,56]. Thus, the subtle limits to behavioural plasticity observed in *P. canadensis* queens could be an example of ‘plasticity-first’ evolution, whereby an initial, environmentally induced phenotype can result in committed polyphenisms that have reduced environmental sensitivity; plasticity may precede and promote the evolutionary origins of complex adaptive traits [3,57]. It is also a plausible proximate mechanism for the evolution of division of labour in simple social groups [3]. Limited plasticity in breeders, but not non-breeders, of cooperative groups is also in line with the predictions of inclusive fitness theory, which explains the evolution of altruism in social groups [58,59]. In a cooperative matrilineal group, selection may be strong for the queen to commit to her role—assuring high indirect fitness for workers—and for the workers to retain plasticity so that they are fully able to exploit direct reproductive strategies opportunistically, through queen succession, usurpation or independent founding. Further studies on how phenotypic specialization may impose limits on the expression of plasticity in a range of cooperative breeding organisms are required at the behavioural and molecular levels to understand the mechanisms and evolution of sociality.

Ethics. The field research was conducted under Autoridad Nacional del Ambiente (ANAM) research permits SE/A-20-12 and SE/A-55-13.

Data accessibility. Data are provided as electronic supplementary materials. Sequence data are available here: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA716699.

Supplementary material is available online [60].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. E.F.B.: conceptualization, formal analysis, investigation, methodology, visualization, writing—original draft, writing—review and editing; C.D.R.W.: data curation, formal analysis, investigation, validation, visualization, writing—original draft, writing—review and editing; D.T.: data curation, investigation; A.N.R.: conceptualization, methodology, supervision, validation, writing—review and editing; S.S.: conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of Interest. We declare we have no competing interests.

Funding. The field research was conducted under Autoridad Nacional del Ambiente (ANAM) research permits SE/A-20-12 and SE/A-55-13. The research was funded by a NERC studentship and Smithsonian Tropical Research Institute pre-doctoral fellowship awarded to E.F.B.; S.S., D.T. and C.D.R.W. were supported by NERC grant NE/M012913/2. For the purpose of open access, the authors have applied a Creative Commons Attribution (CC BY) licence to any Author Accepted Manuscript version arising from this submission.

Acknowledgements. We thank the Smithsonian Tropical Research Institute, particularly W.T. Wcislo and D.M. Windsor, for supporting the fieldwork, the staff at Punta Galeta field station for their support, and L. Bellamy, D. Fabbro, S. Patalano and R. Southon for help in the field. Thanks also to A.F.G. Bourke and J. Bridle for useful comments on an earlier version of this work.

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