Vitellogenin expression in the ovaries of adult honeybee workers provides insights into the evolution of reproductive and social traits

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Running title: *Vitellogenin* in the ovaries of honeybee workers

Abstract

Social insects have two or more female castes that exhibit extreme differences in their reproductive capacity. The molecular basis of these differences is largely unknown. In honeybees the egg yolk protein vitellogenin (Vg), has acquired regulatory functions that go beyond reproduction, including the regulation of aging and task polyethism. Vg is synthesised in the fat body of both queens and workers and in the ovaries of queens. Here we show that *Vg* is also expressed in worker ovaries and that ovary activation and environmental cues (both social and diet) modulate *Vg* expression. Workers that are fed Royal Jelly have higher expression of *Vg* than those not fed Royal Jelly. Surprisingly, we find that *Vg* expression is not corelated with worker ovary activation, which suggests that Vg has non-reproductive functions in worker's ovaries. We also find that Royal Jelly in the diet disrupts the expression of likely regulators of Vg expression: *Vitellogenin receptor* (*VgR*), *Krüppel-homolog 1* (*Kr-h1*), *DNA methyltransferase 3* (*Dnmt3*) and the *Forkhead box O transcription factor* (*FoxO*). These results therefore support the 'reproductive grand plan hypothesis', which argues that genes that had reproductive functions in solitary ancestors have been co-opted for social functions in social insects.

Keywords: queen mandibular pheromone, honey bee, epigenetic, Juvenile hormone, reproduction-longevity tradeoff

1. Introduction

Social insects are characterised by two phenotypically divergent female castes: queens and workers (though there are often worker subcastes). Queens are highly reproductive and have an extraordinarily long lifespan, whereas workers are facultatively sterile and have a short lifespan [1]. Social insect queens are therefore exceptions to the normal situation in animals where there is a trade-off between lifetime reproduction and longevity [2,3]. Understanding of the molecular basis and evolutionary origins of the phenotypic divergence between queen and worker castes, despite their development from identical genomes, are major outstanding questions in sociobiology [4].

In honeybees (*Apis mellifera*) a key environmental difference between adult queens and workers is their diet. Adult queens are fed Royal Jelly, a nutritious food rich in carbohydrates, lipids and protein [5]. Royal Jelly is secreted from the hypopharyngeal and accessory glands on worker's heads [1]. In contrast, adult workers feed directly on stored honey and pollen, or on regurgitated crop contents provided by other workers [1]. Interestingly, if workers are forced to consume Royal Jelly in an artificial diet, they develop a more queen-like phenotype, including ovary activation and increased lifespan [6–8]. Thus, the effects of Royal Jelly seem to be associated with the gene networks that regulate reproduction and longevity [9].

Another key environmental cue for adult honeybee workers is their social environment. Queens release queen mandibular pheromone (QMP), which signals her presence to workers and supresses activation of their ovaries [10]. Furthermore, QMP regulates the expression of reproductive genes, such as *Vitellogenin* (*Vg*) [11], the primary yolk precursor protein in all oviparous animals including insects [12]. When workers are exposed to QMP, *Vg* expression is upregulated [13], which in turn inhibits juvenile hormone (JH) expression [14], thus establishing a dual repressor mechanism between Vg and JH [11].

In honeybees Vg not only acts as a reproductive protein, but is involved in additional social functions. In particular, Vg acts as a precursor for the synthesis of Royal Jelly by workers [15] suggesting that its function has changed from direct maternal care to alloparental care [16]. Vg is also involved in the regulation of behavioral maturation [17,18]. The Vg protein is synthesised in the fat body of most insects [19], released into the haemolymph, and taken up by other tissues via the Vitellogenin receptor (VgR) protein [20,21]. In honeybees *Vg* is expressed in the fat body of both queens and workers [22–24] and in the queen's ovaries [25], but its expression in worker ovaries has not yet been detected [24,25].

Why is Vg, whose primary function is as an egg yolk protein, involved in the regulation of reproduction and behavioural maturation of adult honeybee workers? The Reproductive Ground Plan hypothesis argues that the gene networks that were once involved in the regulation of reproduction in the ancestors of social insects have been coopted to facilitate the regulation of behavioural differences between queen and worker castes [26,27], and even between behavioral phenotypes within worker castes [28]. *Vg* is a gene that appears to have been co-opted in this way [29].

Here we investigate how ovary state and expression of *Vg* are affected by environmental cues that have opposite effects on ovary activation: diet (the presence or absence of Royal Jelly) and social environment (presence or absence of QMP). *A priori*, *Vg* expression is likely to be under environmental regulation by these two factors because its expression is associated with social functions in addition to its canonical reproductive function. If expression of *Vg* in the ovaries of adult workers is affected by these two environmental cues, then this would link a specific gene to the molecular mechanisms underlying caste-specific differences in reproductive capacity and longevity.

2. Material and methods

131
132 **(a) Biological material, cage setup and longevity**
133 We obtained age-matched worker honeybees of standard Australian commercial stock
134 (primarily *A. m. ligustica*) by incubating frames of emerging brood from two source colonies
135 overnight at 34.5 °C. Workers (n = 150) from each colony were randomly allocated to each of
136 four cages [30] (total eight cages) in a three-factor (colony plus the two treatments) factorial
137 design. Four of the cages contained half a queen mandibular pheromone strip (QMP^+) (Phero
138 Tech Inc. Canada), and the other four contained no QMP strip (QMP^-). Pollen and water were
139 provided *ad libitum*. In addition, four of the cages (2 QMP^+ and 2 QMP^-) were fed a Royal
140 Jelly diet of 50% honey:50% fresh Royal Jelly (stored frozen), whereas the other four cages (2
141 QMP^+ and 2 QMP^-) were only fed a Control diet of pure honey. Food was replenished when
142 necessary, and the number of dead workers recorded each day (supplementary table 1). Cages
143 were housed in an incubator at 34.5°C. At seven days of age workers were collected and
144 immediately frozen.
145 In a parallel experiment to assess longevity, two additional source colonies were
146 prepared as above (four cages per colony). Cages were kept under the same conditions.
147 However, the workers were not collected and instead the number of dead workers was recorded
148 daily until all workers had died.
149
150 **(b) Assessment of ovary activation**
151 We dissected and imaged the ovaries from 7-day-old workers under a dissecting
152 microscope (Leica M60). The paired ovaries of the workers were removed and snap frozen.
153 During the dissection the activation state of the ovaries was recorded on a three-point scale: 1
154 - deactivated (thin ovarioles, no visible oocytes), 2 - semi-activated (ridged ovarioles, with
155 oocytes at the vitellarium stage of development) and 3 - activated (mature, white oocytes) [10].
156
157 **(c) Expression analysis**
158 Ovaries were macerated in TRIzol (Invitrogen) and total RNA extracted with the
159 Direct-zol™ RNA Miniprep kit (Zymo Research) according to the manufacturer's instructions.
160 Each sample consisted of four pairs of non-activated ovaries (deactivated and semi-activated).
161 Individual ovaries were combined to obtain a sufficient amount of RNA. Activated ovaries
162 (figure 2 b-d) were not combined as there were a limited number available and because
163 individual ovaries yielded sufficient RNA. The samples were digested with the Turbo DNase
164 Kit (Thermo Fisher Scientific) to remove DNA. RNA concentration was then quantified using
165 a Qubit 2.0 Fluorometer system (Invitrogen). Each RNA sample was diluted to a final
166 concentration of 15 ng/µL with ultrapure water. We used 142.5 ng of each sample to synthesise
167 cDNA using the SuperScript™ III Reverse Transcriptase Kit (Invitrogen) with Oligo (dT)
168 primer. The cDNA was diluted to 2 ng/µL in ultrapure water.
169 To detect the expression of *Vg* and its receptor *VgR* [20,21] in the ovaries of workers
170 (see Table S2 for primer sequences and sources) we first performed conventional PCR. To
171 semi-independently validate *Vg* expression we utilised two sets of *Vg* primers, *Vg*P1 [31] and
172 *Vg*P2 [16], which target different exons within *Vg* (*Vg*P1: exon 3; *Vg*P2: spans exon 5 and 6).
173 PCR reactions were carried out on three different cDNA samples of Royal Jelly fed workers.
174 The cDNA (2 ng) was mixed with 0.2 µL TaqTi enzyme (Fisher Biotec Australia), 3.2 nmol
175 dNTPs, 1.25 pmol of each primer, buffer 1X, 50 nmol $MgCl_2$ in a final volume of 20 µL. The
176 following genes were used as positive controls: ribosomal protein 49 (*RP49*, also known as
177 *rpl32*) and elongation factor 1 alpha (*Ef1α*). The negative control gene was yellow fluorescent
178 protein (*YFP*), which is not present in honeybees. Primer sequences and sources are listed in
179 Table S2. The amplification cycles were: 95 °C for 2 min; 40 cycles of 95°C for 15 sec; 60 °C

for 15 sec; and 72 °C for 30 sec, 72°C for 5 min. RT-PCR products were ran on a 1% agarose gel and imaged using a Bio-Rad ChemiDoc Touch Imaging System.

We quantified the expression of *Vg* and *VgR* in the ovaries of adult workers with RT-qPCR. In addition, we quantified the expression of three genes that are potential regulators of *Vg* expression in the honeybee: *Krüppel-homolog 1* (*Kr-h1*) [11,32,33], *DNA methyltransferase 3* (*Dnmt3*) [34] and the *Forkhead box O transcription factor* (*FoxO*) [24,35–37]. RT-qPCR reactions consisted of 2.5 µL SsoAdvanced™ Universal SYBR® Green Supermix (Bio Rad), 1.25 pmol of each primer, 1 µL diluted cDNA (2 ng) in a total volume of 5 µL, with three technical replicates per biological sample. The reactions were performed using a CFX384 Real-Time System (Bio-Rad) with the cycle conditions: 95 °C for 10 min; 40 cycles of 95°C for 10 sec; 60 °C for 10 sec; and 72 °C for 15 sec. For each gene the melt curve was checked to confirm a single amplification peak. The primer efficiency (Table S2) was calculated based on an amplification curve of 10 points obtained through serial dilution of mixed cDNA samples. To normalise the gene expression we used two reference genes (*RP49* and *Ef1α* [38]) whose expression was stable according to *BestKeeper* software [39]. The relative expression was calculated as in [40] using a relative gene expression formula that takes into account the efficiency of each primer.

(d) Statistical analysis

Ovary activation score was calculated, per sample, as the average of the activation scores of all ovaries included in the sample. Average ovary activation score and gene expression level were analysed as dependent variables using generalised linear mixed models (GLMM), with 'colony' as random effect, and diet and QMP treatments as fixed effects. We used a log function, link = identity and family = Gaussian, to model *Vg*, *VgR* and *Kr-h1* expression. *Dnmt3* and *FoxO* expression and ovary activation score were modelled without applying a log function. Analyses were performed in *R* [41] using the packages lme4, car and lsmeans or GraphPad Prism 7. To compare *Vg* gene expression in activated and non-activated ovaries, we used a two-tailed Student's *t* test after $\log_{10}$ transformation of expression data. Longevity was assessed with survival curves that were statistically compared using Mantel-Cox test. Two-tailed Spearman correlation test was used to assess the correlation between Vg and VgR expression. A *p*-value lower than 0.05 was considered significant for all statistical tests. When appropriate, significance levels for *post-hoc* tests were Bonferroni adjusted for multiple comparisons.

3. Results

(a) High ovary activation and short lifespan in workers fed Royal Jelly

Ovary activation score was significantly higher in workers fed the Royal Jelly diet compared to workers fed the Control diet ($p < 0.001$; figure 1a and supplementary table 3). In addition, ovary activation score was significantly higher in workers not exposed to QMP compared to workers exposed (Figure 1a, $p < 0.05$, see supplementary table 3 for further details). There was no significant interaction between the diet and QMP treatments ($p = 0.14$; figure 1a). For the majority of the treatment combinations there was a significant difference in ovary activation score when comparing both QMP treatment and diet (comparison of least square means, $p < 0.001$ after Bonferroni correction). The two exceptions were: (i) QMP^+ and QMP^- of the Control diet ($p = 0.058$) and (ii) QMP^- of the Control diet and QMP^+ of Royal Jelly diet ($p = 0.26$).

The Royal Jelly diet reduced the lifespan of workers in both colonies ($p < 0.0001$ Mantel-Cox test followed by Bonferroni correction for multiple comparisons, Figure 2). Differences between workers exposed or not to QMP was only found in bees fed Royal Jelly

from source colony A ($p < 0.0001$ Mantel-Cox test followed by Bonferroni correction for multiple comparisons, Figure 2a).

(b) *Vg* expression in the ovaries of workers is affected by their diet and social environment

Contrary to previous studies [24,25] we find that *Vg* is expressed in the ovaries of adult honeybee workers (Figure 3a). *Vg* expression in the ovaries of adult workers is affected by both QMP ($p < 0.05$; figure 3b and supplementary table 4) and diet ($p < 0.001$; figure 3b and supplementary table 4) with no significant interaction between the two main effects ($p = 0.8$). In the presence of both QMP and the Royal Jelly diet, workers had significantly higher expression of *Vg* in their ovaries compared to the other three treatment groups: QMP^+/Control diet (comparison of least square mean, $p < 0.01$ after Bonferroni correction); QMP^-/Control diet ($p < 0.001$) and QMP^-/Royal Jelly diet ($p < 0.01$). In addition, Vg expression was significantly higher in QMP^-/Royal Jelly diet workers compared to workers from QMP^-/Control diet treatment ($p < 0.01$). There was no significant difference in the expression of *Vg* between the two primer sets ($p = 0.76$, figure 3b and supplementary figure 1).

VgR expression is affected by both QMP ($p < 0.05$; figure 3b and supplementary table 4) and diet ($p < 0.001$; figure 3b and supplementary table 4) with no significant interaction between the two main effects ($p = 0.57$). *VgR* expression was significantly higher in the treatment combination of Royal Jelly diet with no QMP compared to all other treatment combinations ($p < 0.01$, figure 3c). In addition, *VgR* expression was significantly higher in the treatment with Royal Jelly diet and QMP^+ compared with the Control diet and QMP^+ ($p < 0.001$).

(c) *Vg* expression is lower in activated ovaries whereas *VgR* is higher

Vg expression was significantly lower in activated ovaries compared to non-activated ovaries ($p = 0.004$; figure 3b inset, supplementary figure 3 inset and table 4). In contrast, expression of *VgR* was significantly higher in activated ovaries compared to non-activated ovaries ($p = 0.0033$; figure 3d inset).

(d) Royal jelly alters the expression of putative *Vg* regulators

Vg expression in the ovaries of workers is not significantly correlated with the expression level of its receptor, *VgR* ($r^2 = 0.16$, $p = 0.15$, supplementary figure 2). To gain insights into the regulatory control of *Vg* transcription in the ovaries of workers, we analysed the expression of three additional potential *Vg* regulators (Figure 4 and supplementary table 5). Firstly, the expression of *Kr-h1* was affected by both QMP exposure ($p < 0.0001$, fig 4a and supplementary table 5) and Royal Jelly diet ($p < 0.0001$, fig 4a and supplementary table 5). We found no interaction between the two main effects ($p > 0.05$). Interestingly, the QMP effect was suppressed in the workers fed Royal Jelly ($p > 0.05$). Secondly, the expression of *Dnmt3* is influenced by QMP presence ($p < 0.0001$; figure 4b and supplementary table 5). We found an interaction between QMP and diet ($p < 0.05$). *Dnmt3* is upregulated in the QMP^+/Control diet in relation to all other groups (comparison of least square mean, $p < 0.01$ after Bonferroni correction). Similar to *Kr-h1*, Royal Jelly suppressed the effects of QMP exposure on *Dnmt3* expression. Thirdly, the expression of *FoxO* was only affected by diet ($p < 0.0001$, figure 4c) with no significant interaction between diet and QMP ($p > 0.05$). *FoxO* expression was higher in QMP^-/Control diet workers compared to those fed Royal Jelly (comparison of least square mean $p < 0.05$ after Bonferroni correction). In addition, *FoxO* expression was lower in QMP^-/Royal Jelly diet treatment compared to QMP^+/Control diet ($p < 0.0001$ after Bonferroni correction).

4. Discussion

Our study demonstrates that *Vg* is expressed in the ovaries of adult honeybee workers. Previously, *Vg* expression was only known in the ovaries of queens, not workers [24,25]. It therefore appears that honeybee workers are similar to many other social and non-social insects that also express *Vg* in tissues other than the fat body [42,43]. However, we find that the environment of a worker (diet and exposure to QMP) influences the expression of *Vg* in its ovaries.

The decreased expression of *Vg* in activated ovaries compared to non-activated ovaries is unexpected for a reproductive protein. We propose two plausible explanations. First, in activated ovaries with mature oocytes the Vg protein is translocated from the haemolymph *via* VgR into the oocytes [20,21]. Translocation of large amounts of Vg into the ovaries to nourish developing oocytes may supress transcription of *Vg in situ*. Second, and more speculatively, it is known that Vg influences the population of micro-RNAs in the honeybee brain and fat body, and by this means Vg may influence gene expression in these tissues [44]. Thus, Vg may be resposible for regulating the microRNA population in ways that control ovary activation state. We therefore suggest that *Vg* has a signalling role in the ovaries of workers, providing additional evidence that the ovary regulates the behaviour and reproduction of worker honeybees [24,45–47].

Feeding Royal Jelly to workers has a major effect on their *Vg* expression (~20 fold higher), which provides an opportunity to investigate the pathways that regulate *Vg* expression in the ovaries. We determined whether genes in physiological, epigenetic and signalling pathways are influenced by the presence of Royal Jelly in the diet. First, *Kr-h1*, a downstream target of JH that is frequently used as a reliable proxy of this hormone [33,48,49], is downregulated in Royal Jelly-fed workers. This suggests that that JH levels are lowered by the presence of Royal Jelly in the diet. Since JH represses *Vg* expression [11,14], this effect is likely to be a factor that unlocks *Vg* transcription. Second, *Dnmt3*, an enzyme involved in DNA methylation, is downregulated in Royal Jelly-fed workers and this diet blocked the effects of QMP. This finding indicates that epigenetic mechanisms may moderate the effects of Royal Jelly in the diet, in both the adult (this study) and larval [50] stages of honeybee workers. Third, *FoxO*, a regulator of *Vg* expression through physical binding to *Vg*'s promoter sequence [35], is mainly regulated by post-translational modification and sub-cellular reallocation [51]. *FoxO* mRNA levels are positively correlated with the activity of FoxO protein [52]. We found that *FoxO* transcription is reduced in adult workers fed Royal Jelly (this study) as has been previously shown in honeybee larvae [53]. We suggest that FoxO negatively regulates *Vg* in honeybees (but see [24]). We note that in general, insects fed highly nutritious diets switch on the insulin/IGF1 pathway, which leads to the inactivation of FoxO activity by translocating it to the cytoplasm [35,52]. Together, these results suggest that Royal Jelly acts through epigenetic, physiological and signalling pathways to boost *Vg* expression.

The diet and social environment of adult honeybee workers have an antagonistic effect on the activation of their ovaries [6,10]. Workers fed a diet containing Royal Jelly activate their ovaries despite the presence of QMP. The Royal Jelly diet appears to counteract the degeneration of the germ cells that normally occurs in the ovaries of workers exposed to QMP (figure 1b, 1c and [54,55]). Our findings indicate that Royal Jelly can block the inhibitory effect of QMP in the ovaries of workers and possibly in queens as well. In line with this hypothesis, *Kr-h1* is downregulated in QMP-exposed workers fed diets lacking Royal Jelly [9, this study] and the same effect is observed for its downstream target JH [56]. Queens have very low levels of JH in their haemolymph [57,58], suggesting that a queen's ovaries are protected from her own pheromone by her diet of Royal Jelly.

The longevity-reproduction trade-off observed in most insects and mammals [2,3,59] is clearly broken in queen honeybees [1]. Reproduction may not be costly for queens since they have unlimited access to a highly nutritious diet, Royal Jelly [60]. If a diet of Royal Jelly increases *Vg* expression in the ovaries of queens, as we have shown here for workers, then high levels of the Vg protein are likely to act as an anti-oxidant [24,61] and for energy storage [62], key factors in promoting longevity in honeybees [60]. While we are not able to experimentally test the effects of Royal Jelly in queens, since this is their sole food, our study provides a starting point to uncover the nutrigenomic effects of Royal Jelly in honeybees. Curiously, while a low-dose diet of Royal Jelly increases the lifespan of honeybee workers [7,8] and other invertebrates [63–65], we found that workers fed Royal Jelly have shorter lifespans. A likely explanation for these contrasting results is the higher concentration of Royal Jelly used in our study [8,59].

Our study reveals that the physiological role of *Vg* in ovaries of adult honeybee workers goes far beyond its primary role as a reproductive protein. These findings therefore provide further support the 'reproductive ground plan hypothesis' [26,27] by revealing new roles of an egg-yolk precursor protein in reproductive and social traits.

Author's contributions

C.A. designed the study, carried out the dissections, survival experiment and molecular lab work, analysed the data and wrote the manuscript; B.P.O. participated in the design of the study, analysed the data and wrote the manuscript; I.R. conceived the study, designed the study, analysed the data, performed the cage experiment and wrote the manuscript. All authors gave final approval for publication.

Funding.

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (2016/15881-0 and 2017/09269-3 to C.A.) and Australian Research Council grant DP180101696 to BPO and Amro Zayed.

Competing interests.

We have no competing interests.

Acknowledgments

The authors thank Ms. Patsavee Utaipanon for helping with the survival experiment.

Figure Legends:

Figure 1. Effect of QMP and Royal Jelly diet on the ovary state of honeybee workers. (*a*) Average ovary activation score from 7-day-old workers treated with QMP and Royal Jelly diet (QMP^+/Control diet N = 64; QMP^-/Control diet N = 62; QMP^+/Royal Jelly diet N = 63; QMP^-/Royal Jelly diet N = 75; different letters represent $p < 0.05$). See supplementary table 2 for further statistical details. (*b*) Semi-activated ovary from a QMP^+/Royal Jelly diet worker which shows that Royal Jelly allows the young germ cells to develop whereas the older germ cells have degenerated (dashed line delineates transition). (*c*) Activated ovaries from a QMP^+/Royal Jelly diet worker.

Figure 2. Effect of Royal Jelly and QMP exposure on the longevity of honeybee workers. Longevity rate of workers from two source colonies, Colony A (*a*) and Colony B (*b*) fed Royal Jelly (purple lines) or control diet (blue lines), exposed to QMP (continuous lines) or not exposed to QMP (dashed lines). Different letters represent statistical significance ($p < 0.05$; see the supplementary tables for further statistical details).

Figure 3. Expression of *Vg* and *VgR* in the ovaries of honeybee workers. (*a*) RT-PCR analysis of three samples (S1, S2 and S3) and a negative control (NC) with no cDNA. RP49 and Ef1α were positive control genes and YFP a negative control gene. (*b*) *Vg* (primer set 1) expression in the ovaries of workers exposed to QMP and fed a Royal Jelly or a control diet. Sample size information: QMP^+/Control diet N = 16; QMP^-/Control diet N = 15; QMP^+/Royal Jelly diet N = 18 and QMP^-/Royal Jelly diet N = 26. The inset box is the QMP^-/Royal Jelly diet samples split into the non-activated ovaries and activated ovaries (non-activated ovaries N = 17; activated ovaries N = 9). (*c*) *VgR* expression under the same treatment groups as *Vg*. Different letters represent statistical significance ($p < 0.05$; see the supplementary tables for further details) and ** represents $p < 0.01$ obtained after two-tailed Student's *t*-tests.

Figure 4. Expression of *Kr-h1*, *Dnmt3* and *FoxO* in the ovaries of honeybee workers. The expression of *Kr-h1* (*a*), *Dnmt3* (*b*) and *FoxO* (*c*) in the ovaries of workers exposed to QMP and fed a Royal Jelly or a control diet. Sample size is the same as in Figure 3. Different letters represent statistical significance ($p < 0.05$; see the supplementary tables for further details) and **** represents $p < 0.0001$ obtained after two-tailed Student's *t*-tests.

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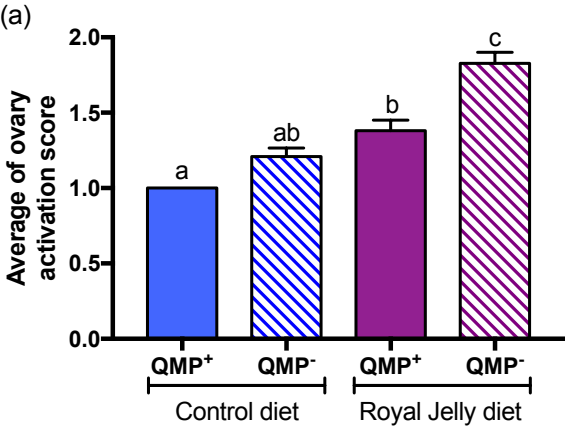
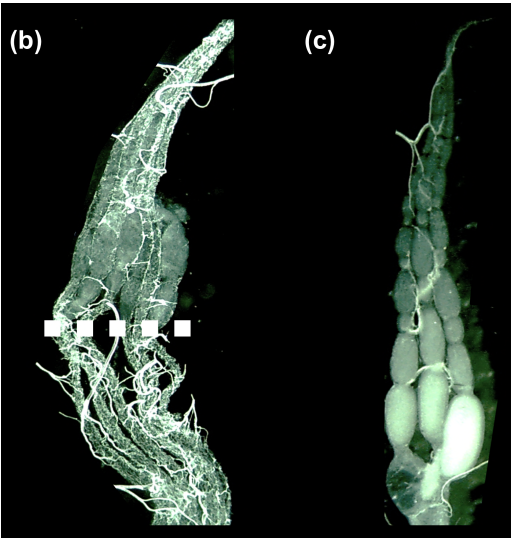
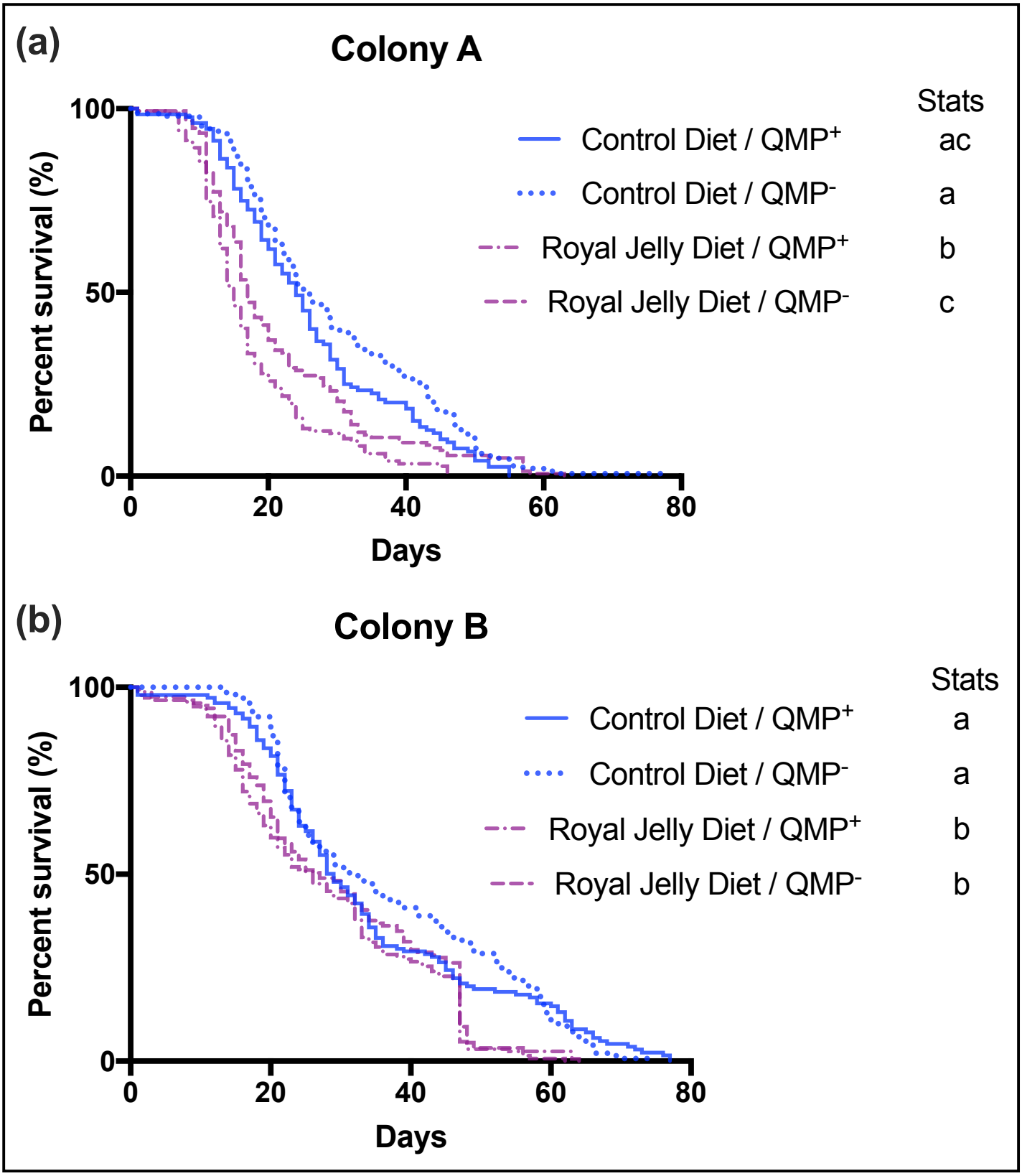
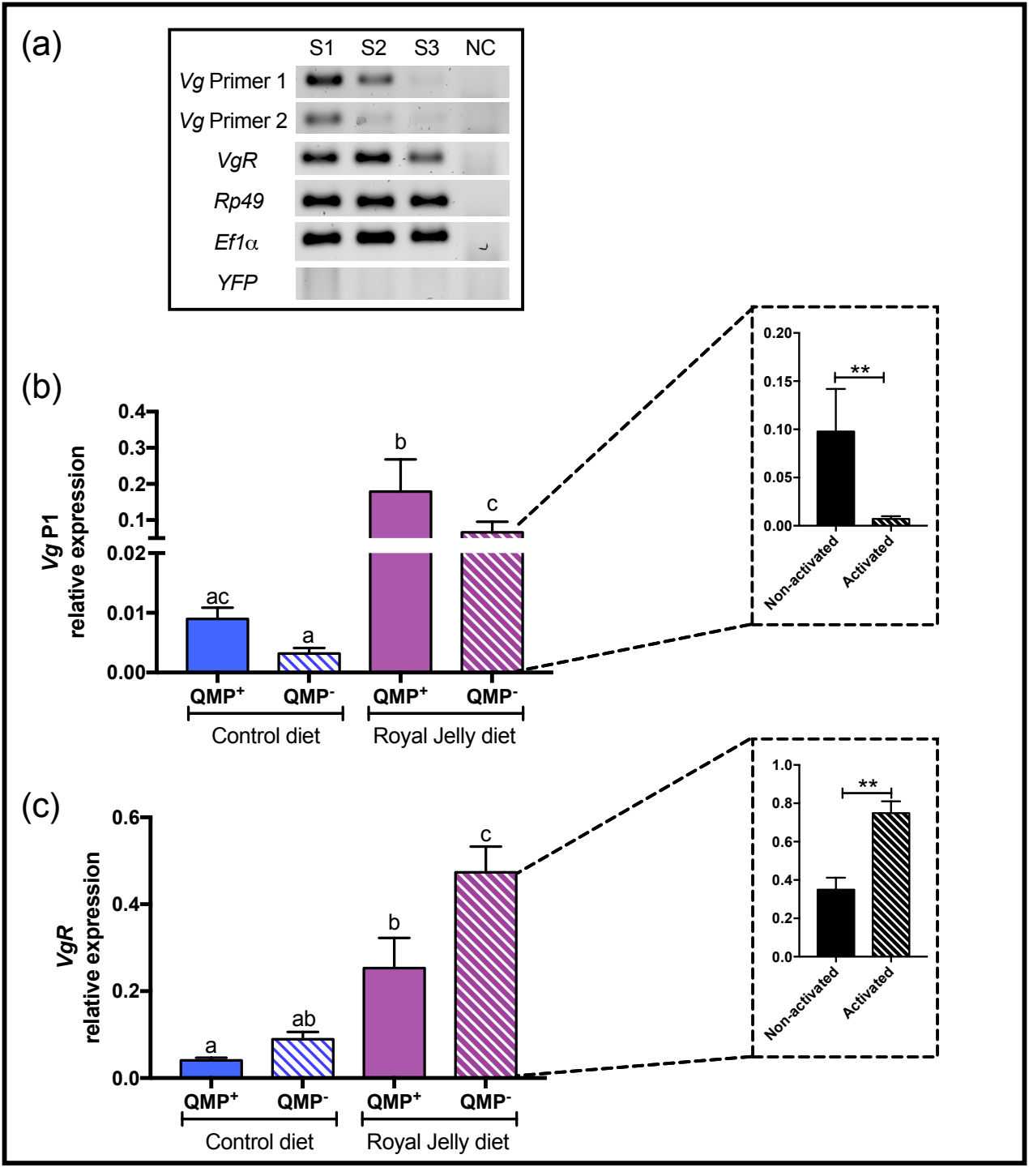
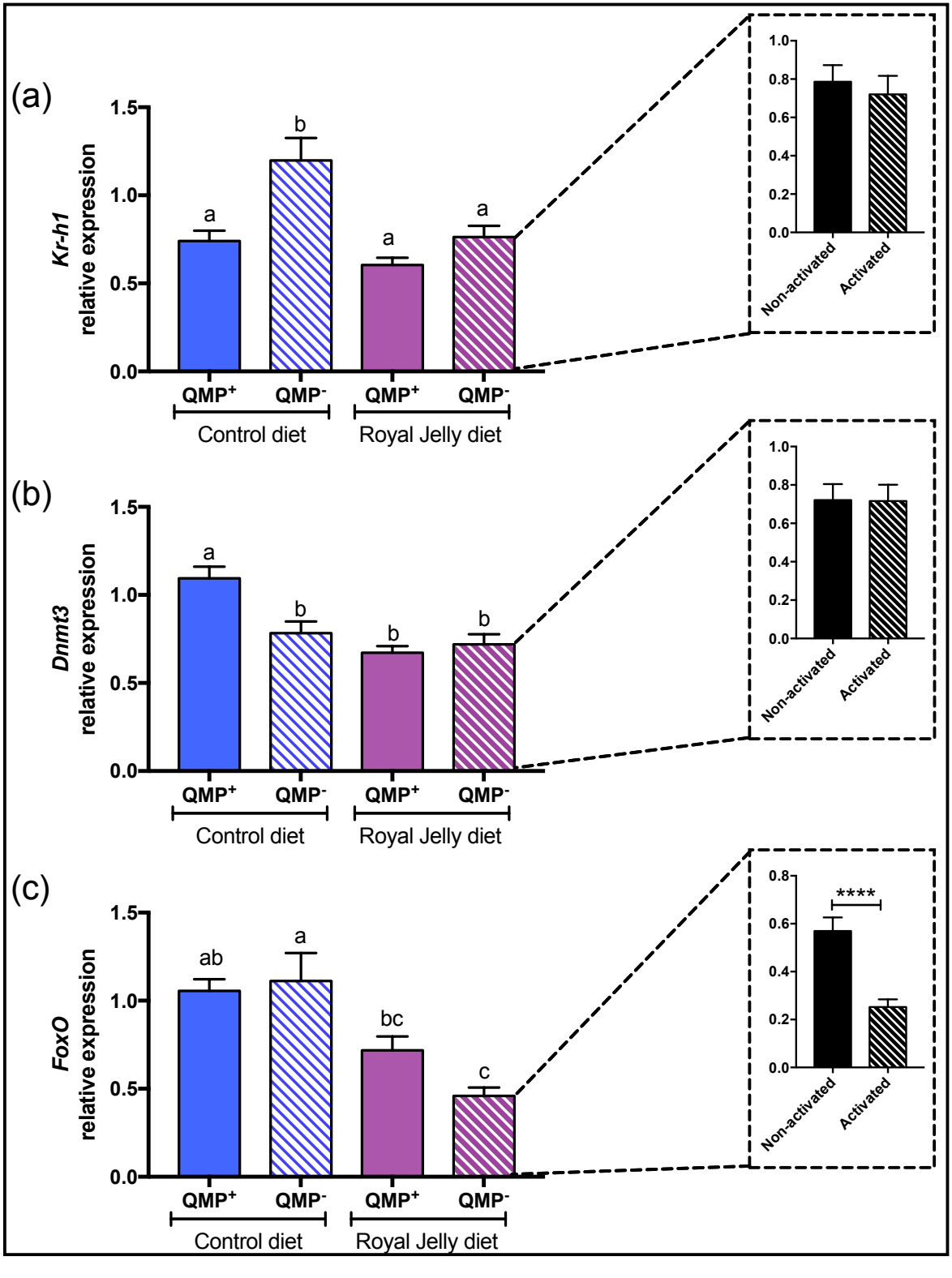

(a)
Average of ovary activation score
2.0
1.5
1.0
0.5
0.0
a
ab
b
c
QMP+
QMP-
QMP+
QMP-
Control diet
Royal Jelly diet


(b)
(c)


(a)
Colony A
Stats
Control Diet / QMP+ ac
Control Diet / QMP- a
Royal Jelly Diet / QMP+ b
Royal Jelly Diet / QMP- c
Percent survival (%)
100
50
0
0
20
40
60
80
Days
(b)
Colony B
Stats
Control Diet / QMP+ a
Control Diet / QMP- a
Royal Jelly Diet / QMP+ b
Royal Jelly Diet / QMP- b
Percent survival (%)
100
50
0
0
20
40
60
80
Days


(a)
S1
S2
S3
NC
Vg Primer 1
Vg Primer 2
VgR
Rp49
Ef1α
YFP
(b)
Vg P1 relative expression
0.4
0.3
0.2
0.1
0.02
0.01
0.00
ac
a
b
c
QMP+
QMP-
QMP+
QMP-
Control diet
Royal Jelly diet
0.20
0.15
0.10
0.05
0.00
**
Non-activated
Activated
(c)
VgR relative expression
0.6
0.4
0.2
0.0
a
ab
b
c
QMP+
QMP-
QMP+
QMP-
Control diet
Royal Jelly diet
1.0
0.8
0.6
0.4
0.2
0.0
**
Non-activated
Activated


(a)
Kr-h1
relative expression
1.5
1.0
0.5
0.0
a
b
a
a
QMP+
QMP-
QMP+
QMP-
Control diet
Royal Jelly diet
1.0
0.8
0.6
0.4
0.2
0.0
Non-activated
Activated
(b)
Dnmt3
relative expression
1.5
1.0
0.5
0.0
a
b
b
b
QMP+
QMP-
QMP+
QMP-
Control diet
Royal Jelly diet
1.0
0.8
0.6
0.4
0.2
0.0
Non-activated
Activated
(c)
FoxO
relative expression
1.5
1.0
0.5
0.0
ab
a
bc
c
QMP+
QMP-
QMP+
QMP-
Control diet
Royal Jelly diet
0.8
0.6
0.4
0.2
0.0
****
Non-activated
Activated