

Proximate influences on eusocial caste behaviour

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Thesis Abstract

Why do some individuals behave as queens and others as workers in eusocial societies? This question has interested ethologists and evolutionary biologists since Darwin. Queens and workers of eusocial sweat bee species are morphologically and developmentally similar, which means that each female is capable of behaving as a queen or a worker. However, few females lay eggs and behave as queens, while the majority of females provision the queen's offspring, rarely lay eggs, and behave as workers. This makes eusocial sweat bee species, such as *Lasioglossum laevisimum*, excellent models to study the underlying environmental (social) and genetic factors that contribute to variation in caste behaviours. My research has focused on describing some of the proximate mechanisms that influence caste behaviours in *L. laevisimum* females.

The social environment of a sweat bee colony, specifically the behaviour of a queen, can have a dramatic impact on worker behaviour. Queens can influence the reproductive behaviour of workers both indirectly and directly. Directly, queens can suppress worker reproduction by physically bullying their workers. In a nesting aggregation at Brock University, almost half of *L. laevisimum* nests became queenless, which provided me with a natural experiment to assess the direct influence by queens on worker behaviour. I took advantage of this natural experiment and compared the ovarian development of workers in queenright and queenless nests. Dissection data showed that a small proportion (17%) of workers developed their ovaries in both queenright and queenless nests. Therefore, even though the queen was no longer present in queenless nests, queenless workers had similar ovarian development scores as workers from queenright nests, which still had a living queen. This suggests that *L. laevisimum* queens exert an early, negative, and strong influence on worker egg-laying behaviour, which lasts even after she is gone. Thus,

the social environment in which workers eclose can have a long lasting impact on their behaviour.

The ovarian ground plan hypothesis (OGPH) suggests that egg-laying and brood provisioning behaviours likely exhibited by solitary ancestors decoupled to be expressed separately in queens and workers of highly eusocial descendants (West-Eberhard 1987, 1996). Furthermore, the OGPH suggests that the molecular mechanisms and gene expression underpinning ancestral egg-laying and brood provisioning behaviours also decoupled. Queens express egg-laying genes more than workers and workers express provisioning genes more than queens. I tested this prediction by comparing queen and worker gene expression levels of two genes, *vitellogenin* and *foraging*, which are associated with egg-laying and provisioning behaviour in other social insects. *Lasioglossum laevissimum* queens had higher *vitellogenin* expression levels than workers, and females with high ovarian development had high *vitellogenin* expression. On the other hand, queens and workers had similar *foraging* expression levels.

Vitellogenin and *foraging* gene expression comparisons between *L. laevissimum* queens and workers highlight two important behavioural characteristics of sweat bee castes. First, in eusocial sweat bees, both queens and workers actively provision brood at some point during the breeding season, which is reflected in the similar *foraging* expression levels of *L. laevissimum* queens and workers in the middle of the breeding season, when both castes are present in nests. Secondly, queens lay eggs while a small proportion of workers have queen-like ovarian development, which is reflected in *vitellogenin* expression differences between castes.

In the final chapter of this thesis I suggest a modification to the OGPH, which applies specifically to eusocial evolution in bee lineages (superfamily Anthophila), The *Anthophila*

ground plan hypothesis. The OGPB suggested that eusocial wasp descendants evolved from a solitary ground plan in which a solitary ancestor provisioned its offspring progressively, and egg-laying and provisioning behaviours occurred nonsimultaneously. In contrast, The Anthophila ground plan hypothesis refers specifically to the ancestral solitary ground plan from which eusocial bee lineages likely evolved, which was a mass-provisioning solitary ancestor that exhibited egg-laying and provisioning behaviour concurrently. From this ground plan, I describe how the biasing of egg-laying and provisioning behaviours, and their molecular mechanisms, in castes of eusocial descendants may have occurred through evolutionary time.

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Overview of Thesis Contents

This thesis consists of 5 total chapters: a general introduction, three data chapters, and a general discussion. Each data chapter is written in manuscript format (tables and figures presented after the text) and will be submitted for publication with minor revisions (Chapters 3 and 4), or is already published (Chapter 2). Since each data chapter is written as an independent study, some definitions, theoretical framework, and relevant scientific importance that are common between studies are stated multiple times.

Chapter 1 is a literature review of the ultimate and proximate explanations for caste phenotypes in eusocial animal species. This chapter also demonstrates the need for proximate descriptions of caste phenotypes in primitively eusocial species.

Chapter 2 has been published in *Insectes Sociaux* (2018) 65: 367–379. This publication is co-authored with Miriam Richards.

Chapter 3 will be submitted with minor revisions to one of the following journals: Molecular Ecology, Behavioral Ecology and Sociobiology, or the Journal of Insect Physiology. This chapter is co-authored with Adonis Skandalis and Miriam Richards

Chapter 4 will be submitted with minor revisions to one of the following journals: Molecular Ecology, Behavioral Ecology and Sociobiology, or the Journal of Insect Physiology. This chapter is co-authored with Adonis Skandalis and Miriam Richards

Chapter 5 is a general discussion and review of the findings from this thesis. In this chapter I hypothesize the process by which egg-laying and provisioning behaviours, and their molecular mechanisms, may have decoupled through evolutionary time to be expressed separately in queens and workers of highly eusocial bee species.

Table of Contents

Chapter 1: General Introduction	1
Eusocial organisation and sociality in Hymenoptera	1
Explanations for eusocial caste phenotypes in Hymenoptera.....	3
<i>Ultimate explanations</i>	3
Why do phenotypes characteristic of eusocial societies evolve?.....	3
What is the phylogenetic history of species with eusocial organisation?.....	4
<i>Proximate explanations</i>	9
Proximate mechanisms of caste phenotypes in highly eusocial societies.....	9
Proximate mechanisms of caste phenotypes in primitively eusocial societies	10
A primitively eusocial sweat bee as a model to describe proximate influences on caste phenotypes	11
Thesis objectives.....	15
Chapter 2: Investigating queen influence on worker behaviour using comparisons of queenless and queenright workers	17
ABSTRACT.....	18
INTRODUCTION.....	19
Direct versus indirect queen influence on worker behaviour	20
Queen influence on worker behaviour in <i>Lasioglossum laevissimum</i>	22
METHODS	24
Study site.....	24
Nest excavations and colony phenology.....	24
Characteristics of adult females	25
Identification of queens and workers	26
Data analysis	27
Data resources	28
RESULTS	29
Colony phenology and brood development	29
Characteristics of queens	30
Characteristics of workers.....	30
Comparisons of queens and workers	31
Inter-species comparisons	31
DISCUSSION	33
Queen influences on worker egg-laying	33
Queen influence on worker mating.....	35
Queen influence on worker ergonomic inputs	35
Life without a queen	36
Interspecific comparisons	37
Conclusions.....	38

Chapter 3: *Vitellogenin* expression in a primitively eusocial sweat bee, *Lasioglossum laevissimum* 50

INTRODUCTION.....	51
<i>Vitellogenin</i> influences phenotypes of solitary and social insects	52
<i>Vitellogenin</i> in a eusocial sweat bee	55
METHODS	58
<i>Lasioglossum laevissimum</i> collections and measurements.....	58
RNA extractions and cDNA preparation	59
<i>Vitellogenin</i> and reference gene primer design for RT-qPCR.....	59
RT-qPCR and reference gene validation	60
Data analyses	62
RESULTS	65
<i>Vitellogenin</i> expression in <i>L. laevissimum</i> females	65
Gynes, Foundresses, and Queens.....	65
Workers.....	67
Queen and worker comparisons.....	68
<i>Vitellogenin</i> expression in <i>L. laevissimum</i> males	69
DISCUSSION	70
<i>Vitellogenin</i> expression in <i>L. laevissimum</i> queens and workers	70
<i>Vitellogenin</i> expression in <i>L. laevissimum</i> queens and workers provides support for the OGPB.....	71
<i>Vitellogenin</i> expression, task polyphenism, and overwintering diapause.....	73
Lack of <i>vg</i> expression in adult <i>L. laevissimum</i> males may stem from age and behaviour ...	75
The importance of multiple reference genes for RT-qPCR data analysis	75
Conclusions.....	76

Chapter 4: *Foraging* expression in a primitively eusocial sweat bee, *Lasioglossum laevissimum* 104

INTRODUCTION.....	105
<i>Foraging</i> gene expression influences foraging behaviour in insects.....	106
Variation in foraging behaviour in castes of eusocial sweat bees	108
<i>Lasioglossum laevissimum</i> as a model to understand how <i>for</i> expression influences foraging and provisioning behaviour.....	111
METHODS	113
<i>Lasioglossum laevissimum</i> collections and morphometrics	113
RNA extractions and cDNA preparation	114
Primer design and PCR amplicon validation for RT-qPCR	114
RT-qPCR protocol	115
Data analysis	115
RESULTS	118
<i>Foraging</i> expression in heads and abdomens	118
Inter-caste variation in <i>for</i> expression.....	118
<i>Foraging</i> expression patterns in queens and workers.....	119
<i>Foraging</i> expression in <i>L. laevissimum</i> males.....	120

DISCUSSION	121
<i>Foraging</i> expression in <i>L. laevissimum</i> queens and workers	121
<i>Foraging</i> expression as a possible primer for foraging activity	124
<i>Lasioglossum laevissimum</i> males have higher <i>for</i> expression than females.....	126
Conclusions.....	127
 Chapter 5: General Discussion	 138
The influence of the social environment on worker reproduction	138
<i>Vitellogenin</i> and <i>for</i> expression levels reflect behavioural and life history differences between queens and workers	140
The decoupling of ancestral egg-laying and brood provisioning phenotypes in bees.....	141
The <i>Anthophila</i> ground plan hypothesis	144
 REFERENCES.....	 148

List of Tables

Chapter 2

Table 2.1. Social variation among ten species of <i>L. (Dialictus)</i> species.....	40
Table 2.2. Factors contributing to variation in worker ovarian development scores.....	41
Table 2.3. Results of the principal components analysis based on social characteristics of 8 <i>L. (Dialictus)</i> species (Table 2.1).....	42
Table S2.1. Numbers of <i>L. laevissimum</i> nests excavated during the 2013, 2014, and 2015 breeding season in St. Catharines, Ontario, Canada.....	49

Chapter 3

Table 3.1. General <i>vg</i> expression patterns in insects with respect to life history traits such as development, sex, diapause, and ovarian development.....	78
Table 3.2. Sample sizes of each <i>L. laevissimum</i> category used in head and abdomen <i>vg</i> expression comparisons.....	79
Table 3.3. Sequences, locations, and amplicon sizes of primer sets used for each gene in RT-qPCR analyses.....	80
Table 3.4. The effects of breeding season phenology, age, the timing of overwintering-diapause, and differential foraging behaviour on <i>vg</i> expression in <i>L. laevissimum</i> gynes, foundresses, and queens.....	81
Table 3.5. The effect of breeding season phase (A.) and foraging behaviour (B.) on <i>vg</i> expression in worker heads and abdomens.....	82
Table 3.6. The effect of caste on <i>vg</i> expression.....	83
Table 3.7. Factors contributing to variation in <i>vg</i> expression levels in queen and worker heads (Head) and abdomens (Abdomens).....	84
Table 3.8. Correct classifications of females to caste using a Linear Discriminant Analysis (LDA) with only physical traits and an LDA using only head and abdomen <i>vg</i> expression levels.....	85
Table 3.9. Detectable <i>vg</i> expression in <i>L. laevissimum</i> female and male heads and abdomens.....	86

Table S3.1. Results and methods from studies that investigate *vg* expression or Vg titer levels during the life cycles of insect species.....96

Chapter 4

Table 4.1. *Foraging (for)* expression patterns with respect to foraging phenotypes in social Hymenopteran species.....128

Table 4.2 *Lasioglossum laevissimum* categories used in head and abdomen *for* expression comparisons.....129

Table 4.3. The effect of breeding season phase, caste, sex, and collection method on *for* expression in heads, thoraces, and abdomens.....130

Table 4.4. Factors contributing to *for* expression levels in queen and worker heads (Head) and abdomens (Abdomens).....131

List of Figures

Chapter 1

Figure 1.1. The ovarian ground plan hypothesis (OGPH; West-Eberhard 1987, 1996)7

Figure 1.2. The typical life cycle of a eusocial sweat bee in Canada. Each number next to an arrow represents the corresponding phase that occurs between the two dates.....13

Chapter 2

Figure 2.1. Contents of the *L. laevissimum* nests in 2013, 2014, and 2015.....43

Figure 2.2. Size variation among queens, queenless workers, and queenright workers from phases 2 and 3.....44

Figure 2.3. Mandibular wear scores of queens, queenless workers, and queenright workers from phases 2 and 3.....45

Figure 2.4. Ovarian development scores of queens, queenless workers, and queenright workers from phases 2 and 3.....46

Figure 2.5. Variation in worker size and ovarian development throughout the breeding season.....47

Figure 2.6. Interspecific social variation among eight species of *L. (Dialictus)*.....48

Chapter 3

Figure 3.1. Predicted *vg* expression levels in *L. laevissimum* males and females at different points during the breeding season.....87

Figure 3.2. Validation of *gapdh*, *actin*, and *rpP2* of suitable reference genes reference genes for head and abdomen gene expression comparisons.....88

Figure 3.3. Intra-individual comparison of head and abdomen *vg* expression levels of *L. laevissimum* females.....89

Figure 3.4. The effects of breeding season phenology, age, the timing of overwintering-diapause, and differential foraging behaviour on *vg* expression levels in the heads and abdomens of *L. laevissimum* gynes, foundresses, and queens.....90

Figure 3.5. The effect of breeding season phase on <i>vg</i> expression levels in worker heads (Head) and abdomens (Abdomen).....	91
Figure 3.6. <i>Vitellogenin</i> expression levels in the heads (Head) and abdomens (Abdomens) of nest workers (collected from nest excavations) and foraging workers (caught on the wing while actively foraging).....	92
Figure 3.7. The effect of cast on head (Head) and abdomen (Abdomen) <i>vg</i> expression levels....	93
Figure 3.8. The effect of ovarian development scores and caste on <i>vg</i> expression levels in queen and worker heads (Head) and abdomens (Abdomen).....	94
Figure 3.9. Linear Discriminant 1 score generated from a Linear Discriminant Analysis (LDA) with only physical traits (top) and an LDA using only <i>vg</i> expression levels (bottom) for each <i>L. laevissimum</i> female.....	95

Chapter 4

Figure 4.1. Validation of three reference genes used in <i>L. laevissimum</i> for expression comparisons.....	132
Figure 4.2. Comparisons of head and abdomen <i>for</i> expression levels. (A) <i>Lasioglossum laevissimum</i> samples that had <i>for</i> expression measurements in their head and abdomen.....	133
Figure 4.3. The effect of breeding season phase on normalized <i>for</i> expression levels in foundresses, queens, and gynes.....	134
Figure 4.4. Normalized <i>for</i> expression levels with respect to the provisioning behaviour of workers collected mid-day compared to workers collected from nests in the morning.....	135
Figure 4.5. The effect of caste on <i>for</i> expression levels.....	136
Figure 4.6. The effect of sex on normalized <i>for</i> expression levels. Early spring foundresses, spring foundresses, summer queens, workers, and gynes are included in the female category.....	137

Chapter 5

Figure 5.1. The relationship between <i>vg</i> expression and <i>for</i> expression levels in <i>L. laevissimum</i> queen and worker heads and abdomens.....	143
Figure 5.2. The Anthophila ground plan hypothesis.....	145

Chapter 1: General Introduction

This thesis is about the environmental (social) and genetic factors that influence caste phenotypes of a eusocial sweat bee, *Lasioglossum (Dialictus) laevissimum* (Smith, 1853). Castes of this species are morphologically and developmentally similar, and females are capable of behaving as a queen or a worker (Yanega 1989; Schwarz et al. 2007). This means that *L. laevissimum* is an excellent model to investigate the environmental and genetic factors that influence differential caste phenotypes of adult females. My research takes advantage of field and laboratory techniques to investigate the proximate mechanisms that influence caste phenotypes of *L. laevissimum*. In chapter two I describe the natural history and social characteristics of a *L. laevissimum* aggregation located at Brock University to investigate how the presence or absence of a queen in a nest influences traits associated with reproduction in workers. In chapters three and four I use real-time quantitative PCR (RT-qPCR) to investigate how the head and abdomen expression levels of two target genes, *vitellogenin* and *foraging*, correlate with phenotypes typically associated with queens or workers, specifically, the extent to which a female develops her ovaries and whether or not a female is actively foraging.

Eusocial organisation and sociality in Hymenoptera

The term eusociality refers to an evolutionarily derived form of animal social organisation, in which groups of conspecifics are characterised by overlapping generations, cooperative parental care, and reproductive division of labour (Batra 1966; Michener 1969, 1974; Wilson 1971). In eusocial groups, few individuals are reproductive while the remaining majority of individuals are temporarily or permanently non-reproductive, and help raise the offspring of reproductives. Darwin (1859) first recognized the difficulty of explaining the

evolution of non-reproductive helpers by individual selection, since non-reproductive helpers do not produce their own offspring and cannot propagate their non-reproductive helper traits directly. Unsurprisingly, without an understanding of Mendelian inheritance and modern genetics, Darwin did not provide an adequate explanation for the evolution of non-reproductive helpers in eusocial societies. Moreover, the question as to why an individual sacrifices its own reproduction and helps another individual to rear offspring continues to be a source of fascination to evolutionary-minded geneticists.

Eusocial insects, specifically those from the order Hymenoptera (bees, wasps, and ants), dominate the ecosystems they inhabit and are likely the most specialised animals on the planet (Wilson 1971). Eusociality has several independent evolutionary origins in this order and hymenopteran species have incredible interspecific variation in social organisation, which make them ideal subjects to understand the evolution and elaboration of eusociality (Hunt and Toth 2017; Peters et al. 2017; Wcislo and Fewell 2017; Taylor et al. 2018). Species range in social complexity from solitary and subsocial life histories, in which females raise offspring alone, to the aforementioned eusocial groups, in which non-reproductive females (workers) help raise the offspring of few reproductive females (queens: Michener 1969, 1974; Wilson 1971). Species in this order also vary in the type of eusocial organisation they exhibit. Some species form highly (advanced) eusocial groups, which have developmentally and morphologically distinct queens and workers (Michener 1974; Sumner et al. 2018). In contrast to highly eusocial taxa, species that form primitively eusocial groups have behavioural castes, which means that queens and workers are developmentally and morphologically similar and each female is capable of exhibiting behaviours typical of the queen or worker caste (Michener 1974; Yanega 1989; Schwarz et al. 2007; Sumner et al. 2018). Behaviours often associated with primitively eusocial

queens are initiating a nest, mating, and ovipositing, while behaviours often associated with workers include provisioning, nest construction, and nest guarding.

Explanations for eusocial caste phenotypes in Hymenoptera

As with any phenotype (physical trait or behaviour exhibited by an organism), the specific phenotypes of the individuals that make up eusocial groups can be understood with ultimate and proximate explanations (Tinbergen 1963; Kapheim 2018). Ultimate explanations deal with the functional purpose (fitness) of a phenotype among individuals, specifically why does a phenotype evolve, and the phylogenetic history of a phenotype. Proximate explanations focus on the causal mechanisms that generate phenotypes within individuals via development (ontogeny) and in response to immediate internal and external cues (e.g. environmental and genetic). Since the mid-2000s, the field of “sociogenomics” has focused on describing the ultimate and proximate causes of traits characteristic of individuals that make up eusocial groups (Robinson et al. 2005). Studies investigating these ultimate and proximate causes often focus on insects in the order Hymenoptera (Rehan and Toth 2015; Toth and Rehan 2017; Kapheim 2018).

Ultimate explanations

Why do phenotypes characteristic of eusocial societies evolve?

There are several evolutionary drivers that may have contributed to each evolutionary origin of eusociality (Lin and Michener 1972; Michener 1974; Crespi and Ragsdale 2000; Wade 2001; Wilson and Holldobler 2005; Bourke 2011). Eusociality likely evolved under different ecological conditions via positive selection on a combination of individual phenotypes: the helping behaviour of non-reproductives, the manipulation of daughters by mothers, and cooperation by both reproductive and non-reproductive individuals for shared benefit. First, the

helping behaviour exhibited by non-reproductives (alloparental care) can be explained in part by kin selection (Hamilton 1964; Bourke 2011). If an individual can pass on more genes identical by descent by helping to raise the offspring of relatives than by raising their own offspring, then these behaviours can evolve. Secondly, positive selection on mothers to manipulate the behaviour of their daughters can also explain the evolution of helping behaviours by non-reproductives (Lin and Michener 1972; Michener 1974; Wade 2001). Mothers that manipulate their daughters to help rear siblings instead of reproducing themselves can accrue higher fitness than if they were to raise offspring alone, therefore maternal manipulation and the resulting helping behaviours of non-reproductives can evolve. Finally, the evolution of helping behaviours by non-reproductives can also be explained by the shared benefit of living in a group under a variety of ecological constraints (Lin and Michener 1972; Crespi and Ragsdale 2000; Wilson and Holldobler 2005). Selection may promote cooperation and helping behaviours if resources such as nesting locations are limited and non-reproductives are in a better position to take over a nest by staying, helping, and foregoing reproduction. If nests are at a high risk of parasitism or predation then reproductives that tolerate the presence of a non-reproductive guard(s) may accrue higher fitness than if they were to live alone.

What is the phylogenetic history of species with eusocial organisation?

Eusociality has evolved multiple times independently in the Hymenoptera. It has one evolutionary origin in ants (Formicidae; Gadau et al. 2013), one origin in the Crabronidae (Mathews 1991), two origins in the Vespidae (once in the Stenogastrinae and once in Polistinae + Vespinae; Piekarski et al. 2018), and four origins in bees; two in Apidae (Cardinal and Danforth 2011; Rehan et al. 2012) and two in Halictidae (Gibbs et al. 2012). With all of these origins, it is widely accepted that eusocial descendants evolved from solitary antecedents

(Wilson 1971; Lin and Michener 1972; Linksvayer and Wade 2005; Peters et al. 2017; Toth and Rehan 2017).

For each of the evolutionary origins, eusociality may have evolved from solitary ancestors via the following evolutionary sequences: the parasocial (polygynous family) route, the familial (subsocial) route, or by nest-mate waiting behaviour (Wheeler 1922; Wilson 1971; Lin and Michener 1972; Michener 1974; West-Eberhard 1978; Schwarz et al. 2011). The parasocial route describes eusocial organisation as evolving from communal colonies (Wilson 1971; Michener 1974), which are groups of related or unrelated females that nest together and do not cooperate in parental care. Over generations caste roles may evolve within these groups and female lifespan may extend long enough to overlap with the lifespans of their own daughters or nieces (Wilson 1971; Michener 1974). The evolution of eusocial organisation via the familial route begins with a single female initiating and remaining in a single nest (Wilson 1971). Over evolutionary time, female lifespan may extend so that mothers live long enough to see their daughters eclose, and these daughters remain in the nest to help their mother raise more offspring (Wilson 1971). Finally, eusocial organisation may evolve from nest-mate waiting behaviour (Schwarz et al. 2011). In this evolutionary sequence daughters may opt to remain in the nest, refraining from reproduction or parental care (waiting), with the potential to take over the nest later. Eusocial organisation may evolve under ecological constraints when a forage-now strategy results in greater inclusive fitness compared to the waiting strategy (Schwarz et al. 2011). These evolutionary sequences suggest that different types of social organisation represent transitional states between solitary and highly eusocial behaviour, one of these transitional states is primitive eusociality (Rehan and Toth 2015; Taylor et al. 2018).

Ground plan hypotheses have hypothesized proximate mechanisms for the evolutionary process by which solitary ancestors may have evolved into highly eusocial descendants. The ovarian ground plan hypothesis (OGPH) first suggested that the expression of the egg-laying and provisioning phases of an ancestral solitary wasp's life cycle decoupled to be expressed separately in queens and workers of highly eusocial descendants (West-Eberhard 1987, 1996; depicted in Fig.1.1, pg. 7). In solitary taxa, a single female exhibits both egg-laying and provisioning behaviours required to rear offspring. In highly eusocial taxa, physical traits and behaviours associated with the egg-laying are expressed in queens, while physical traits and behaviours associated with provisioning are expressed in workers. In primitively eusocial species, which may represent a transitional state between solitary and highly eusocial species, egg-laying is expressed more in queens than workers, and provisioning is expressed more in workers than queens when both castes are present in a colony (Rehan and Toth 2015; Toth and Rehan 2017; Sumner et al. 2018; Taylor et al. 2018). Since primitively eusocial taxa have behavioural castes, and females are capable of exhibiting egg-laying and provisioning behaviours, a proportion of queens and workers should overlap and express both egg-laying and provisioning behaviours (Fig.1.1, pg. 7). Primitively eusocial species vary from weakly to strongly eusocial, depending on how well queens control worker reproduction (Breed 1976; Packer and Knerer 1985; Wyman and Richards 2003; Peso and Richards 2010). Therefore, in weakly eusocial species, the overlap of queens and workers expressing both egg-laying and provisioning behaviour should be large. In contrast, in strongly eusocial species, the overlap of queens and workers expressing both egg-laying and provisioning behaviour should be small.

The OGPH also proposed that the expression of genes and molecular pathways associated with egg-laying and provisioning behaviours decoupled to be expressed separately in highly

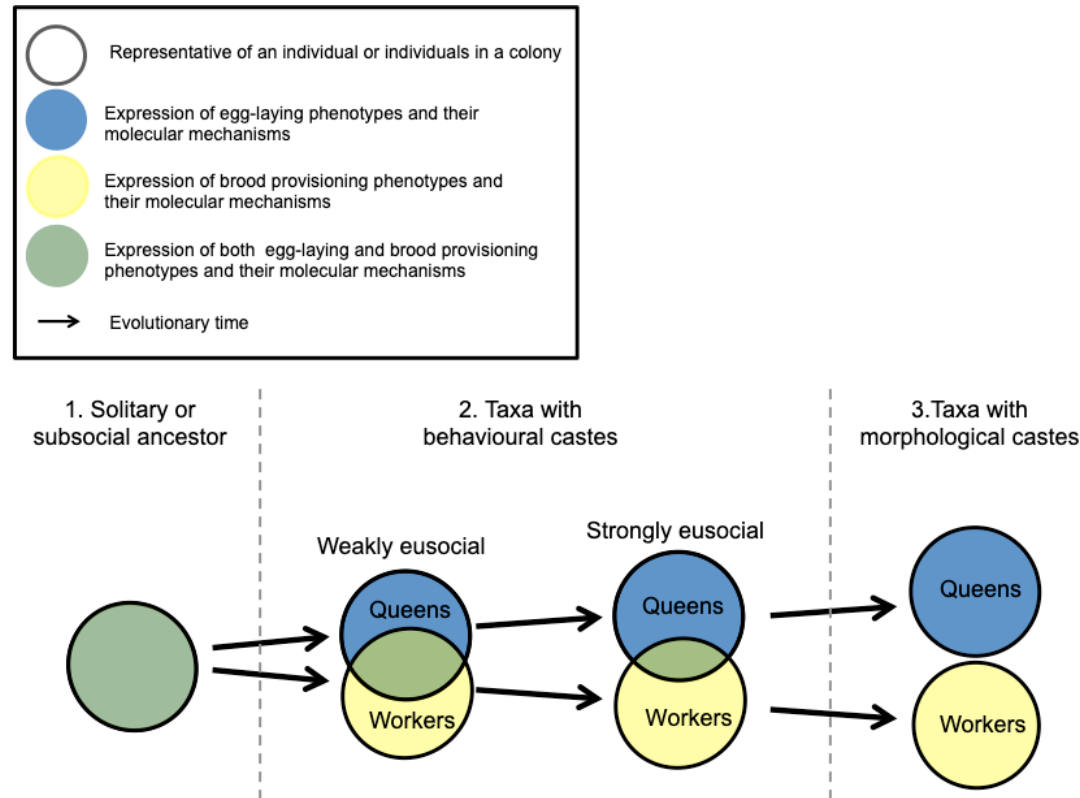


Figure 1.1. The ovarian ground plan hypothesis (OGPH; West-Eberhard 1987, 1996). The OGPH, suggests that the decoupling of ancestral solitary egg-laying and brood provisioning behaviours, and the molecular mechanisms underpinning them, became expressed separately in queens and workers of highly eusocial descendants. Phenotypes associated with egg-laying include ovarian development and ovipositioning, and phenotypes associated with brood provisioning include foraging and collecting provisions. Outlined circles indicate a representative individual or individuals in a colony for a given taxa. The shaded colour of each circle indicates the expression of egg-laying (blue), brood provisioning (yellow), or both (green) phenotypes, and the underlying expression of genes associated with those behaviours, for the representative individual(s). 1. In a solitary or subsocial ancestor both phenotypes / molecular mechanisms are expressed in each individual and are required to rear offspring. 2. In eusocial species with behavioural castes individuals are capable of expressing both phenotypes, queens express more egg-laying phenotypes / molecular mechanisms than workers, which express more brood provisioning phenotypes / molecular mechanisms than queens when both castes are in a colony. Taxa with behavioural castes are displayed as weakly or strongly eusocial. In, weakly eusocial species, queens have weak control over worker reproduction. In strongly eusocial species, queens exhibit strong control over worker reproduction. 3. Eusocial species with morphological castes have complete decoupling of egg-laying and brood provisioning phenotypes; queens express egg-laying phenotypes / molecular mechanisms and workers express brood provisioning phenotypes / molecular mechanisms

eusocial queens and workers (Fig.1.1, pg. 7; West-Eberhard 1987, 1996). Queens should have higher expression levels of genes associated with egg-laying than workers, while workers have higher expression levels of genes associated with provisioning than queens. This idea has since been expanded upon with the conserved genomic toolkit hypothesis, which suggests that the evolutionary decoupling of molecular pathways associated with egg-laying and provisioning behaviours occurred with a similar assortment of orthologous genes and pathways across independent social lineages (Toth and Robinson 2010; Rehan and Toth 2015).

In the last 15 years, hypotheses similar to the OGPH have used the idea of decoupling a ‘solitary ground plan’ to explain the evolution of caste phenotypes in social Hymenoptera species (Toth and Rehan 2017). The maternal heterochrony hypothesis describes the evolution of sibling care as an evolutionary rearrangement of the timing of solitary maternal care (Linksvayer and Wade 2005; Johnson and Linksvayer 2010). The reproductive ground plan hypothesis (RGPH) and modified RGPH propose that differences in foraging behaviour of worker honeybees evolved from the same ground plan that produced honeybee castes (West-Eberhard 1996; Amdam et al. 2006a; Oldroyd and Beekman 2008). Finally, the diapause ground plan hypothesis describes the evolution of *Polistes* castes from differences in female bivoltine diapause physiology (Hunt and Amdam 2005; Hunt 2006, 2012). Fundamentally, each of these hypotheses proposes that the different phenotypes displayed by queens and workers of eusocial descendants, and the proximate mechanisms that influence them, evolved via the evolutionary decoupling of solitary phenotypes. Therefore, to understand how eusocial castes evolved we need a comprehensive understanding of the proximate mechanisms that influence caste phenotypes in species whose sociality represent transitional states between solitary behaviour and advanced eusociality.

Proximate explanations

A major focus of entomologists and ethologists studying eusocial species has been describing the proximate mechanisms that influence some individuals to behave as queens and others as workers. In the last 10 years alone, there have been a large number of comparative genomic and transcriptomic studies describing the genetic influences on various phenotypes associated with queen or worker castes in primitively and highly eusocial species (e.g. Woodard et al. 2011, 2013; Toth et al. 2014; Harrison et al. 2015; Morandin et al. 2016; Jones et al. 2017; Okada et al. 2017). These lists of differentially expressed genes correlate with variation in behaviours such as egg-laying, provisioning, foraging, and aggression. Recent attention has focused on describing the proximate mechanisms that influence phenotypes of individuals in species whose social organisation may represent a transitional state between solitary and advanced eusociality (Rehan and Toth 2015; Toth and Rehan 2017; Sumner et al. 2018). However, we have a much better understanding of the proximate mechanisms that influence caste phenotypes in highly eusocial species.

Proximate mechanisms of caste phenotypes in highly eusocial societies

Developmental, genetic, and environmental mechanisms that influence differential caste phenotypes have been described in detail for highly eusocial species with developmental castes. In these species, such as ants and some species of bees and wasps, caste determination begins during the larval stages, as a result of nutritional differences between individuals (Haydak 1970; Wheeler 1986; Cnaani et al. 1997). In honeybees, adult workers feed royal jelly to all larvae for the first three days, after which worker-destined larvae are switched to a diet of honey and pollen whereas queen-destined larvae remain on a royal jelly diet (Haydak 1970). These dietary differences result in honeybee queens that are morphologically and behaviourally distinct from

workers. Additionally, a large proportion of detected gene transcripts (~48%) are differentially expressed between honeybee queen and worker destined larvae, and these differences persist in adults, demonstrating a gene expression influence on differential caste phenotypes (Grozinger et al. 2007; Chen et al. 2012). Honeybee worker behaviour is also influenced by environmental signals such as queen mandibular pheromone (QMP), which is produced by the queen and induces workers to remain non-reproductive in the presence of an extant, reproductive, and QMP-producing queen (Slessor et al. 1988).

Proximate mechanisms of caste phenotypes in primitively eusocial societies

Compared to highly eusocial species, we know relatively little about the proximate mechanisms that influence caste traits in species with behavioural castes (Rehan and Toth 2015; Toth and Rehan 2017; Sumner et al. 2018; Taylor et al. 2018). Queens and workers of primitively eusocial species are morphologically similar and therefore undergo similar development from egg to adult. However, some individuals might be biased toward behaviours typical of queens or workers based on the amount and quality of provisions they receive as larvae (Boomsma and Eickwort 1993; Richards and Packer 1994; Kapheim et al. 2011). Queen-destined larvae receive larger provisions than worker-destined larvae; therefore queen-destined larvae become larger adults than worker-destined larvae, putting some individuals at a size advantage or disadvantage during physical conflict. This is important since environmental conditions, such as who else is in a nest, can influence which individuals behave like queens and which behave like workers. In primitively eusocial paper wasp (Polistinae) and sweat bee (Halictidae) societies, caste determination is at least partially influenced by physical interactions between conspecifics (Michener and Brothers 1974; Jandt et al. 2014; Kapheim et al. 2016).

Therefore, the social environment of a nest can have a large influence as to which individuals become reproductive, since larger individuals have an advantage during physical conflict.

The molecular mechanisms that influence caste traits in species with behavioural castes have only recently come to the forefront of insect sociogenomic research (Toth and Rehan 2017; Sumner et al. 2018). In primitively eusocial species, queens and workers differ very little in the proportion of detected gene transcripts that are differentially expressed (~1%; Patalano et al. 2015; Standage et al. 2016). Moreover, a higher proportion of differentially expressed genes are up-regulated in workers compared to queens (Ferreira et al. 2013; Berens et al. 2015; Jones et al. 2017). However, the expression patterns of specific genes that might underlie caste phenotypes are still poorly described in species with behavioural castes (Toth and Rehan 2017; Sumner et al. 2018). Therefore, research investigating the proximate mechanisms that influence caste traits in primitively eusocial societies is desperately needed. Since castes of primitively eusocial species differ behaviourally, studies need comprehensive inter- and intra-caste comparisons of individuals through a complete breeding season. This approach would provide valuable information regarding caste determination and provide descriptions of proximate mechanisms that may have undergone an evolutionary decoupling during social evolution, as proposed in ground plan hypotheses.

A primitively eusocial sweat bee as a model to describe proximate influences on caste phenotypes

There are several hymenopteran families whose social organisation may represent a transitional state between solitary and eusocial behaviour. Sweat bees (family Halictidae) are one of these families and are excellent models to describe the environmental and genetic influences on caste phenotypes since castes of eusocial sweat bee species are morphologically and

developmentally similar, and newly eclosed females are behaviourally totipotent, meaning they are capable of behaving as a queen or a worker (Yanega 1989; Schwarz et al. 2007; Sumner et al. 2018). Halictid castes show incredible inter- and intra-caste variation of important phenotypes characteristic of eusocial castes such as ovarian development, ovipositioning, provisioning, and foraging. This is easily exemplified by the typical life cycle of a eusocial sweat bee in Canada, which can be described in discrete phases (Fig.1.2, pg. 13). During spring (phase 1), foundresses (queens) emerge from their hibernacula, initiate a nest independently, provision brood cells, and lay eggs, which eventually become their first brood of workers in the summer (phases 2 and 3; Schwarz et al. 2007). After these workers eclose in phase 2, queens remain in their nests and lay eggs on provisions provided by workers. This means that over the course of a breeding season, queens transition from expressing provisioning and egg-laying in spring to expressing only egg-laying in summer. Workers vary in egg-laying behaviour. In many species, a proportion of workers lay eggs even in the presence of a reproductive queen, while in other species workers do not (Breed 1976; Packer and Knerer 1985; Wyman and Richards 2003; Schwarz et al. 2007). During summer and early fall (phases 3 and 4), a second brood of males and future queens (gynes) eclose. Males leave the nest, forage for themselves and search for mates. Gynes mate, feed, overwinter, and initiate a nest the following spring, becoming new queens.

The caste phenotypes of eusocial sweat bees are influenced by environmental and molecular factors. Queens can influence whether or not workers become egg layers by manipulating their provisions before an egg is ever laid (Boomsma and Eickwort 1993; Richards and Packer 1994; Kapheim et al. 2011). By providing small larval provisions, queens can make small workers, which can render workers susceptible to physical manipulation by a larger and more aggressive queen (Michener and Brothers 1974; Pabalan et al. 2000; Kapheim et al. 2016).

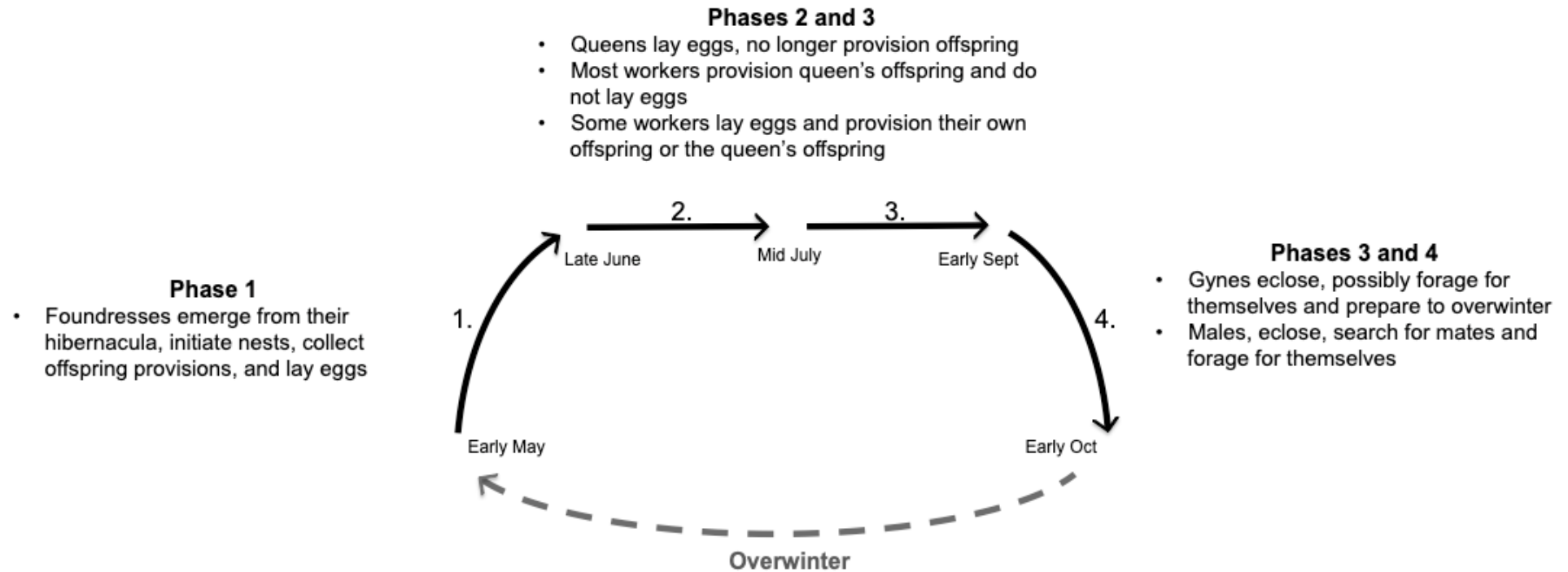


Figure 1.2. The typical life cycle of a eusocial sweat bee in Canada. Each number next to an arrow represents the corresponding phase that occurs between the two dates. Foundresses emerge from their hibernacula and provision their first brood (workers) during spring (phase 1). During summer (phases 2 and 3), queens remain in the nest, no longer provision offspring, and lay eggs for a second brood. This second brood is provisioned by workers, some of which provision and lay their own eggs. During summer / early fall (phases 3 and 4) the second brood of males and future queens (gynes) eclose, mate, and possibly forager for themselves. Gynes then overwinter and initiate a nest the following spring, becoming new queens

Queens can also manipulate worker behaviour by limiting the availability of males in the first brood. Therefore, workers are less likely to mate, and in some species this results in workers that are less likely to develop their ovaries (Breed 1976; Packer and Knerer 1985; Wyman and Richards 2003). Local weather conditions can also influence the size and number of workers a queen produces, which can impact how well a queen can limit egg laying by her workers (Richards and Packer 1996)

The molecular mechanisms that influence egg-laying and parental care phenotypes are not well studied using castes of sweat bee species. Only a couple of studies have described molecular mechanisms that influence queen and worker egg-laying behaviour. In a tropical sweat bee, *Megalopta genalis*, queens express higher concentrations than workers of Vitellogenin, a protein that is essential for ovarian development (Kapheim et al. 2012). Reproductive *M. genalis* females (solitary and social) have higher levels of Juvenile hormone than non-reproductive females. Moreover, Juvenile hormone is expressed in higher concentrations in queens compared to workers of highly eusocial species as well (Smith et al. 2012).

Lasioglossum laevissimum is a eusocial sweat bee in the subgenus *Dialictus*. It has been studied previously in Calgary, Alberta (Packer 1992), and Cape Breton, Nova Scotia (Packer et al. 1989). In both of these locations, *L. laevissimum* populations exhibit obligate primitive eusociality and have high levels of worker ovarian development. This suggests that queens have weak control of worker egg-laying behaviour, which provides excellent opportunities for inter- and intra-caste comparisons of the environmental and molecular influences on caste phenotypes. Therefore, *L. laevissimum* queens and workers can be used to investigate the proximate mechanisms that influence caste traits in a species with behavioural castes.

Thesis objectives

The major objective of this thesis is to investigate underlying environmental (social) and genetic factors that contribute to observable variation in caste phenotypes of a primitively eusocial sweat bee, *L. laevissimum*.

In chapter two, I describe the colony phenology, social characteristics, and individual morphometric data for a *L. laevissimum* aggregation located at Brock University, St. Catharines, Ontario. I take advantage of a natural experiment, the occurrence of nests with a queen (queenright) and nests without a queen (queenless), to investigate the influence of the social environment on a worker trait associated with egg-laying, ovarian development. Queenright and queenless nests were excavated throughout the breeding season and the physical traits of queenright and queenless workers were compared to investigate the direct influence that queens may have over worker reproduction.

In chapters three and four, I use the ‘candidate gene approach’ (Fitzpatrick et al. 2005) to investigate the association between the expression levels of specific genes and the expression of caste traits. One of these traits was the level of ovarian development, which can be used to estimate future egg-laying behaviour since ovarian development is essential for laying eggs. In chapter three I compare *vitellogenin* gene expression between castes to investigate whether or not queens and workers differ in the expression levels of a gene associated with egg-laying. Another caste trait was whether or not an individual was caught while actively foraging, which is necessary for provisioning brood. In chapter four I compared queen and worker expression levels of the *foraging* gene, which is associated with foraging and provisioning behaviour in other insects. For both of these chapters I designed gene specific primers of target and reference genes used in real time quantitative-PCR (RT-qPCR) experiments in a non-model organism.

Finally, in chapter five I take the results from chapters two, three, and four, and provide a description of some of the proximate mechanisms that influence caste phenotypes in a species with behavioural castes. I demonstrate that the important difference between queens and workers in *L. laevissimum*, is differential egg-laying, not differential provisioning behaviour. Furthermore, the expression of genes associated with these two phenotypes reflects this argument. I argue for a modification to the ovarian ground plan hypothesis, which applies specifically to the evolution of eusocial behaviour in bees and possibly other eusocial animals. I hypothesize that during the evolution of eusocial bee taxa, the expression of egg-laying phenotypes and their molecular mechanisms may have become biased towards queens and away from workers through evolutionary time. Provisioning behaviour became differentially expressed between queens and workers when eusocial taxa with developmental castes evolved a life history trait in which nest initiation required both queens and workers.

Chapter 2: Investigating queen influence on worker behaviour using comparisons of queenless and queenright workers

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Author contributions: DNA and MHR designed the experiment. DNA collected, measured specimens, and analyzed the data. MHR provided equipment. DNA wrote and MHR edited the manuscript.

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ABSTRACT

Female eusocial sweat bees are capable of behaving as queens or workers. Relatively few females become queens, and those that do can directly manipulate the reproductive behaviour of other females in the nest. We collected *Lasioglossum (Dialictus) laevissimum* workers from nests with and without queens (queenright and queenless nests, respectively) to investigate the influence queens exert on worker behaviour via direct manipulation. Overall, very few *L. laevissimum* workers (17%) had developed ovaries in Ontario, but queenright and queenless workers were equally likely to have developed ovaries and worn mandibles. However, queenless workers were more likely to be mated than queenright workers. These results suggest first, that queens inhibit egg-laying in most, but not all workers, and second, that queen behaviour during the first few days of workers' adult lives exerts a lasting influence on worker behaviour. We also compared social traits of *L. laevissimum* and other *Dialictus* species using principal components analysis. A strong correlation between worker reproduction and male availability suggests that queen manipulation of the worker brood sex ratio has evolved as an indirect mechanism for queens to discourage worker reproduction.

INTRODUCTION

Newly eclosed females of obligately eusocial sweat bees (family Halictidae) are totipotent, meaning that they are capable of expressing the entire range of behaviours typical of queens or workers (Yanega 1989, 1990; Schwarz et al. 2007). Any newly eclosed female should be able to found a nest, construct brood cells, guard the nest entrance, mate, lay eggs, and provision brood (Schwarz et al. 2007). Despite this flexibility, in most eusocial colonies, foundress queens more or less monopolize egg-laying, while workers provide the ergonomic inputs necessary for raising the queen's offspring (e.g. construction of brood cells and foraging for larval provisions). Most workers are non-reproductive, exhibiting no ovarian development even if they have mated, and thus they are altruistic helpers raising queens' offspring (Kukuk and May 1991; Wyman and Richards 2003; Richards et al. 2015). However, in many species, at least some workers are reproductive: they have well developed ovaries and often are mated, meaning that they can produce either male or female offspring (Breed 1976; Packer and Knerer 1985; Wyman and Richards 2003; Schwarz et al. 2007). Successful reproduction by workers has been documented using genetic analyses for several species of sweat bees (Mueller et al. 1994; Packer and Owen 1994; Richards et al. 1995, 2005; Paxton et al. 2002; Soro et al. 2009)

The behaviour of sweat bee queens, altruistic workers, and reproductive workers has different fitness outcomes. Queens that can successfully manipulate workers into helping raise brood, have higher fitness than if they themselves provided the ergonomic inputs for brood production (Crespi and Ragsdale 2000; Wade 2001; Richards et al. 2005). Therefore, it is advantageous for queens to exploit their own daughters to help produce siblings. From the workers' perspective, laying eggs results in higher fitness than helping the queen to raise siblings (Richards et al. 2005; Kapheim et al. 2015), so it is advantageous for workers to produce their

own offspring, if they can. This may often result in queen-worker conflicts, which queens are likely to win. If workers cannot reproduce because of queen interference, they can still accrue indirect fitness by behaving altruistically (Wenseleers et al. 2004; Ratnieks and Helantera 2009; Bourke 2011).

Direct versus indirect queen influence on worker behaviour

Species and populations of eusocial sweat bees vary from weakly to strongly eusocial, depending on how well queens control worker reproduction (Breed 1976; Packer and Knerer 1985; Wyman and Richards 2003; Richards et al. 2010). Species with strong queen control (“strongly eusocial”) tend to have large numbers of small workers, few males in the worker brood, a small proportion of mated workers, and few workers with ovarian development (Schwarz et al. 2007). In contrast, species with weak queen control (“weakly eusocial”) tend to have small numbers of large workers, a high proportion of males in the worker brood, a high proportion of mated workers, and a high proportion of workers with ovarian development (Schwarz et al. 2007).

Foundress queens influence the behaviour of workers both indirectly and directly. Indirectly, queens manipulate worker reproductive behaviour by manipulating the amount and quality of larval provisions, which determines worker size (Boomsma and Eickwort 1993; Richards and Packer 1994; Kapheim et al. 2011). Pollen provisions for workers are fewer and contain less sugar than those provided to gynes, so workers are often small in size and have few fat stores (Boomsma and Eickwort 1993; Richards and Packer 1994). The small size of workers makes them susceptible to bullying by larger queens. In general, the larger the size difference between queens and workers, the better queens are at preventing workers from laying eggs (Richards and Packer 1996; Strohm and Bordon-Hauser 2003). A second indirect mechanism by

which sweat bee queens manipulate worker behaviour is by modifying the availability of males. When queens produce fewer males early in the colony cycle, workers are less likely to mate, and in some species, are less likely to develop their ovaries (Breed 1976; Packer and Knerer 1985; Wyman and Richards 2003). Even if they do develop their ovaries, an unmated worker cannot produce daughters. Unmated workers can produce their own male offspring, help rear male and female siblings produced by the queen, or help rear nephews produced by their sisters.

Sweat bee queens can also suppress worker reproduction directly by physically manipulating workers. Sweat bee queens are typically the most aggressive females in nests (Michener and Brothers 1974; Pabalan et al. 2000; Kapheim et al. 2016). The majority of aggressive behaviours in *Megalopta genalis* nests are directed from queens toward workers (Kapheim et al. 2016). In fact, the presence of a queen in a nest is enough to prevent *M. genalis* workers from developing their ovaries (Smith et al. 2009). *Lasioglossum zephyrum* females also show a similar pattern; the queen is the most aggressive and most reproductive female in the nest (Michener and Brothers 1974). This means that the direct aggressive actions of the queen can suppress worker reproduction and promote altruistic behaviour. The death or disappearance of a queen eliminates the direct manipulation she exerts on her workers, which can result in workers developing their ovaries, mating, and foraging more. In some populations, queen disappearance leads to a single worker developing her ovaries (a replacement queen) or an overall increase in worker ovarian development (*M. genalis*, *Augochlorella aurata*, *L. zephyrum*, and *L. malachurum* from Greece), while in others (*L. imitatum* and *L. malachurum* from Austria), workers have similar ovarian development scores in queenright and queenless nests (Michener and Wille 1961; Michener and Brothers 1974; Mueller 1991; Richards 2000; Wyman and Richards 2003; Smith et al. 2009; Soro et al. 2009). Queenless workers in *L. zephyrum* colonies

are more receptive to mating opportunities than queenright workers (Greenberg and Buckle 1981). *Halictus ligatus* workers forage more when their own reproductive opportunities are highest (Richards 2004), while *L. malachurum* workers from queenless nests have less wing wear than queenright workers, which suggests they fly less (Soro et al. 2009).

Environmental factors can influence how effectively queens directly manipulate worker behaviour (Schwarz et al. 2007). Local ecological conditions such as weather influence the number and size of workers a queen produces. For instance, in rainy conditions, queens raise small numbers of small-bodied workers (Richards and Packer 1996). Consequently, they are better able to police worker reproduction and fewer workers develop their ovaries. Under better conditions, queens produce large numbers of large-bodied workers, but are then unable to completely prevent worker reproduction as a high proportion of workers develop their ovaries (Richards and Packer 1996).

Queen influence on worker behaviour in *Lasioglossum laevissimum*

In some eusocial sweat bees, foundress queens frequently die before the end of the breeding season (Breed 1975; Mueller 1996). This creates a natural experiment on queen manipulation, in which we can compare the behaviour of workers in queenless versus queenright nests. In general, the death of a queen should provide workers with more opportunities to produce their own offspring, rather than raising the queen's brood. We used this approach to study worker behaviour in the weakly eusocial sweat bee, *Lasioglossum (Dialictus) laevissimum* nesting in an aggregation in southern Ontario, where almost half of nests become queenless prior to cessation of brood production.

Lasioglossum laevissimum was previously studied in Calgary, Alberta (Packer 1992), where it exhibits high levels of worker mating and ovarian development that suggests relatively

weak, direct queen control of worker behaviour. In Alberta, production of a high proportion of males in the worker brood also suggests relatively weak, indirect manipulation of worker behaviour through sex ratio manipulation. In the current study, we investigate queen influence on the reproductive behaviour of workers by comparing workers in queenless and queenright nests in Ontario. In contrast to Alberta, in Ontario, far fewer workers have developed ovaries and no males are produced in the worker brood. The contrast between Alberta and Ontario bees suggests intraspecific variation in the mechanisms by which queens prevent worker reproduction, and that in Ontario, queens suppress worker reproduction by preventing worker oviposition. We tested this hypothesis by comparing workers from queenright nests to those from queenless nests. Queenless workers should be more likely than queenright workers to raise their own offspring, and so should have more ovarian development and be more likely to mate. Furthermore, if queenless workers perform all the ergonomic inputs required for their own reproduction, they should have more wing and mandibular wear than queenright workers, which can share the ergonomic load required for the queen's reproduction. On the other hand, without direct manipulation by the queen, queenless workers may spend less time foraging and excavating nest tunnels; if so, then queenless workers should have less wing and mandibular wear than queenright workers.

METHODS

Study site

The study site was a garden on the north side of a three-walled courtyard (open towards the south) at Brock University, St. Catharines, Ontario, Canada (N 43° 07' 10'', W 79° 14' 49''). Study dates were 17 April to 10 September 2013, 21 April to 9 September 2014, and 12 April to 11 September 2015.

Nest excavations and colony phenology

In total, 70 nests (19 in 2013, 8 in 2014, and 43 in 2015; Supplementary Table 2.1, pg. 49) were excavated from the edges of the aggregation. Nest excavation data were pooled over years because annual sample sizes were small. Nests were excavated early in the morning to ensure that all occupants were inside. Talcum powder was blown down nest entrances to enhance the visibility of tunnels. Adult females, males, and young larvae were collected directly into liquid preservative (RNA preservative), whereas older larvae and pupae were collected into paraffin-lined containers or microcentrifuge tubes, and raised to adulthood in the laboratory prior to storage. Two nests, nest 174 excavated in week 14 and nest W excavated in week 17 of 2015, were very large (each contained 29 occupants including adults, developing brood, and provision masses) and probably represented two nests accidentally excavated together; the contents of both nests (adults, developing brood, and provision masses) were excluded from analyses.

The colony cycle in St. Catharines comprises four phases and is comparable to that of populations in Alberta and Nova Scotia (Packer et al. 1989; Packer 1992). During phase 1, overwintered foundresses initiate nests and provision Brood 1, which in St. Catharines, consists only of workers. During phase 2, first brood workers provision brood while queens remain in

their nests laying eggs. During phase 3, Brood 2 (gynes and males) emerges, while workers continue to forage and queens remain in the nest. During phase 4, worker provisioning continues after the reproductive brood has emerged.

Characteristics of adult females

Adult females were measured and dissected using a stereomicroscope equipped with an eyepiece micrometer at 8 to 66x magnification. Head width (HW) was measured as the distance across the widest part of the head, including the compound eyes. Wing length (WL) was measured as the length of the costal vein. Head width and wing length were positively correlated ($r=0.57$, $n=153$, $p<0.001$), therefore we used only head width as a measure of body size. Wing and mandibular wear scores are indicators of how much effort females expend in foraging and nest excavation behaviours (Packer 1992; Packer et al. 2007; Richards et al. 2010, 2015). Mandibular wear (MW) was scored from 0 (pristine condition) to 5 (completely worn with no apical tooth remaining). Wing wear (WW) was also scored from 0 (unworn wing margins) to 5 (damage along the entire wing margin). As 80% of females had WW=0 ($n=158$), there was little variation in wing wear, so we did not include this variable in the analyses.

Ovarian development (OD) was scored by giving each developing oocyte a fractional value based on its size relative to a fully developed oocyte (1, $\frac{3}{4}$, $\frac{1}{2}$, or $\frac{1}{4}$) and summing all fractions. An ovarian development score of 0 was assigned to females with thin, transparent ovaries or thickened ovaries but no developing oocytes. Females with at least one $\frac{1}{2}$ -developed oocyte were considered to have large ovaries (Packer 1992). The spermatheca was difficult to locate when empty but was opaque and easy to find when full, so we were confident in designating females as mated but less confident in designating them as unmated.

Identification of queens and workers

Classifying adult females as queens, workers, or gynes was simple early in the colony cycle, but more complex later. All phase 1 females were classified as queens. During phase 2, all mated females were classified as queens and unmated females were classified as workers. By phase 3 males had emerged, so mating no longer distinguished queens from workers, so we used the following rules to classify adult females as gynes, queens, or workers. If the largest female in a nest was worn, mated, and had the highest OD, she was classified as the queen. Females with $OD > 0$ or $MW > 0$ were classified as workers, while those with neither mandibular wear ($MW = 0$) nor developed ovaries ($OD = 0$) were designated as gynes. Workers excavated from nests with live queens were classified as queenright, while workers excavated from nests without queens were classified as queenless. Given our identification criteria, it is unlikely that a replacement queen (born in the worker brood) was misidentified as a foundress queen. During phase 2, there were no adult males around the aggregation or in nests; therefore mated females must have overwintered. During phase 3, a replacement queen (i.e. a worker) could only have been misidentified as a foundress queen if she was the largest female in the nest, had mated, and had enough time to acquire substantial wear and ovarian development. In total, we examined 24 foundress queens (10 in phase 2 and 14 in phase 3), 36 queenright workers (8 in phase 2 and 28 in phase 3), and 101 queenless workers (2 in phase 2, 39 in phase 3, and 60 in phase 4).

We calculated the percent queen-worker size difference as $((\text{queen HW} - \text{worker HW}) / \text{queen HW}) \times 100$. This proportional difference was calculated using queens and workers from the same nest. If there was more than one worker in a nest then one worker was randomly selected for the comparison.

Data analysis

Non-parametric statistics were used for caste comparisons of head width, mandibular wear, and ovarian development among colony phases and colony status (queenright vs. queenless), because mandibular wear and ovarian development were not normally distributed (Shapiro-Wilks test of normality). All statistical analyses are reported in text.

A general linear model was used to quantify how mandibular wear, mating status, and colony status influenced ovarian development scores (response variable). We included the interaction terms of mandibular wear and colony status as well as mating status and colony status in the model because sweat bee ovarian development is known to vary with wear (Richards 2003; Richards et al. 2015) and mating status (Richards 2001; Richards et al. 2015). We confirmed that the data fit the assumptions of general linear models by plotting the residuals against the fitted values and visualizing the normal quantile-quantile plot of the residuals. The relationship between ovarian development and each independent variable was not expected to change from year to year, so collection year was not included in the model. Variation in ovarian development among colony phases was examined in the intra-caste comparisons.

A principal components analysis (PCA) was used to compare social traits of *L. laevissimum* with several other members from the same subgenus, *Dialictus* (Table 2.1, pg. 40), based on summaries provided by Breed (1976) and Packer (1992), as well as *L. aeneiventre* data from Wcislo et al. (1993). The variables used in this comparison were the proportion of mated workers, queen-worker size dimorphism, proportion of fecund workers, number of females per nest, proportion of males in Brood 1, and queen longevity (described in Breed 1976). Queen longevity was scored as follows: when queens are almost always replaced as 1, when queens are sometimes replaced as 2, or when queen replacement was rare (or unknown) as 3. Populations

for which data was available for all variables were used in the PCA. Principal components with eigenvalue ≥ 1 were considered to be statistically significant. Three species names have recently changed (Gibbs 2010, 2011); *L. versatum* was studied as *L. rohweri* (Breed 1975), *L. trigeminum* was studied as *L. versatum* (Michener 1966), and *L. gotham* was studied as *L. laevissimus* (Batra 1987; and referred to as *L. near laevissimum* in Packer 1992). In light of these name changes, the identity of *L. laevissimum* individuals from the aggregation at Brock University were confirmed by Jason Gibbs, Thomas Onuferko, and DNA barcodes.

All statistical analyses were carried out using R version 3.3.0 running under R-Studio version 0.99.902. The *lm* command was used for the general linear model and the *prcomp* command was used for the PCA. PCA biplots were visualized using the *fviz_pca_biplot* command from the R package *Factoextra* version 1.0.5.

Data resources

Data underpinning the analyses in this study was deposited in the Brock University Digital Repository

RESULTS

Colony phenology and brood development

During phase 1 (late April to late June), foundresses foraged to provision Brood 1, and only one foundress was ever observed entering or leaving each nest (based on observations of at least 50 nests). After foundress foraging ended, there was a lull in aboveground activity lasting several weeks.

Phase 2 (late June to mid-July) began with an escalation in foraging activity, as Brood 1 workers emerged. Multiple females (workers) were observed leaving and returning to nests, often with another female guarding the nest entrance. Nests excavated during phase 2 contained queens, workers, developing larvae, and provision masses (Fig.2.1, pg. 43). Queenright nests contained an average of 0.8 ± 0.6 workers ($n = 10$ excavated nests). One queenless nest excavated had 2 workers and 3 nests had no workers. No adult males were found in excavated nests during phase 2, and no males were seen flying around the aggregation.

Phase 3 (mid-July to early September) began with the emergence of Brood 2 adults (males and gynes). Excavated nests contained queens, workers, gynes, males, provision masses, larvae, and pupae (Fig.2.1, pg. 43). Excavated nests with queens contained 2.0 ± 1.4 adult workers ($n = 14$ nests), and queenless nests contained 1.5 ± 1.6 workers ($n = 26$; Kruskal-Wallis $X^2 = 1.97$, $df = 1$, $p = n.s.$). Over the whole Brood 2 production period (phases 2 and 3), there was an average of 1.5 ± 1.6 workers in queenright nests ($n = 24$) and 1.4 ± 1.5 workers in queenless nests ($n = 30$; Kruskal-Wallis $X^2 = 0.42$ $df = 1$, $p = n.s.$). Only 44% of nests ($n = 54$ nests) contained a queen during Brood 2 production, and phase 3 nests were less likely to have queens (14/40 nests) than phase 2 nests (10/14 nests; $X^2 = 4.20$, $df = 1$, $p = 0.041$).

Phase 4 (early September – early October) was characterized by continued worker provisioning of brood. All phase 4 nests were queenless, containing workers, gynes, males, and provision masses but no larvae or pupae (Fig.2.1, pg. 43).

Characteristics of queens

In general, queens were larger than workers (mean HW = 1.67 ± 0.08 mm; Fig.2.2, pg. 44). Most queens had highly worn mandibles (MW median 4; Fig.2.3, pg. 45). Almost all queens (87.5%, $n = 24$) had at least one $\frac{1}{2}$ -developed oocyte, and the median ovarian development score was 2.5 (Fig.2.4, pg. 46). All queens were mated. There were no differences in queen size or ovarian development between phases 2 and 3 (HW - Kruskal-Wallis $X^2 = 0.38$, $df = 1$, $p = \text{n.s.}$; MW - Kruskal-Wallis $X^2 = 0.21$, $df = 1$, $p = \text{n.s.}$; OD - Kruskal-Wallis $X^2 = 1.76$, $df = 1$, $p = \text{n.s.}$).

Characteristics of workers

Worker traits changed over time, especially in Phase 4. Phase 4 workers were larger (Fig.2.5, pg. 47; Kruskal-Wallis $X^2 = 11.88$, $df = 2$, $p = 0.003$), had less mandibular wear (Kruskal-Wallis $X^2 = 5.95$, $df = 2$, $p = 0.051$), lower total OD scores (Fig.2.5, pg. 47; Kruskal-Wallis $X^2 = 5.70$, $df = 2$, $p = 0.058$), and were less likely to be mated than earlier in the colony cycle (32/63 in phase 3 vs. 20/60 phase 4 respectively; Fisher's exact test $p = 0.068$). Of 133 dissected workers, 23 (17.3%) had at least one $\frac{1}{2}$ -developed oocyte and 24 had total OD ≥ 0.75 , the minimum OD score in queens (Fig.2.5, pg. 47).

Since there were no queenright workers in phase 4, we investigated how colony status (queenright vs. queenless), size, wear, and mating status influenced worker ovarian development during phases 2 and 3 (Table 2.2, pg. 41). Workers with more mandibular wear and those that were mated had higher OD, and this relationship did not differ between queenright and queenless

workers. In phases 2 and 3, a similar proportion of queenless (28%) and queenright (21%) workers had OD scores greater than or equal to 0.75 (11/40 vs. 7/33 respectively; Fig.2.5, pg. 47; $X^2 = 0.12$, $df = 1$, $p = n.s.$).

Comparisons of queens and workers

If queens directly manipulate worker behaviour then queenless workers (released from queen control) potentially could be queen-like. Comparisons are limited to phases 2 and 3, as no queens were found during phase 4. Queens were larger than workers, regardless of colony status (Fig.2.2, pg. 44; Kruskal-Wallis $X^2 = 13.77$, $df = 2$, $p = 0.001$), and queens were $4.6\% \pm 3.2\%$ larger than their own workers ($n=21$ comparisons). All three groups had similar mandibular wear scores (Fig.2.3, pg. 45; Kruskal-Wallis $X^2 = 5.11$, $df = 2$, $p = n.s.$). Queens had higher ovarian development than either group of workers (Fig.2.4, pg. 46; Kruskal-Wallis $X^2 = 42.15$, $df = 2$, $p < 0.001$). All queens were mated, compared to 65% of queenless and 18% of queenright workers (26/40 vs. 6/33 respectively; Fisher exact test $p < 0.001$); so queenless workers were more like queens with respect to mating status.

Inter-species comparisons

Social traits of Ontario and Alberta *L. laevissimum* populations are compared to those of nine additional species from the same subgenus, *Dialictus*, in Table 2.1, pg. 40. The first 3 principal components had eigenvalues >1 and explained 88.7% of the variation in the data set (Table 2.3, pg. 42). PC1, which explained 43.1% of the variance among populations, was most strongly influenced by queen-worker size difference and colony size, which were positively correlated. PC2 was most strongly influenced by the proportion of fecund workers and the proportion of males produced in the worker brood, which were also positively correlated. PC3 was most strongly influenced by queen longevity. The two *L. laevissimum* populations were

strongly differentiated along factor 2 (Fig.2.6, pg. 48), with Ontario *L. laevissimum* producing no males in the worker brood and having far fewer workers with large ovaries (Table 2.1, pg. 40). Interspecific variation was greater along PC1 (Fig.2.6, pg. 48); species with larger colonies had a larger queen-worker size difference (Table 2.3, pg. 42).

DISCUSSION

Queen influences on worker egg-laying

The main indicators of worker reproduction in sweat bee studies are the proportions of workers with developed ovaries and the proportions of workers mated. In Ontario *L. laevissimum*, only about 17% of workers have well developed ovaries, far fewer than in Alberta, where about 63% of workers have developed ovaries, and lower than almost all other populations from the same subgenus, *Dialictus* (Table 2.1, pg. 40). In contrast, 40% of Ontario *L. laevissimum* workers are mated, which is slightly higher than in Alberta (35%) and the second highest proportion of any *Dialictus* population (Table 2.1, pg. 40). Within *L. laevissimum*, variation in the proportion of fecund workers suggests that Ontario queens are highly effective at preventing worker ovarian development and probably monopolize oviposition of reproductive brood (Brood 2). While the majority of Alberta workers have developed ovaries, genetic analyses suggest they rarely produce offspring (Packer and Owen 1994). Thus in both Ontario and Alberta, queens likely lay most of the eggs that produce males and gynes, but the mechanism by which they interfere with workers is clearly different.

We predicted that if *L. laevissimum* queens manipulate worker behaviour through continuous aggression, then workers with queens should have less ovarian development, be less likely to mate, and possibly have less worn mandibles than queenless workers. Only one of these three predictions was supported: queenright workers were less likely than queenless workers to mate, but equally likely to have developed ovaries or worn mandibles. This suggests that queens only influence whether workers mate, but do not influence whether workers develop their ovaries or how much work they do. However, a more likely explanation is that queens exert their influence on workers during a sensitive period in the first few days after worker eclosion, with

long-lasting effects. In a Greek *L. malachurum* population queens suffered artificially early mortality due to insecticide spraying, this resulted in unusually high numbers of workers with developed ovaries (Richards 2000; Wyman and Richards 2003). Early queen manipulation of worker behaviour with permanent effects would explain why the proportions of altruistic and reproductive workers are similar in queenright and queenless nests of *L. laevissimum*, as well as *L. imitatum* and *L. malachurum* (Michener and Wille 1961; Soro et al. 2009).

Queen suppression of worker reproduction shortly after worker emergence would suggest that the oldest workers are the most likely to become non-reproductive altruists, because the oldest workers are the most likely to emerge into colonies with large, viable queens. Younger workers would be more likely to emerge in colonies with dead or dying queens. If queens mainly focus suppressive influence on their oldest workers, then within populations, we would expect the proportion of reproductive workers to correlate with colony size. This pattern was observed in a *H. ligatus* population in which brood production varied from year to year (Richards and Packer 1995). When queens produced large numbers of large-bodied workers, there was more worker reproduction than when queens produced fewer, smaller workers (Richards and Packer 1996).

The hypothesis that queens mostly suppress reproduction by early-emerging workers is consistent with the observation that in many eusocial sweat bees, there are often reproductive workers in colonies with queens (Kukuk and May 1991; Richards and Packer 1995, 1996; Wyman and Richards 2003; Richards et al. 2015). Evidently, queens allow some workers to reproduce. Although we often refer to how effectively queens prevent worker reproduction, this is based on the assumption that it is in a queen's interest always to monopolize oviposition. However, sweat bee queens lay large eggs and their egg-laying capacity may often be

outstripped by the ability of workers provision brood cells faster than a queen can lay eggs on them. Under these conditions, queens should favour worker egg-laying (Kukuk and May 1991).

Queen influence on worker mating

In Ontario *L. laevissimum*, proportionately more queenless than queenright workers were mated. Queens potentially influence worker mating both directly and indirectly. How queens can prevent workers from mating outside the nest is not at all clear, but in laboratory colonies, *L. zephyrum* queenright workers resisted mating outside of the nest, but queenless workers mated (Greenberg and Buckle 1981). Indirectly, queens can influence mating behaviour by controlling the sex ratio of Brood 1. In our study population, no males at all are produced in Brood 1, as is also true of *L. gotham*, *L. imitatum*, and *L. versatum* (Table 2.1, pg. 40). However, in other populations, including Alberta *L. laevissimum*, substantial numbers of males are produced in Brood 1. The timing of worker mating may influence whether a worker opts to develop her ovaries. Interestingly, mated workers were more likely to have high ovarian development scores, a pattern observed in some eusocial sweat bee populations but not others (Richards 2001; Packer et al. 2007; Albert and Packer 2013; Richards et al. 2015). This suggests that mating status may contribute to, but does not control, whether or not a sweat bee worker develops her ovaries.

Queen influence on worker ergonomic inputs

If eusocial sweat bee workers are more likely to work when directly forced by queens, then we expect queenright workers to be more worn than queenless ones. Alternatively, if sweat bee workers are more likely to work when provisioning their own brood, then queenless workers should be more worn. We found that queenright and queenless workers had similar amounts of mandibular wear, which means they likely engaged in similar amounts of brood cell and tunnel excavation. However, workers with high ovarian development had higher mandibular wear

scores, regardless of the queen's presence in the nest, which suggests that in *L. laevissimum*, reproductive workers do more excavation because they need brood cells in which to lay eggs. In contrast, comparisons of *L. malachurum* workers show that queenright workers had more wing wear than queenless workers (Soro et al. 2009), but had similar levels of ovarian development, which suggests that workers with ovarian development were not working more. In general, it is not clear whether sweat bee workers invest more in their own offspring or in queen offspring, as altruistic workers are more worn in some populations (Richards 2001; Richards et al. 2015) and less worn in others (Richards 2004).

Life without a queen

In all three *L. laevissimum* populations studied so far, new offspring provisions were found in nests well after males and gynes eclosed (Packer et al. 1989; Packer 1992). It is unclear why workers continue collecting provisions so late in the breeding season, since it is unlikely that these produce brood before the onset of cold weather in autumn. However, if workers are still alive in autumn, they probably simply continue provisioning brood until they die.

It is possible that some of the workers produced late in Brood 2 were meant to be gynes but became workers instead (Packer et al. 1989). In southern Alberta, the emergence of Broods 1 and 2 overlapped in nests with more than one queen, so some of the workers produced late in Brood 2 may have been gynes that became workers instead (Packer et al. 1989). In our study population, phase 4 workers were larger, less worn, less likely to be mated, and had less ovarian development compared to workers in phase 3. There were also large females with little wear but developed ovaries. Together these observations suggest that large females that emerge late in summer may become either workers or gynes. In other eusocial sweat bees, worker-sized queens

are found occasionally, and these are worker brood females that become queens rather than workers (Wyman and Richards 2003; Richards et al. 2015).

The intraspecific variation in social characteristics between Ontario and Alberta *L. laevissimum* populations contrasts with that observed in *L. malachurum* populations. Populations of *L. malachurum* from the United Kingdom to Greece show very little variation in the number of males produced in the worker brood (0% - 2.3%), but wide variation in the proportion of reproductive workers (3% - 61%; Packer and Knerer 1985; Wyman and Richards 2003). *Lasioglossum malachurum* also exhibits clinal variation in several social traits, including the proportion of reproductive workers, the number of workers in the first brood, the number of worker broods, and queen-worker size difference (Packer and Knerer 1985; Wyman and Richards 2003). It seems likely that within species, queens adjust the colony social environment to reduce worker reproductive behaviour.

Interspecific comparisons

We investigated the relationships among behavioural traits related to colony social organisation among *Dialictus* species with a principal components analysis. In general, species with large colonies composed of small workers had fewer reproductive workers, fewer mated workers, and fewer males in the worker brood, which is consistent with strong queen control of worker behaviour (Schwarz et al. 2007). The relationships among social characteristics were consistent with Breed's (1976) original analysis. In our analysis, the size of colonies and queen-worker size difference explained more variation among *Dialictus* species (loaded more on PC1) than the proportion of fecund workers. The proportion of males in the worker brood correlated best (loaded in the same direction on PC2) with the proportion of fecund workers. Furthermore, the proportion of fecund workers and queen longevity were only weakly correlated on a single

principal component axis (PC3), which suggests that worker reproduction is not dependent on the total life span of their queen. These results are consistent with the PCA results for *Lasioglossum (Evylaeus)*; Wyman and Richards 2003) in which the proportion of males in the worker brood was also significantly correlated with the proportion of fecund workers. This supports the hypothesis that queen manipulation of the worker brood sex ratio has evolved as an indirect mechanism to discourage worker reproduction. Although unmated workers can lay eggs, they cannot produce daughters. Producing females earlier than males creates temporal bias in the reproductive brood sex ratio and sets up a situation in which worker inclusive fitness is enhanced more by producing sisters than sons (Hamilton 1964; Trivers and Hare 1976; Richards et al. 1995). *Dialictus* queens that provide workers with a favorable sex ratio may be more successful at manipulating worker reproductive behaviour because workers have a fitness incentive to stay and maintain the current sex ratio.

Conclusions

In Ontario *L. laevissimum*, queens likely exert their long-lasting suppressive influence on worker reproduction in the first few days after worker eclosion. Furthermore, a small proportion of reproductive workers occur in queenright nests, probably because queens exert their influence on their first (oldest) workers, and the queen's ability to influence new workers fades as the colony size increases.

Lasioglossum laevissimum queens from Ontario and Alberta both monopolize oviposition of reproductive brood. However, Ontario queens prevent most of their workers from developing their ovaries in the first place; Alberta queens do not prevent worker ovarian development, but do prevent worker egg-laying. This suggests that intraspecific variation in queen manipulation of worker behaviour is mainly due to direct behavioural interference. In contrast, comparisons

among *Dialictus* species suggest that the proportion of fecund workers is lowest when queens limit worker access to males. Thus, interspecific variation may be more strongly related to indirect queen manipulation of worker behaviour.

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Table 2.1. Social variation among ten species of *L. (Dialictus)* species. Terms in parentheses are used in the principal components analysis of the eight species with complete information (Fig.2.6, pg. 48). Data for the southern Ontario *L. laevisissimum* population are from the current study. Data for the remaining *L. (Dialictus)* populations were summarised by Breed (1976) or Packer (1992). Note that taxonomic revisions (Gibbs 2010, 2011) have resulted in three name changes: *L. versatum* was originally studied as *L. rohweri* (Breed 1975), *L. trigeminum* was originally studied as *L. versatum* (Michener 1966), and *L. gotham* was studied as *L. laevisissimus* (Batra 1987; and referred to as *L. near laevisissimum* in Packer 1992).

	<i>laevisissimum</i> (laeO)	<i>laevisissimum</i> (laeA)	<i>aeneiventre</i>	<i>gotham</i>	<i>imitatum</i> (imit)	<i>lineatum</i> (line)	<i>rhytidophorum</i> (rhyt)	<i>trigeminum</i> (trig)	<i>umbripenne</i> (Damitas) (umbr)	<i>umbripenne</i> (Turrialba)	<i>versatum</i> (vers)	<i>zephyrum</i> (zeph)
Location	Ontario, Canada	Alberta, Canada	San José Costa Rica	Maryland, USA	Kansas, USA	New York, USA	Panará, Brazil	Kansas, USA	Puntarenas, Costa Rica	Cartago, Costa Rica	Kansas, USA	Kansa, USA
% mated workers (Mated)	39.1	35	68	-	2.5	20	12.9	3	2.5	3.5	37.9	8
% queen-worker size difference (Size)	4.6	7	2	9	9.9	4.4	6	11.9	16.9	10.0	10	9.1
% fecund workers (Fecund)	17.3	63.3	59	53.6	12	28.3	28	25	32	24	9	38
No. females per nest (Number)	2.5	3.5	2.7	6.7	8.1	7	3.8	29.6	75.6	16.7	4.9	14.3
% males in the worker brood (Males)	0	42.6	-	0	0	25	41	5	8	-	0	14
Queen longevity (Long)	3	3	-	3	3	1	2	2	3	2	2	1
Reference	This study	(Packer 1992)	(Wcislo et al. 1993)	Batra 1987	(Michener and Wille 1961)	(Eickwort 1986)	(Michener and Lange 1958)	Michener 1966	(Wille and Orozco 1970)	(Eickwort and Eickwort 1971)	Breed 1975	(Batra 1966)

Table 2.2. Factors contributing to variation in worker ovarian development scores. Workers with high ovarian development had high mandibular wear and were more likely to have mated, regardless of colony status (queenright vs. queenless).

Effects	Coefficient	d.f.	F	p	Adjusted R ²
Model	1.62	65	2.98	0.012	0.14
Head width	-1.23	1	1.13	0.29	
Mandibular wear	0.16	1	7.00	0.010	
Mating status (mated)	0.64	1	8.61	0.005	
Colony status	0.28	1	0.03	0.87	
Mandibular wear * Colony status	-0.04	1	0.19	0.67	
Mating status * Colony status	-0.38	1	0.89	0.35	

Table 2.3. Results of the principal components analysis based on social characteristics of 8 *L. (Dialictus)* species (Table 2.1, pg. 40). Only the first three principal components had an eigenvalue ≥ 1 .

Variable	Factor loading scores		
	PC1	PC2	PC3
% Mated workers	0.41	0.24	-0.48
% Queen-worker size difference	-0.58	-0.16	-0.07
% Fecund workers	0.19	-0.70	-0.17
No. females per nest	-0.54	-0.29	-0.05
% Males in worker brood	0.37	-0.58	0.03
Longevity of queen influence	-0.17	0.01	-0.86
Eigenvalue	2.58	1.61	1.13
Proportion of variance	43.1	26.8	18.8
Cumulative proportion	43.1	69.9	88.7

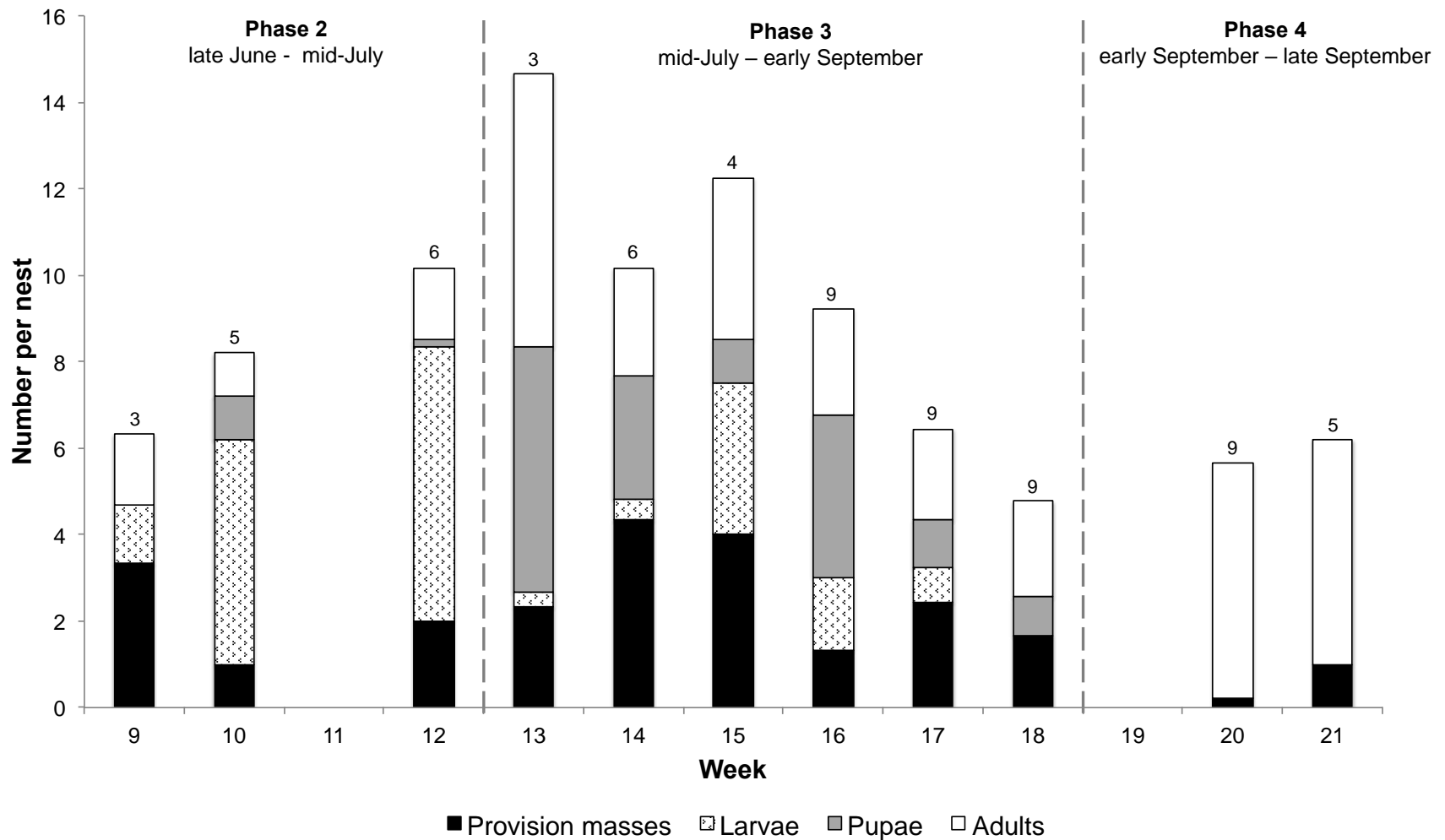


Figure 2.1. Contents of the *L. laevissimum* nests in 2013, 2014, and 2015. Workers emerged and began foraging during phase 2, while males and gynes emerged during phase 3. During phase 4, brood were still being provisioned but no larvae or pupae were found. Numbers above the bars represent the numbers of nests excavated each week. Week 9 was the third week in June and week 15 began at the end of July

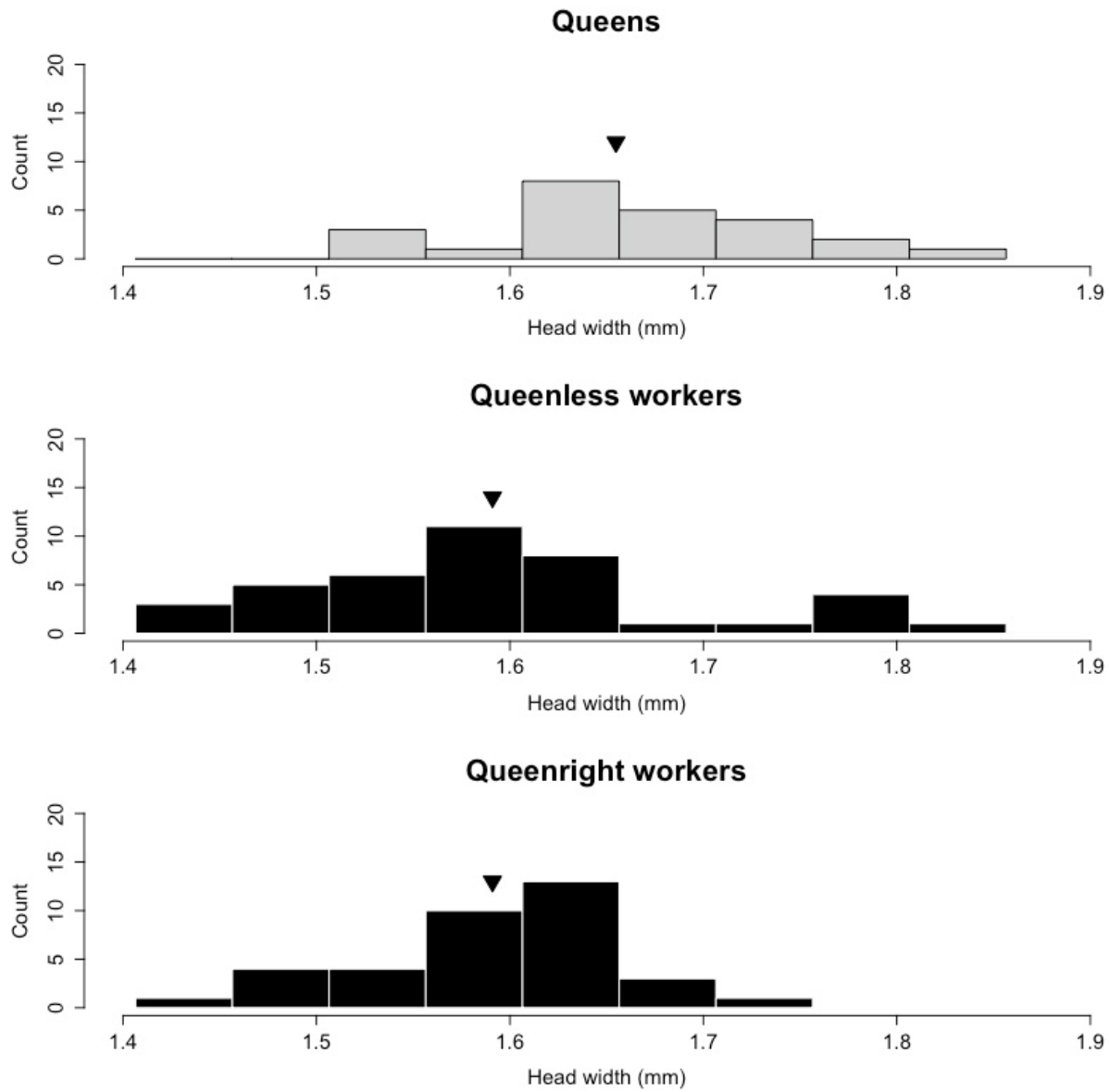


Figure 2.2. Size variation among queens, queenless workers, and queenright workers from phases 2 and 3. Median head widths for each group are indicated with a triangle

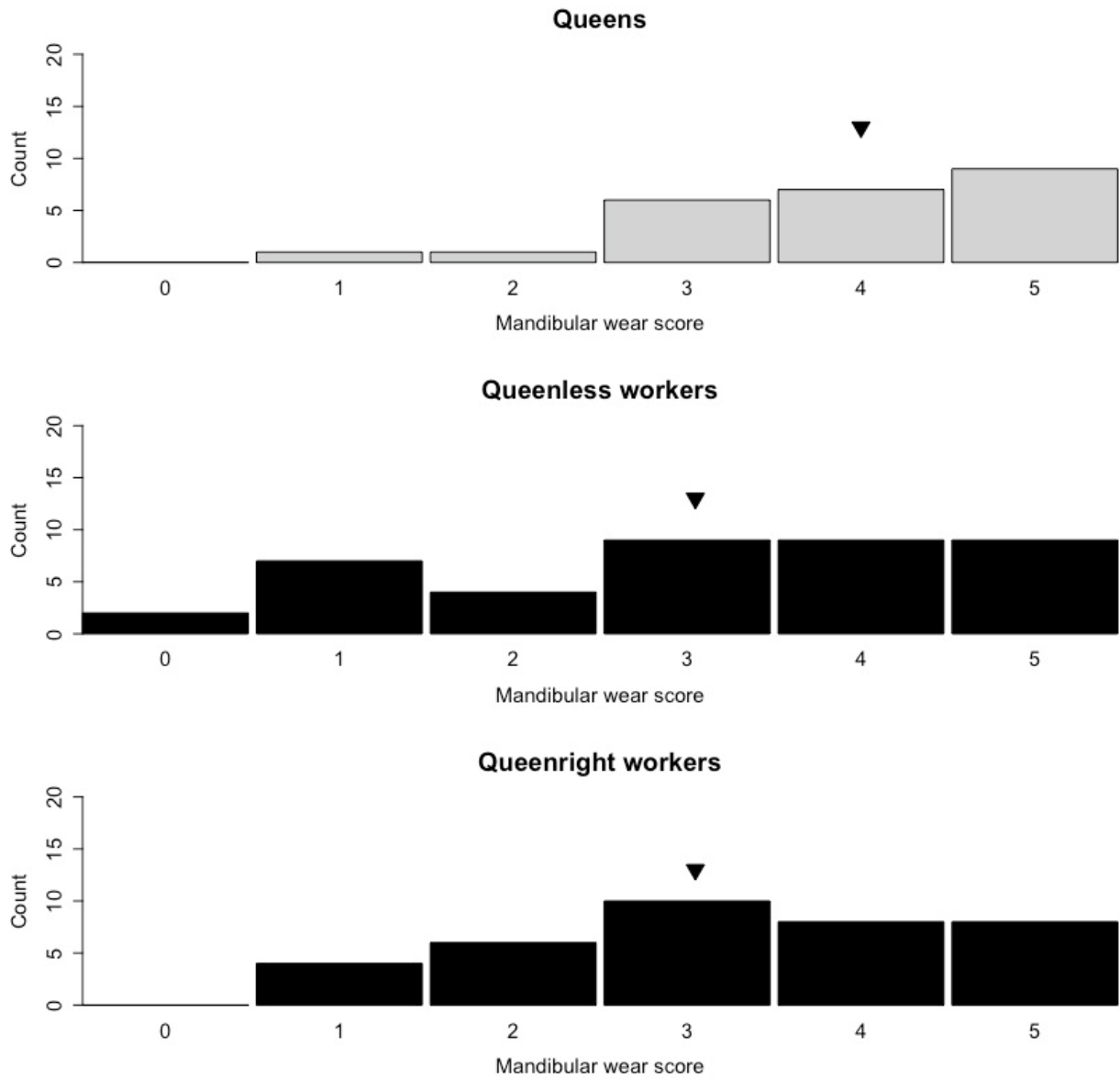


Figure 2.3. Mandibular wear scores of queens, queenless workers, and queenright workers from phases 2 and 3. Median mandibular wear scores for each group are indicated with a triangle

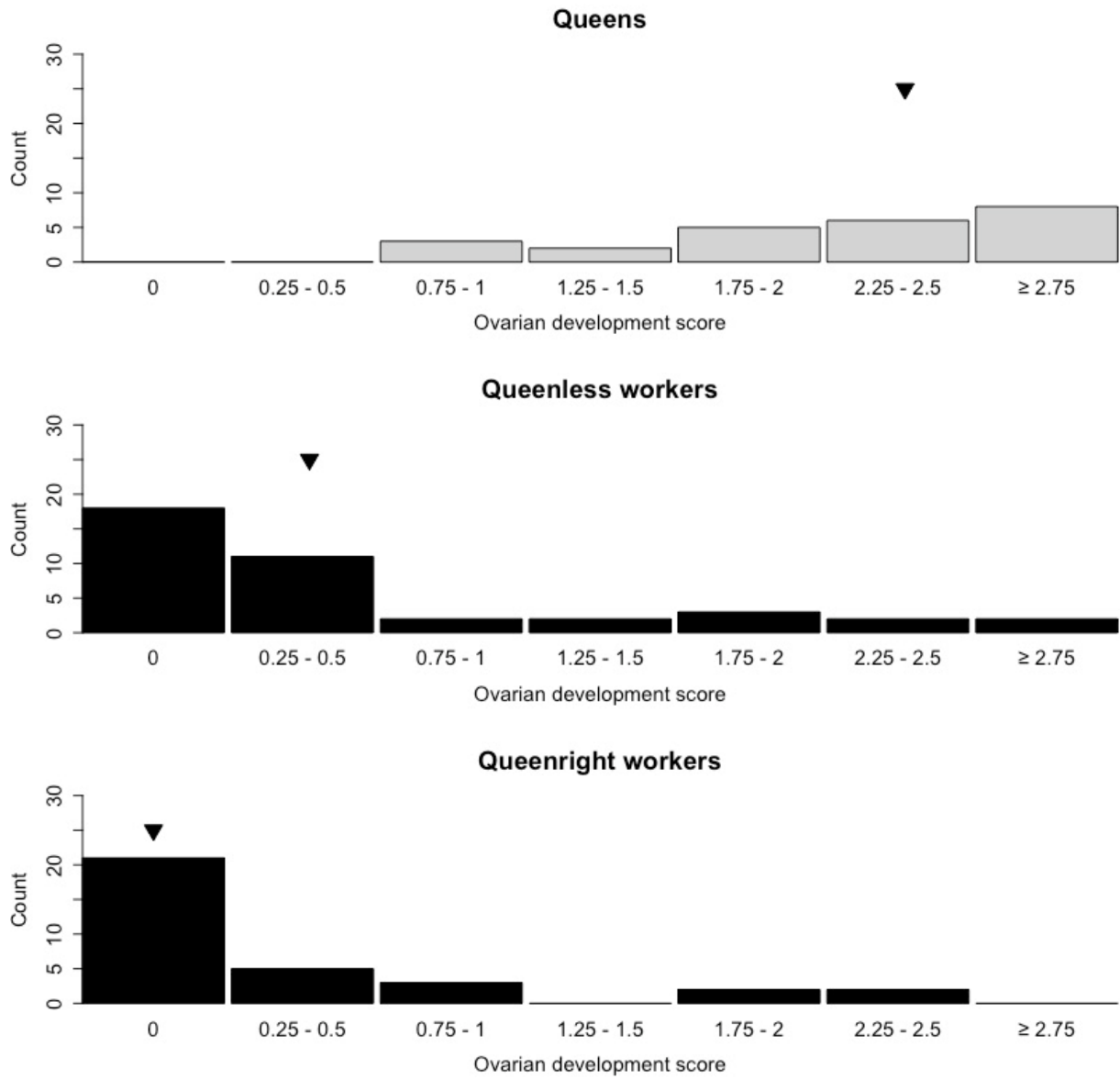


Figure 2.4. Ovarian development scores of queens, queenless workers, and queenright workers from phases 2 and 3. Median ovarian development scores for each group are indicated with a triangle

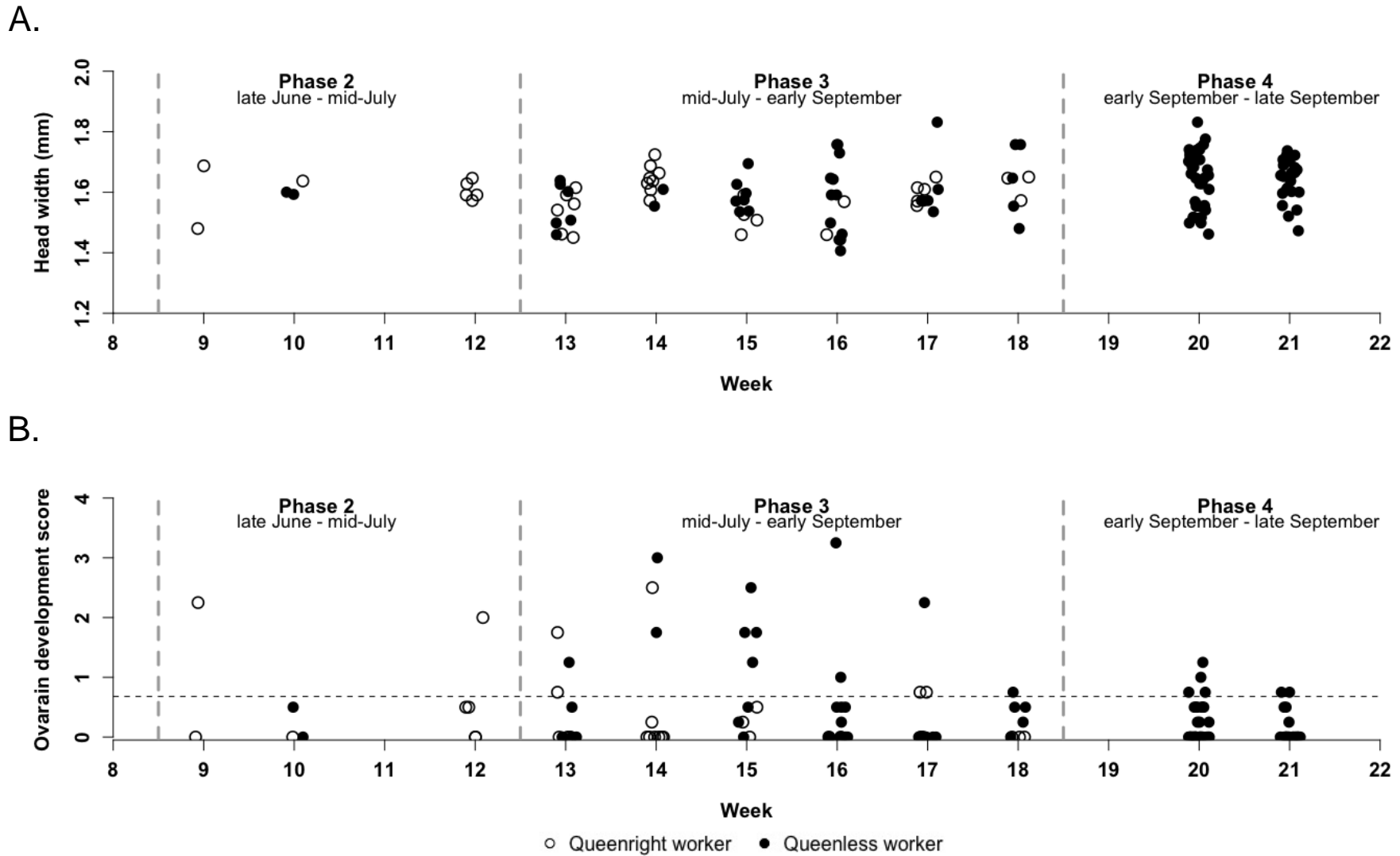


Figure 2.5. Variation in worker size and ovarian development throughout the breeding season. Open circles represent queenright workers and solid circles represent queenless workers **A.** Variation in head width. In general, worker head widths were largest in phase 4 **B.** Worker ovarian development scores compared to the minimum queen score (OD=0.75; dashed horizontal line)

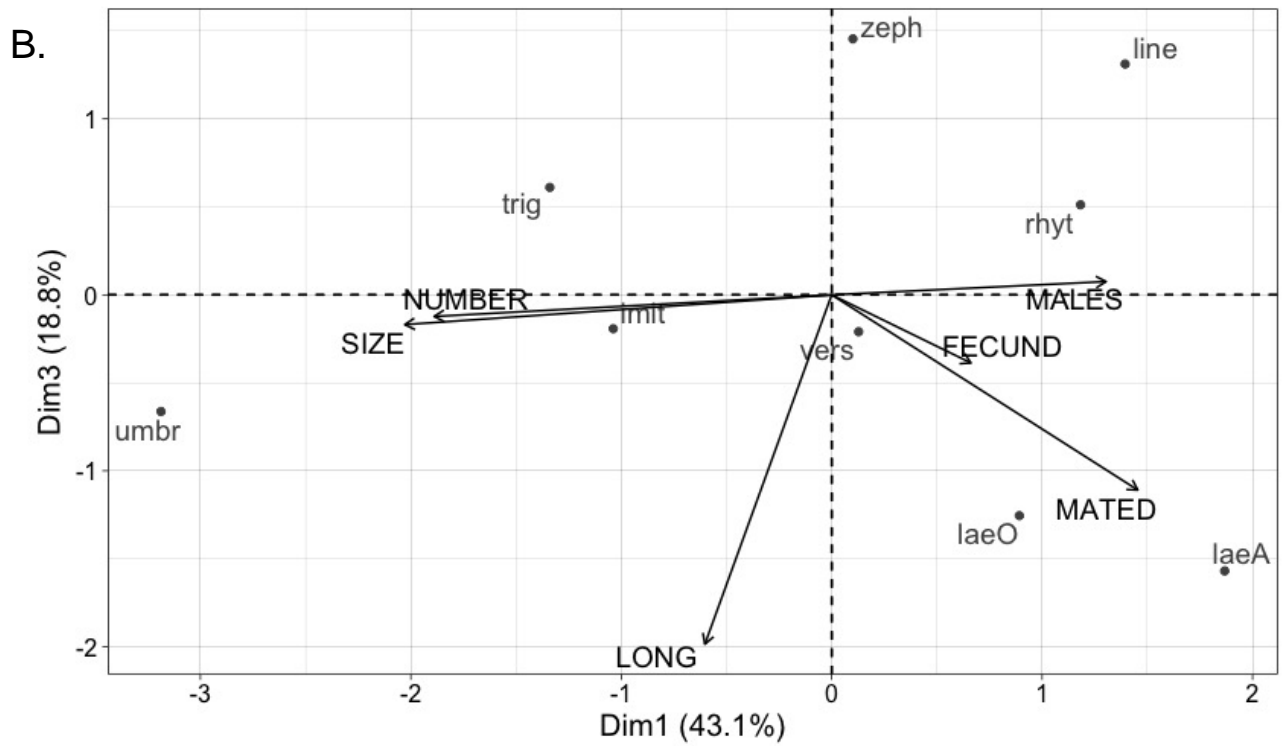
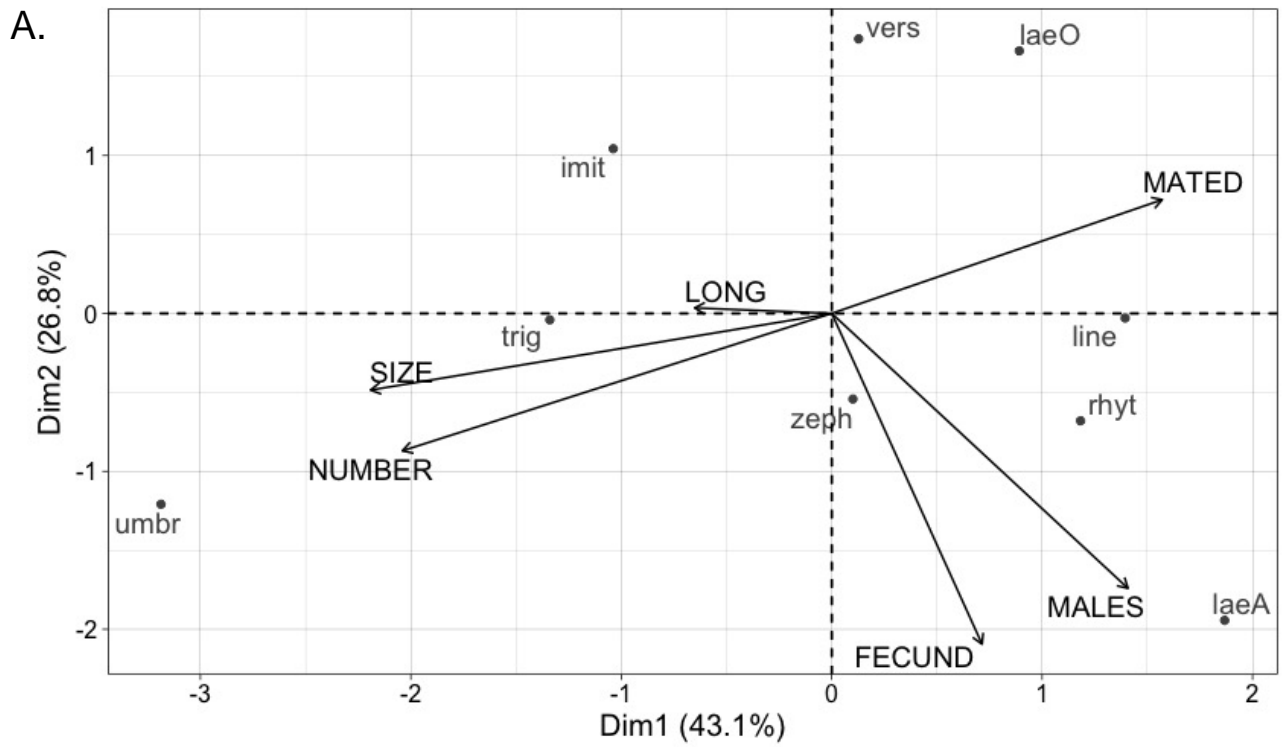


Figure 2.6. Interspecific social variation among eight species of *L. (Dialictus)*. The six variables used in the analysis are presented in Table 2.1, pg. 40. Vectors (arrows) represent the influence of each variable on the significant principal components **A** PC1 and PC2, **B** PC1 and PC3. Variables were mated (proportion of mated workers), size (proportional queen-worker size difference), fecund (proportion of fecund workers), number (number of females per nest, including queens), males (proportion of males in the worker brood), and long (longevity of queens relative to colony lifespan). Further details of the PCA analysis can be found in Table 2.3, pg. 43

Table S2.1. Numbers of *L. laevissimum* nests excavated during the 2013, 2014, and 2015 breeding season in St. Catharines, Ontario, Canada. Nest excavations began after workers started foraging (week 9) and continued until the end of the breeding season. Week 9 was the third week in June and week 15 began the end of July.

Week	No. nests excavated per year			Total
	2013	2014	2015	
9			3	3
10			5	5
11				
12	6			6
13			3	3
14	3		4	7
15			4	4
16		5	4	9
17	5		5	10
18	5		4	9
19				
20		3	6	9
21			5	5

Chapter 3: *Vitellogenin* expression in a primitively eusocial sweat bee, *Lasioglossum laevissimum*

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Author contributions: DNA, AS, and MHR designed the experiment. DNA and AS designed *L. laevissimum* gene specific primers. DNA collected and measured specimens, measured gene expression, and analyzed the data. AS and MHR provided equipment. DNA wrote the manuscript and AS and MHR edited the manuscript.

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INTRODUCTION

Animals that display eusocial organisation are characterized by cooperative brood care, overlapping generations, and a reproductive division of labour (Batra 1966; Wilson 1971; Michener 1974). In eusocial Hymenoptera, one or a few females (queens) lay eggs while the remaining majority (workers) rarely lay eggs and instead, help to raise the offspring of the queen(s). Primitively eusocial insects have morphologically similar castes that differ behaviourally, while highly eusocial species have developmentally and morphologically distinct queens and workers (Michener 1974; Sumner et al. 2018). Regardless of the type of eusociality, the regulation of egg-laying among individuals is at the heart of understanding eusocial organisation, because at least one individual in the group needs to produce offspring for any group member to accrue fitness, directly or indirectly (Fletcher and Ross 1985).

In hymenopteran species, as with other oviparous insects, laying eggs first consists of a female devoting nutrients, otherwise used for her own metabolic needs, to her ovaries. These nutrients are used to supply developing eggs with yolk, which then acts as sustenance for a developing embryo (Sappington and Raikhel 1998; Tufail et al. 2014). Depending on the species, a female lays an egg (oviposits) in either a pre-constructed brood cell with food provisions necessary for the larva to reach adulthood, or lays an egg that will be actively provisioned as the larva grows and develops (Michener 1974, 2000; Cowan 1991).

The ovarian ground plan hypothesis (OGPH) suggests that egg-laying and brood provisioning phenotypes exhibited by solitary ancestors decoupled to be expressed separately in queen and worker castes of highly eusocial descendants (West-Eberhard 1987, 1996; depicted in Chapter 1 Fig.1.1, pg. 7). In primitively eusocial species, which may represent an intermediate stage between solitary and highly eusocial behaviour, a newly eclosed female is capable of

behaving as a queen or a worker (Yanega 1989; Schwarz et al. 2007; Rehan and Toth 2015). In these species, queens and workers can express both egg-laying and brood provisioning phenotypes, though queens lay more eggs than workers (Michener 1974; Schwarz et al. 2007; Sumner et al. 2018). The OGPB also suggested that the expression of genes and molecular mechanisms underpinning ancestral egg-laying and brood provisioning phenotypes decoupled (West-Eberhard 1987, 1996). Genes associated with egg-laying are expressed more in queens than workers, and genes associated with brood provisioning are expressed more in workers than queens. Therefore, descriptions of the molecular underpinnings of egg-laying or provisioning phenotypes in species that represent transitional stages between solitary and eusocial behaviour will provide insights into the evolution of queens and workers (Sumner et al. 2006; Rehan and Toth 2015; Toth and Rehan 2017; Taylor et al. 2018). Specifically, these descriptions may reveal the sequence in which the molecular mechanisms underpinning egg-laying and provisioning phenotypes decoupled through evolutionary time. Furthermore, the decoupling of these molecular mechanisms may have occurred independently in multiple evolutionary lineages with similar assortments of orthologous genes (Toth and Robinson 2010; Rehan and Toth 2015).

***Vitellogenin* influences phenotypes of solitary and social insects**

Vitellogenin (*vg*) is a gene whose mRNA expression and protein (Vg) titer are consistently associated with variation in solitary and social insect phenotypes, particularly egg-laying. *Vitellogenin* encodes a phosphoglycolipoprotein, a large lipid transfer protein, and is conserved among a wide range of organisms including insects, nematodes, and vertebrates (Chen et al. 1997; Sappington and Raikhel 1998; Tufail and Takeda 2008; Tufail et al. 2014). In insects, Vg consists of five conserved domains: the N-terminal domain (N-sheet), a polyserine linker region, an α -helical domain, large β -sheets that form a lipid-binding cavity, and a vWFD

domain at the C-terminus (Tufail and Takeda 2008; Salmela et al. 2016). This conserved protein is a transporter of lipids, carbohydrates, and metals, and is the precursor to the major yolk protein, vitellin, in developing oocytes (Sappington and Raikhel 1998; Tufail and Takeda 2008; Tufail et al. 2014).

Vitellogenin is expressed in response to nutritional signaling cascades, such as Insulin/Insulin-like signaling (IIS), Target of Rapamycin (TOR) signaling, and juvenile hormone (JH) signaling (Hansen et al. 2004; Smykal and Raikhel 2015; Corona et al. 2016; Kapheim and Johnson 2017). In insects, *vg* is primarily expressed and translated in fat bodies located in the head, thorax, and abdomen; however, expression in tissues such as the brain, male and female reproductive tracts, and salivary glands is not uncommon (Guidugli et al. 2005b; Colonello-Frattini et al. 2010; Amsalem et al. 2014; Roy-Zokan et al. 2015; Jedlicka et al. 2016). After translation, Vg is secreted to the hemolymph where it eventually binds to the vitellogenin receptor (VgR) and is taken up by tissues via receptor-mediated endocytosis (Sappington and Raikhel 1998; Tufail and Takeda 2009; Arrese and Soulages 2010). Molecules such as lipids bind to Vg's lipid-binding cavity and are brought into cells with Vg.

In insects, well-known functions of Vg are to facilitate the transport of lipids and carbohydrates from fat bodies to other tissues, such as the ovaries and brain, during ovarian development, juvenile development, and before overwintering diapause (Sappington and Raikhel 1998; Tufail and Takeda 2008). These functions are supported by *vg* expression and Vg titers during the life cycles of many insect species (Supplementary Table S3.1, pg. 96 and summarized in Table 3.1, pg. 78). In accordance with one of Vg's roles as a vitellin precursor, increased head and abdomen *vg* expression, and hemolymph Vg titers are associated with increased ovarian development in insects (Engles 1974; Robinson et al. 1991; Scott et al. 2005; Toth et al. 2009;

Kapheim et al. 2012; Tokar et al. 2014; Lockett et al. 2016; Xiao et al. 2016). *Vitellogenin* expression patterns also reflect insect developmental stages and the timing of overwintering diapause. During development of holometabolous insects, which develop in discrete stages, *vg* is expressed in whole body samples of male and female larvae, decreases at the start of pupation, increases at eclosion, and then fluctuates in adult males and females, with females having higher expression than males (Piulachs et al. 2003; Guidugli et al. 2005b; Li et al. 2010; Colonello-Frattini et al. 2010; Lee et al. 2015; Roy-Zokan et al. 2015). With respect to overwintering diapause, *vg* is expressed in brains, ovaries, and fat bodies prior to diapause, decreases during diapause, and then increases again after diapause (Kawakami et al. 2009; Jedlicka et al. 2016; Liu et al. 2016).

Vitellogenin expression has been studied extensively with respect to important differences in caste phenotypes of bees with developmental castes (e.g. *Bombus sp.* and *Apis sp.*; Table S3.1, pg. 96). The connection between *vg* expression and phenotypes of eusocial castes is obvious since the hallmark difference between queens and workers is differential egg-laying, and *Vg* is required to produce developing eggs. *Vitellogenin* mRNA expression and *Vg* titers are positively correlated with ovarian development in bees (Cardoen et al. 2011; Kapheim et al. 2012; Harrison et al. 2015; Jedlicka et al. 2016; Lockett et al. 2016). In eusocial bees, *vg* expression is also associated with phenotypes such as diapause, aggression, longevity, and foraging. In *Bombus terrestris*, queens collected before their workers have eclosed and queens in diapause both express *vg* in their heads (Amsalem et al. 2015a). Interestingly, in *B. terrestris* workers, high levels of aggressive interactions explain high levels of *vg* expression levels better than ovarian development (Amsalem et al. 2014; Padilla et al. 2016). In honeybees, high head and abdomen *vg* expression is associated with increased longevity of queens, and low *vg*

expression is associated with the onset of foraging behaviour in workers (Amdam and Omholt 2003; Amdam et al. 2004, 2006b; Corona et al. 2007; Nelson et al. 2007; Page et al. 2012). However this result may be specific to honeybees because in bumblebees *vg* expression levels are similar in nest and foraging bumblebee workers (Amsalem et al. 2014).

***Vitellogenin* in a eusocial sweat bee**

To test the evolutionary process posited by the OGP, in which solitary antecedents evolved into eusocial descendants, we need descriptions of molecular mechanisms that influence caste phenotypes in species whose sociality may represent transitional stages. These descriptions should reveal how molecular mechanisms underpinning caste traits decoupled through evolutionary time to be expressed separately in queens and workers. The social organisation of primitively eusocial sweat bees (family Halictidae) represents one of these transitional stages (Rehan and Toth 2015; Toth and Rehan 2017). Castes of eusocial sweat bee species are morphologically similar, and newly eclosed females are behaviourally totipotent, meaning they are capable of behaving as queens or workers (behavioural castes; Yanega 1989; Schwarz et al. 2007; Sumner et al. 2018).

Few studies have investigated *vg*'s influence on egg-laying phenotypes in primitively eusocial species with behavioural castes (e.g. *Polistes sp.*), and only one study has used a primitively eusocial sweat bee (Table S3.1, pg. 96). In the tropical sweat bee, *Megalopta genalis*, *Vg* protein titers are higher in queens than workers (Kapheim et al. 2012). Interestingly, newly eclosed *M. genalis* females are capable of breeding solitarily. These solitary females have *Vg* titers comparable to those of queens (Kapheim et al. 2012), which suggests that high *vg* expression is associated with high ovarian development first and caste second. To further

investigate the *vg*'s role in caste determination or ovarian development in castes of transitional species, more studies are needed that utilize primitively eusocial sweat bees.

The objective of this study was to investigate the relationship between *vg* expression and traits of queens and workers in a eusocial sweat bee. To fulfill this objective, I compared the *vg* expression levels of *Lasioglossum laevissimum* from southern Ontario, Canada (Awde and Richards 2018). As with other eusocial sweat bee species in temperate climates, the breeding season of *L. laevissimum* can be understood in discrete phases (described in Chapter 1; Fig.1.2, pg. 13). *Lasioglossum laevissimum* queens emerge from their hibernacula during spring (phase 1) and provision their first brood (workers), which eclose in early summer (phase 2). Queens then remain in the nest for the rest of the summer (phases 2 and 3), laying eggs provisioned by their workers. The second brood, which ecloses in mid to late summer (phase 3), comprises males and future queens (gynes). These gynes mate, feed, overwinter, and initiate nests the following spring, becoming new queens. For the most part workers do not lay eggs; however 17% of *L. laevissimum* workers do develop their ovaries and may lay eggs during phases 2 and 3 (Awde and Richards 2018).

Based on *vg*'s roles in other insects, I assumed that some of the functions of *vg* in *L. laevissimum* are to facilitate the transport of lipids and carbohydrates from fat bodies to other tissues during development, before diapause, and during ovarian development. I hypothesized that in *L. laevissimum*, high *vg* expression results in high levels of ovarian development and lipid transport. Therefore, *vg* expression levels should reflect an individual's caste, reproductive status, life stage, and sex. Furthermore, the OGPB suggests that the expression of genes associated with egg-laying phenotypes should be biased towards queens compared to workers in species whose social organization may represent a transitional stage between solitary and

eusocial behaviour. Therefore, I made several predictions with respect to *vg* expression in *L. laevissimum* individuals (Fig.3.1, pg. 87). I predicted that females with higher ovarian development scores should have higher *vg* expression; since queens have higher ovarian development than workers (Awde and Richards 2018), they should also have higher *vg* expression than workers. Furthermore, I predicted that females collected prior to diapause should have similar *vg* expression levels as females collected after diapause, and I predicted that females should have higher *vg* expression than adult males.

METHODS

Lasioglossum laevisimum collections and measurements

Lasioglossum laevisimum females and males were collected on the wing or from nest excavations, immediately put into RNA preservative (RNAlater; Ambion), and stored shortly after at -80°C until they were measured, dissected, and their RNA extracted. All samples were collected from an aggregation located at Brock University, Ontario, Canada. Criteria used to identify each female to caste were described in Awde and Richards (2018). Spring foundresses were collected on the wing during phase 1. Summer queens, workers, and gynes were collected from nest excavations during phases 2 and 3. Older larvae and pupae collected during nest excavations were placed into paraffin-lined containers or microcentrifuge tubes, raised to adulthood in the laboratory, then stored in RNAlater < 24 hours after they eclosed. Females that eclosed in the laboratory were classified as newly eclosed gynes. In addition, workers and males and were collected on the wing during phases 2 and 3. I also collected early spring foundresses from their hibernacula ~2 weeks before phase 1 began by excavating 30x30x30cm soil cubes from areas in the aggregation that had nests the previous summer.

Lasioglossum laevisimum females and males were measured and dissected under RNA preservative using a stereomicroscope. Size measurements and ovarian development scores were used in part to assign each female to caste (see Chapter 2 for details), and to quantify their influence on normalised *for* expression in analyses described below. Head width (HW) was measured as the distance across the widest part of the head, and ovarian development (OD) was scored by summing the fractional value of each developing oocyte relative to a fully developed egg in the ovary. Queen and worker samples were purposely chosen for gene expression comparisons based on their OD scores so that a range of OD scores were represented. After an

individual was measured and dissected, I removed the head and abdomen (gut removed) from the thorax and stored each body part separately in the freezer until RNA extraction. Samples sizes of each *L. laevissimum* category used in head and abdomen gene expression analyses can be found in Table 3.2, pg. 79.

RNA extractions and cDNA preparation

RNA was extracted from each body part separately using a Total RNA Purification kit (Norgen Biotek Corp.) following the manufacturer's protocol. Total RNA was eluted to a final volume of 50 µl with water. RNA (5 µl at 100 ng – 500 ng; nanodrop and spectrophotometer) from each sample was converted into single-stranded cDNA (20 µl final volume) using SuperScrip III Reverse Transcriptase reagents and protocol (Invitrogen), RNase Inhibitor (BioShop), dNTP mix (BioShop), and Oligo dTVN₂₀ primers (Sigma-Aldrich).

***Vitellogenin* and reference gene primer design for RT-qPCR**

I designed gene specific primers for *vg*, *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*), *actin*, and *acidic ribosomal protein P2* (*rpP2*), for use with *L. laevissimum* cDNA in real time quantitative-PCR (RT-qPCR). These primers amplified small (75 -200 bp) regions of each gene. *Gapdh*, *actin*, and *rpP2* were chosen as potential normalizing reference genes for RT-qPCR because of their role in general cellular functions such as metabolism, forming the cytoskeleton, and protein synthesis. Furthermore these genes are often used as reference genes in RT-qPCR studies with other bee species (Park et al. 2006; Li et al. 2010; Hornáková et al. 2010; Reim et al. 2013; Salmela et al. 2016; Katarzyńska-Banasik et al. 2017). Reference genes are used in RT-qPCR analyses to control for technical and biological variation in starting mRNA concentrations and quality between samples, and to normalize target gene expression (Livak and Schmittgen 2001; Vandesompele et al. 2002; MIQE guidelines in Bustin et al. 2009).

To design primers for *L. laevissimum* cDNA templates, I retrieved mRNA and gDNA sequences from GenBank for *gapdh*, *actin*, and *rpP2* from other bee species (*Apis sp.*, *Bombus sp.*, *Megachile rotundata*, and *Osmia cornifrons*, etc.). I also received *L. albipes* mRNA sequences of these genes through personal communication with Dr. Sarah D. Kocher (Princeton University). Sequences of each gene were aligned using ClustalOmega (Sievers et al. 2011). Primers were designed in areas of high similarity by hand or using the software program Primer-BLAST (Primer3; Ye et al. 2012). To avoid amplifying gDNA, either the forward or reverse primer for each gene-specific primer set was located on an exon–intron boundary, or primers were located on different exons so I could differentiate gDNA or mRNA PCR amplicons based on size. I validated the identity of PCR amplicons using polyacrylamide gels to confirm their expected size. Furthermore, I sequenced the *L. laevissimum* PCR amplicons (Genome Québec) and verified their identity using BLAST. Amplicon sequences will be uploaded to NCBI and made available for public use. Primer sequences, relative locations within each gene, and *L. laevissimum* cDNA amplicon sizes are provided in Table 3.3, pg. 80.

RT-qPCR and reference gene validation

Gene expression was analyzed by RT-qPCR with a BioRad CFX96™ PCR Detection System. The expression of all 4 genes was measured for each cDNA sample on the same plate, with separate reactions for each primer set. On each plate I also included a no-template control with each primer set to ensure that amplification was not the result of contamination in the reagents. In each reaction, 4 µl aliquots of *L. laevissimum* cDNA were used in a 20 µl mix that contained 10 µl of KAPA SYBR FAST Universal qPCR Master Mix (2x), 0.25 µM of the forward and reverse gene-specific primer, and topped up with water. The RT-qPCR was performed using the following thermal cycling program: initial denaturation at 95°C for 3 min,

then 31 cycles of 95°C for 10 sec, 58°C for 10 sec, and 72°C for 15 sec. Reactions were performed in technical triplicates for each sample, with each set of gene specific primers, excluding the no-template controls. Quantification cycle (C_q) values from the triplicate reactions were averaged to give a single value for each gene with each sample. C_q values represent the PCR cycle at which amplification switched from the exponential to logarithmic phase. If the standard deviation of the three replicates was $>0.5 C_q$, then the replicate furthest from the median was excluded to account for technical variation between replicates. A melt-curve analysis was included after every completed RT-qPCR run (ramping from 65°C to 95°C in 0.5°C steps every 5 sec) to confirm the identity of each amplicon and verify that amplification was not the result of primer dimerization or gDNA contamination. Samples were only included in analyses if they had detectable levels of all three reference genes (see Data analyses section for one exception). For sample sizes of each *L. laevissimum* category see Table 3.2, pg. 79.

I quantified *vg* expression by normalizing C_q values of *vg* to the C_q values for the three internal reference genes (Livak and Schmittgen 2001). Using multiple reference genes provides a more accurate assessment of relative target gene expression than using a single reference gene, which can lead to inaccurate assessments (Vandesompele et al. 2002; Huggett et al. 2005; MIQE guidelines in Bustin et al. 2009). Therefore, I first validated *gapdh*, *actin*, and *rpP2* as suitable reference genes in *L. laevissimum* head and abdomen samples. If two genes are valid reference genes then the expression ratio of the two genes should be consistent between samples (Vandesompele et al. 2002). This means that expression values of valid reference genes should be correlated, regardless of experimental factors (Pfaffl et al. 2004). In *L. laevissimum* samples, *gapdh*, *actin*, and *rpP2* expression values had significant positive correlations in pair-wise comparisons with each other using head and abdomen samples (Fig.3.2, pg. 88). Correlations

between the three genes were tighter using abdomen samples (Pearson correlation coefficient ranged between $r = 0.91 - 0.95$) compared to head samples (Pearson correlation coefficient ranged between $r = 0.60 - 0.78$). All three genes were validated as suitable reference genes in head and abdomen samples separately using the free online tool RefFinder, which takes the expression values of each sample and generates the outputs of four algorithms commonly used to assess potential reference genes: geNorm (Vandesompele et al. 2002), Normfinder (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004), and the comparative ΔC_q method (Silver et al. 2006).

Data analyses

I used two methods to compare *vg* expression levels between *L. laevissimum* categories. First, I compared the proportion of samples that had detectable levels of *vg* between categories with Fisher's exact tests. In these analyses, the amplification of three reference genes provided a technical threshold for reliably detecting mRNA expression. Therefore I used the criterion that samples were only included in analyses if they had detectable expression levels of all three reference genes. This criterion was useful for all *L. laevissimum* categories except one, newly eclosed gyne heads, in which expression of all three reference genes was detected in only 2 of 6 samples (Table 3.2, pg. 79). Although there was low detectability of one or more reference genes in some other samples, only in newly eclosed gyne heads did applying the criterion change my interpretation of the subsequent *vg* expression result. Therefore, I present two sets of results for newly eclosed gyne heads: the proportion that had detectable levels of *vg* expression in samples that expressed *gapdh* and *rpp2* and the proportion that had detectable levels of *vg* in samples that expressed all three reference genes. For statistical comparisons between newly eclosed gyne heads and other female categories, values for newly eclosed gynes are the proportion with detectable levels of *vg* expression in samples that expressed *gapdh* and *rpp2*.

Secondly, I compared normalized *vg* expression values between *L. laevissimum* categories. *Vitellogenin* expression was normalized to the geometric mean of the three reference genes [Normalized *vg* expression (ΔC_q) = geometric mean of reference gene expression (C_q) – *vg* expression (C_q)]. *Vitellogenin* expression was normalized only in body parts that had detectable levels of all three reference genes. If a sample had detectable levels of all three reference genes and no detectable *vg* expression, I set the C_q value of *vg* to 31, representing the highest C_q value in our RT-qPCR reaction and thus, a null expression level, similar to Morandin et al. (2014). Kruskal-Wallis tests were used to compare normalised *vg* expression between *L. laevissimum* categories. Newly eclosed gyne heads were excluded from these statistical comparisons since values for all three reference genes were only available for 2 newly eclosed gyne heads.

I used two general linear models to quantify the effect of size, ovarian development, and caste on normalized *vg* expression (response variable) in each body part separately. Head width was included as the first predictor variable in each model to account for variation in *vg* expression that may be the result of size difference between queens and workers. I included caste in the model after OD to account for variation in *vg* expression that may result from a caste effect above and beyond differences in OD. I included the interaction term of ovarian development and caste in the model because *vg* expression may vary differently with OD scores depending on caste.

Two Linear Discriminant Analyses (LDA) were used to investigate how accurately *vg* expression values predict the caste of *L. laevissimum* females. I used data from 14 queens and 23 workers, all of which had complete data consisting of values for HW, MW, OD, normalised *vg* head expression, and normalised *vg* abdomen expression. The *lda* function in Rstudio (package: MASS) was used to perform each LDA, which calculated a discriminating component

from the variables for the predefined groups (queens or workers). The formula [Caste ~ HW+MW+OD] was used to assess the accuracy of using only physical traits and the formula [Caste ~ normalised vg head expression + normalised vg abdomen expression] was used to assess the accuracy of using only vg head and abdomen expression levels. I used the argument *CV=TRUE* with each formula to generate leave-one-out cross-validation (jackknifed) predictions. I then assessed the accuracy of these predictions by comparing them to classifications based on size and OD, and also to my own classification of each female, which was extensively covered in Chapter 2. The accuracy of each LDA was assessed by comparing the proportion of correct predictions with a Fisher's exact test.

RESULTS

Vitellogenin expression in *L. laevissimum* females

To investigate how expression varied between heads and abdomens, I compared expression levels of *L. laevissimum* females that had values for both body parts. Females with high *vg* expression levels in their head had high expression levels in their abdomen (Fig.3.3A, pg. 89; Pearson correlation coefficient $r = 0.62$, $df = 48$, $p < 0.001$). Furthermore, head and abdomen samples had similar *vg* expression levels (Fig.3.3B, pg. 89; Paired t-test; $t = 1.53$, $df = 49$, $p = 0.09$).

Gynes, Foundresses, and Queens

I investigated the effect of breeding season phenology on *vg* expression by comparing *vg* expression levels of gynes, foundresses, and queens collected at different points during the breeding season (Table 3.4, pg. 81; Fig.3.4, pg. 90). In heads, >70% of gyne, spring foundress, and summer queen samples had detectable levels of *vg* compared to ~50% of newly eclosed gyne and early spring foundress samples; these proportions were not significantly different (Table 3.4, pg. 81; Fisher's exact test $p = 0.17$). Quantitatively, head *vg* expression levels did vary through the breeding season, with spring foundresses and summer queens having higher *vg* expression levels than gynes and early spring foundresses (Fig.3.4, pg. 90; Kruskal-Wallis $\chi^2 = 10.71$, $df = 3$, $p = 0.01$). In abdomens, there was a significant difference in the proportion of samples that had detectable levels of *vg* between gynes, foundresses, and queens (Table3.4, pg. 81; Fisher's exact test $p = 0.005$). A smaller proportion of newly eclosed gynes and early spring foundresses had detectable levels of *vg* in their abdomens compared to gynes, spring foundresses, and summer queens. Similar to heads, spring foundress and summer queen abdomens had higher *vg*

expression levels compared to gynes and early spring foundresses (Fig.3.4, pg. 90). But, unlike heads, abdomen *vg* expression levels were statistically similar between gynes, foundresses, and queens (Kruskal-Wallis $\chi^2 = 5.03$, $df = 4$, $p = 0.28$).

The effect of age on *vg* expression levels was assessed by comparing newly eclosed gynes (< 24 hours old) to adult gynes (2 days to several weeks old; Table 3.4, pg. 81; Fig.3.4, pg. 90). In heads, 5/9 newly eclosed gynes had detectable levels of *vg* expression, which was a similar proportion to adult gynes (7/10 heads; Fisher's exact test $p = 0.65$). I did not compare head *vg* expression levels between newly eclosed gynes and adult gynes statistically because only 2 newly eclosed gynes had detectable levels of all three reference genes. In those 2 samples, *vg* expression levels did fall within the range of *vg* expression levels of adult gyne heads (Fig.3.4, pg. 90). In abdomens, a smaller proportion of newly eclosed gynes had detectable levels of *vg* (2/6 abdomens) than adult gynes (11/11 abdomens; Table 3.4, pg. 81; Fisher's exact test $p = 0.006$). Furthermore, adult gynes had higher abdomen *vg* expression than newly eclosed gynes (Kruskal-Wallis $\chi^2 = 3.27$, $df = 1$, $p=0.07$), but this difference was not statistically significant. Therefore, gynes that were two days to several weeks old were more likely to have high levels of *vg* expression in their abdomens than gynes < 24 hours old, while both age categories had similar *vg* expression in their heads.

I investigated how *vg* expression varied with respect to the timing of overwintering diapause by comparing females pre-diapause (newly eclosed gynes and adult gynes) to females post-diapause (foundresses and queens). In heads and abdomens, the proportion of females with detectable levels of *vg* expression did not differ between pre- and post-diapausing females (Table 3.4, pg. 81; Heads - Fisher's exact test $p = 0.30$; Abdomens - Fisher's exact test $p = 0.74$). Furthermore, *vg* expression levels were statistically similar between these two groups as well

(Fig.3.4, pg. 90; Heads - Kruskal-Wallis $\chi^2 = 3.30$, $df = 1$, $p = 0.07$; Abdomens - Kruskal-Wallis $\chi^2 = 0.24$, $df = 1$, $p = 0.62$). However, post-diapausing females did have higher head *vg* expression levels than pre-diapause females, which was largely driven by the high expression levels of spring foundresses and summer queens.

To assess variation in *vg* expression with respect to differential foraging behaviour, I compared *vg* expression between spring foundresses and summer queens. Spring foundresses were collected while actively foraging in spring, while summer queens were collected from nest excavations in summer and were no longer active foragers. In heads and abdomens, a similar proportion of spring foundresses and summer queens had detectable levels of *vg* expression (Table 3.4, pg. 81; Head - Fisher's exact test $p = 0.51$; Abdomen - Fisher's exact test $p = 1$). On average, summer queens had higher *vg* expression levels than spring foundresses in head and abdomen samples, however these differences were not statistically significantly different (Fig.3.4, pg. 90; Heads - Kruskal-Wallis $\chi^2 = 1.83$, $df = 1$, $p = 0.18$; Abdomens - Kruskal-Wallis $\chi^2 = 0.78$, $df = 1$, $p = 0.38$).

Workers

Vitellogenin expression was compared in two ways in workers. First I assessed how *vg* expression varied with respect to phases of the breeding season (Table 3.5A, pg. 82; Fig.3.5, pg. 91). A similar proportion of phase 2 and phase 3 workers had detectable levels of *vg* expression in their heads (Fisher's exact test $p = 0.68$) and abdomens (Fisher's exact test $p = 0.70$). Workers collected in phases 2 and 3 had similar *vg* expression levels in their heads (Kruskal-Wallis $\chi^2 = 0.18$, $df = 1$, $p = 0.67$), and abdomens (Kruskal-Wallis $\chi^2 = 0.79$, $df = 1$, $p = 0.37$).

Secondly, I assessed variation in worker *vg* expression with respect to foraging behaviour by comparing workers collected from nest excavations (nest workers) to workers collected on the

wing while actively foraging (Table 3.5B, pg. 82; Fig.3.6, pg. 92). In heads, a similar proportion of nest and foraging workers had detectable levels of *vg* (Table 3.5B, pg. 82; Fisher's exact test $p = 1$). In abdomens, a higher proportion of nest workers had detectable levels of *vg* than foraging workers, but this difference was not statistically significantly higher. (Table 3.5B, pg. 82; Fisher's exact test $p = 0.16$). Head and abdomen expression levels were higher in nest workers than foraging workers (Fig.3.6, pg. 92). Nest workers had significantly higher *vg* expression in their abdomens (Kruskal-Wallis $\chi^2 = 6.89$, $df = 1$, $p = 0.009$), but not heads (Kruskal-Wallis $\chi^2 = 0.95$, $df = 1$, $p = 0.33$).

Queen and worker comparisons

Based on the OGP, genes associated with egg-laying, such as *vg*, should have higher expression levels in queens compared to workers. Therefore, I investigated the effect of caste on *vg* expression by comparing *L. laevissimum* queens (spring foundresses and summer queens) and workers (phases 2 and 3). Queens were more likely than workers to have detectable levels of *vg* expression in their heads and abdomens (Table 3.6, pg. 83; Heads - Fisher's exact test $p < 0.001$; Abdomens - Fisher's exact test $p = 0.08$). The effect of caste on the detectability of *vg* expression was stronger in heads than abdomens. Furthermore, queens had higher head and abdomen *vg* expression levels than workers (Fig.3.7, pg. 93; Heads - Kruskal-Wallis $\chi^2 = 12.52$, $df = 1$, $p < 0.001$; Abdomens - Kruskal-Wallis $\chi^2 = 3.46$, $df = 1$, $p = 0.06$). Once again, the effect of caste on *vg* expression was stronger in heads than abdomens.

On average, *L. laevissimum* queens had higher ovarian development scores than workers (Chapter 2 - Fig.2.4, pg. 46), which was also the case for samples used in head and abdomen gene expression comparisons (Queen and worker head comparison - Kruskal-Wallis $\chi^2 = 25.56$, $df = 1$, $p < 0.001$; queen and worker abdomen comparison - Kruskal-Wallis $\chi^2 = 26.20$, $df = 1$, p

< 0.001). Therefore I investigated whether or not queens and workers differed in *vg* expression after accounting for any effect of ovarian development. Queens and workers with high ovarian development scores had high *vg* expression levels in both head and abdomen samples (Fig.3.8, pg. 94). There was no caste effect on *vg* expression levels after accounting for variation influenced by ovarian development scores (Table 3.7, pg. 84).

Finally, castes of primitively eusocial species are morphometrically similar and require phenological, behavioural, and dissection data to identify with confidence (Schwarz et al. 2007). Since queens and workers had different *vg* expression levels, I performed two LDAs to investigate whether or not *vg* gene expression levels could be used to classify *L. laevissimum* females as queens or workers with as much accuracy as physical traits alone (Table 3.8, pg. 85; Fig.3.9, pg. 95). Using *vg* alone, 72% of females were correctly assigned to caste. The percentage of females accurately assigned to caste using an LDA with only physical traits was 83% of females, which was a statistically similar proportion as the LDA using only *vg* expression values (Fisher's exact test $p = 0.40$).

***Vitellogenin* expression in *L. laevissimum* males**

Since the major role of *Vg* in insects is as the precursor to vitellin in egg production (Table S3.1, pg. 96), I predicted that *vg* expression would be higher in females than males. Differences between females and males were evaluated by comparing the proportion of samples with detectable levels of *vg* between gynes and males that likely eclosed in phase 3 (Table 3.9, pg. 86). In males, 0/9 heads and 0/11 abdomens had detectable levels of *vg* expression. In gynes, 7/10 heads and 11/11 abdomens had detectable *vg*. Therefore, a higher proportion of females had detectable levels of *vg* than males in their heads (Fisher's exact test $p = 0.003$) and abdomens (Fisher's exact test $p < 0.001$).

DISCUSSION

Vitellogenin expression in *L. laevissimum* queens and workers

Based on *vg*'s roles in other insect taxa as a transporter of lipids and carbohydrates during ovarian development, juvenile development, and before diapause (Sappington and Raikhel 1998; Tufail and Takeda 2008), I hypothesized that in *L. laevissimum*, high *vg* expression results in high levels of ovarian development and lipid transport, and should reflect an individual's caste, reproductive status, life stage, and sex (Table 3.1, pg. 78). The OGPB suggests that the expression of genes associated with egg-laying, such as *vg*, should be biased towards queens compared to workers. An important characteristic of *L. laevissimum* colonies, as with other primitively eusocial sweat bees, is that the queen is the dominant egg-layer in their nest (Schwarz et al. 2007; Awde and Richards 2018). Therefore, I predicted that queens would have higher *vg* expression levels than workers. This prediction was borne out; *vg* expression was higher in *L. laevissimum* queens compared to workers. This result is consistent with Vg titers in castes of another sweat bee, *M. genalis*, in which queens have higher Vg levels than workers (Kapheim et al. 2012). Since *vg* expression and Vg function as the vitellin precursor in developing eggs (Sappington and Raikhel 1998; Tufail and Takeda 2008; Tufail et al. 2014), I also predicted that *L. laevissimum* females with high ovarian development scores would have high *vg* expression. This prediction was also borne out; *vg* expression had a strong positive correlation with ovarian development scores in *L. laevissimum* queens and workers, consistent with the positive correlation between ovarian development and *vg* expression or Vg titers in other insect species (Engles 1974; Robinson et al. 1991; Scott et al. 2005; Toth et al. 2009; Kapheim et al. 2012; Tokar et al. 2014; Lockett et al. 2016; Xiao et al. 2016).

Since sweat bee queens and workers differ behaviourally, not developmentally, I also predicted that after accounting for variation in ovarian development, caste would not explain *vg* expression in *L. laevissimum* females. Higher *vg* expression in queens than workers was due to their higher ovarian development; there seems to be no caste effect beyond the variation explained by ovarian development. This result shows that gene expression associated with egg-laying reflects the post-imaginal determination of caste differences between queens and workers, which develop after eclosion, rather than during larval development (Yanega 1989; Schwarz et al. 2007; Sumner et al. 2018).

***Vitellogenin* expression in *L. laevissimum* queens and workers provides support for the OGPB**

Vitellogenin expression patterns in *L. laevissimum* queens and workers provide support for the OGPB. The OGPB suggests that ancestral solitary egg-laying and parental care phenotypes, and the molecular mechanisms underpinning them, decoupled to be differentially expressed in queens and workers of highly eusocial descendants (West-Eberhard 1987, 1996). Primitive eusociality, as demonstrated by the colony social organisation of species like *L. laevissimum*, may represent a transitional state between solitary and highly eusocial behaviour (Rehan and Toth 2015; Toth and Rehan 2017). *Vitellogenin* expression in *L. laevissimum* was higher in queens than workers. Although queens are the dominant egg-layers in their nests, there is some overlap in ovarian development scores between queens and workers (Chapter 2; Fig.2.4, pg. 46). This phenotypic characteristic of *L. laevissimum* castes was also evident in *vg* expression comparisons. *Vitellogenin* expression was correlated with ovarian development scores; since worker and queen ovarian development scores overlap, the highest worker *vg* levels overlapped with the lowest queen *vg* levels. This result supports the hypothesis that gene

expression associated with an egg-laying phenotype is skewed towards egg laying females in eusocial colonies.

Vitellogenin expression patterns are consistent with the fact that newly eclosed females are behaviourally totipotent, and caste differences do not stem from developmental differences. This is in contrast to *vg* expression levels of bumble bees, *Bombus terrestris*, which have developmental castes (Amsalem et al. 2014; Padilla et al. 2016). In *B. terrestris*, queens have higher *vg* expression than workers, and *vg* expression has a strong positive correlation with ovarian development in queens, but not in workers (Amsalem et al. 2014; Padilla et al. 2016). Worker *vg* expression is positively correlated with the number of aggressive behaviours a worker gives and receives, rather than a worker's level of ovarian development. In *L. laevissimum*, both queens and workers had a strong positive correlation between ovarian development and *vg* expression. This contrast between *L. laevissimum* and *B. terrestris* demonstrates that the expression of *vg* is correlated with ovarian development in queens and workers of a species with behavioural castes; however, in a species with developmental castes, *vg* expression correlates with ovarian development in queens but not workers.

A major difference between sweat bee queens and workers is differential egg-laying behaviour; queens lay more eggs than workers (Michener 1974; Schwarz et al. 2007). Therefore, I investigated whether or not *vg* expression levels, which are associated with egg-laying, could be used to classify *L. laevissimum* females as queens or workers with as much accuracy as physical traits alone. Both gene expression and physical traits correctly predicted the caste classification of ~3/4 of females. Considering that queens had higher *vg* expression levels than workers, and that *vg* expression levels can be used to successfully predict which females are queens, this result is further evidence that the expression of a gene associated with egg-laying is

skewed towards queens compared to workers. Additionally, both gene expression and physical traits misidentified ~1/4 of females to the correct caste, which may be explained by the fact that identifying sweat bee females to caste requires phenological and behavioural data.

***Vitellogenin* expression, age and task polyphenism, and overwintering diapause**

Polyphenism refers to the phenomenon by which a single genome can produce multiple discrete phenotypes in response to different extrinsic factors (Simpson et al. 2011). This is often achieved via differential gene expression. Therefore, I used intra-caste comparisons of *vg* expression to investigate how gene expression may influence changes in *L. laevissimum* behaviour, specifically whether or not an individual was an active forager. Through a breeding season, *L. laevissimum* queens exhibit age polyphenism; foundresses forage and provision brood in spring, while older queens cease foraging activity in summer and remain in the nest (Awde and Richards 2018). Additionally, workers exhibit task polyphenism; actively foraging workers were collected on the wing and compared to workers collected from nests that were not foraging. In *L. laevissimum*, *vg* expression was higher in workers that were not foraging compared to workers that were actively foraging. Furthermore, *vg* expression showed a similar trend in queens; non-foraging summer queens had higher *vg* expression than foraging spring foundresses.

In *L. laevissimum*, non-foraging queens and workers have higher *vg* expression than foraging queens and workers. In honeybees, *vg* expression is higher in non-foraging workers than foraging workers (Amdam and Omholt 2003; Guidugli et al. 2005a; Amdam et al. 2006b; Nelson et al. 2007; Page et al. 2012). In the case of honeybee workers, age-related task polyphenism refers to the pattern in which newly eclosed workers primarily do tasks inside the colony, such as cleaning brood cells and tending to brood, but switch to tasks outside the nest (e.g. foraging) at about 3 weeks of age (Seeley and Kolmes 1991). In social insect literature, this

transition is often referred to as age polyethism (e.g. see Robinson 1992 and Fahrbach 1997; but also Colgan et al. 2011). Regardless of the term, *vg* expression in honeybee workers appears to be associated with behaviour more so than age since knockdown of *vg* by RNAi causes early foraging in young workers (Nelson et al. 2007). This is similar to *vg* expression levels in *L. laevissimum*, which are most likely associated with behaviour rather than age since foraging and non-foraging workers are of similar age.

In insects, *vg* expression is expressed before overwintering diapause, decreases during diapause, and then increases again after diapause (Adams et al. 2002; Kawakami et al. 2009; Jedlicka et al. 2016). In *L. laevissimum*, *vg* expression levels were similar between females pre- and post-diapause. Interestingly, early spring foundresses, which had exited diapause and were preparing to exit their hibernacula at the beginning of spring, had *vg* expression levels similar to those of gynes (pre-diapause). This presents a contrast to other insect species. In *B. terrestris*, a stink bug (*Perillus bioculatus*), and a spider mite (*Tetranychus urticae*), *vg* expression is suppressed in females during diapause (Adams et al. 2002; Kawakami et al. 2009; Jedlicka et al. 2016), most likely because oogenesis is also suppressed (Adams et al. 2002). It's important to note that early spring foundresses were no longer in diapause and some early spring foundresses did have developed ovaries (median OD score = 0.5, range 0-1, n = 7). Therefore, it is reasonable to suggest that *vg* was expressed because diapause had ended and oogenesis was underway or soon to be underway in these females. It is also important to point out that *vg* was detected in gynes prior to diapause, in the absence of ovarian development. *Vitellogenin* expression in pre-diapause gynes provides support for the hypothesis that a function of *Vg* in *L. laevissimum* is to facilitate the transport of lipids in preparation for overwintering diapause.

Lack of *vg* expression in adult *L. laevissimum* males may stem from age and behaviour

I predicted that adult females and males would both have detectable levels of *vg* expression since both sexes express *vg* in other insects (Table S3.1, pg. 96; e.g. Colonello-Frattini et al. 2010; Roy-Zokan et al. 2015). In *L. laevissimum*, females were more likely to have detectable levels of *vg* than males, whereas no male head or abdomen samples had detectable levels of *vg* expression. This is in stark contrast to adult males of the burying beetle, *Nicrophorus vespilloides*, which express *vg* in their brains (Roy-Zokan et al. 2015). Differences in male *vg* expression between these two species may stem from life history differences. First, parental care in burying beetles is biparental (Scott 1998), whereas in *L. laevissimum*, as in most other hymenopteran species, males do not provide parental care to their offspring (Suzuki 2013). Secondly, *L. laevissimum* males may not require *vg* several days after eclosion. In male honeybees, *vg* expression drastically decreases within the first few days after males eclose (Piulachs et al. 2003; Colonello-Frattini et al. 2010). This might be because males do not require *vg* for lipid storage since fat bodies are absent in male abdomens only a couple of days after eclosion (Haydak 1957; Hrasnigg and Crailsheim 2005). Furthermore, male *B. terrestris* use sugar as their main source of energy, not lipids (Surholt et al. 1988). Therefore, *L. laevissimum* males might not have detectable levels of *vg* expression as adults because they do not take part in paternal care or require lipid storage for their energy requirements as adults.

The importance of multiple reference genes for RT-qPCR data analysis

The contrasting *vg* expression patterns in the heads of newly eclosed *L. laevissimum* females when using 3 or 2 reference genes illustrates the importance of using and validating multiple reference genes. Multiple reference genes provide a more accurate normalization of target gene expression compared to using a single reference gene (Vandesompele et al. 2002;

Huggett et al. 2005; Bustin et al. 2009). In this chapter, the use of multiple reference genes provided me with the opportunity to evaluate how target gene expression patterns can be influenced by which reference genes are used. Of the samples that had detectable expression levels of all three reference genes, I detected no *vg* expression in the heads of newly eclosed females. However, when I included samples that had detectable expression levels of only two reference genes (*gapdh* and *rpp2*), regardless of whether or not *actin* expression levels were detectable, *vg* expression levels were detectable in 55% of heads of newly eclosed females. This second result is consistent with *vg* expression patterns of female bees during development, which express *vg* throughout development and after eclosion (Guidugli et al. 2005b; Li et al. 2010; Colonello-Frattini et al. 2010). Interestingly, if I had used only *actin* as an internal reference gene, I would have interpreted these results very differently and concluded that newly eclosed females had very low levels of mRNA expression and no *vg* expression. However, because I used multiple reference genes, the results suggest that newly eclosed females have lower *actin* expression levels compared to other female categories. This result is troubling considering some gene expression studies on bumblebee and honeybee developmental stages use *actin* as the only internal reference gene (Guidugli et al. 2005b; Li et al. 2010; Colonello-Frattini et al. 2010). Furthermore, most gene expression studies that use social insects do not report how individuals without detectable expression levels of target or reference genes were handled. This example provides a demonstration of the importance of using and reporting the validation of multiple internal reference genes for expression studies (Vandesompele et al. 2002).

Conclusions

Vitellogenin expression reflects the caste of a *L. laevissimum* female. Queens had higher *vg* expression than workers. Therefore, *vg* expression levels skew towards queens compared to

workers in a species that exhibits primitive eusociality, which may represent a transitional state between solitary behaviour and highly eusocial organization. This result provides support for the OGPB with respect to the expression of a gene associated with egg-laying.

The expression of *vg* in gynes, which do not have ovarian development, provide an important reminder that *vg*'s role in insect physiology is much larger than simply a yolk protein precursor. I was able to provide data that supports *Vg*'s function as a transporter of lipids and amino acids before diapause by sampling individuals traditionally ignored by social insect gene expression studies. This would not have been possible without accounting for individual variation (not pooling samples) and purposeful sampling of discrete female categories.

Table 3.1. General *vg* expression patterns in insects with respect to life history traits such as development, sex, diapause, and ovarian development. These expression patterns in relation to several phenotypes are summarised from *vg* literature, which can be found in Table S3.1, pg. 96

Factor	<i>Vg</i> expression levels
Developmental stages	<input type="checkbox"/> <input type="checkbox"/> High in larvae <input type="checkbox"/> <input type="checkbox"/> Low in pupae <input type="checkbox"/> <input type="checkbox"/> High in newly eclosed males and females
Sex	<ul style="list-style-type: none"> • Vary in adult males • Vary in adult females; generally higher than males
Diapause	<input type="checkbox"/> <input type="checkbox"/> High in females preparing to overwinter <input type="checkbox"/> <input type="checkbox"/> Low in females while overwintering
Ovarian development	<input type="checkbox"/> <input type="checkbox"/> High in females with high ovarian development

Table 3.2. Sample sizes of each *L. laevissimum* category used in head and abdomen *vg* expression comparisons. The number of samples with detectable expression of all three reference genes and the number of samples with detectable expression levels of just two reference genes (*gapdh* and *rpP2*) are provided for each category. Both head and abdomen measurements are available for some, but not all individuals sampled. Therefore, the total number of individuals sampled in each category is provided, as well as the number of samples with detectable expression in both their head and abdomen

Category	Three reference genes with detectable expression levels				Two reference genes with detectable expression levels (<i>gapdh</i> and <i>rpP2</i>)			
	No. of individuals	Head	Abdomen	Both Head and Abdomen	No. of individuals	Head	Abdomen	Both Head and Abdomen
Early spring foundresses (before Phase 1)	7	6	4	3	7	6	5	4
Spring foundresses (Phase 1)	18	12	14	8	21	17	19	15
Summer queens (Phase 2 and 3)	10	7	9	6	11	11	10	10
Workers (Phase 2)	20	16	16	12	23	18	19	14
Workers (Phase 3)	11	10	11	10	11	10	11	10
Newly eclosed gynes (Phase 3)	6	2	6	2	12	9	6	5
Gynes (Phases 3 and 4)	12	10	11	9	12	12	12	12
Males (Phase 3)	14	9	11	6	17	15	12	10
Total	98	72	82	56	114	98	94	80

Table 3.3. Sequences, locations, and amplicon sizes of primer sets used for each gene in RT-qPCR analyses

Gene	Primer location		Forward primer sequence 5'- 3'	Reverse primer sequence 5'- 3'	cDNA amplicon size
	Forward primer	Reverse primer			
<i>vg</i>	exon 2	exon 2:3	CGCCTCTGCCCCGACTACG	CTCGGCTTGTGGAGAATTTTCGTAAGG	171 bp
<i>gapdh</i>	exon 2:3	exon 3	ACCATGGACAAGGCTTCGGCTC	GATGGGTCATAAGCATCCAAG	75 bp
<i>actin</i>	exon 1	exon 2	GCGGCTCTCGAGTCTCGCTTC	GTGGTCGACCGACGATGGATGGG	191 bp
<i>rpP2</i>	exon 2	exon 2:3	CGTGGCCGCTTATCTTTTGGC	TGCCAATTTCTCGCGTCCTTGT	153 bp

Table 3.4. The effects of breeding season phenology, age, the timing of overwintering-diapause, and differential foraging behaviour on *vg* expression in *L. laevissimum* gynes, foundresses, and queens. The proportion of samples with detectable *vg* expression are presented out of the number of samples with detectable mRNA levels. Almost all gyne, spring foundresses, and summer queen head and abdomen samples had detectable *vg* expression, while a smaller proportion of newly eclosed gyne and early spring foundress heads and abdomens had detectable *vg* expression

Body Part	Newly eclosed gynes (Phase 3 –eclosed in the lab)	Gynes (Phases 3 and 4)	Early spring foundresses (before Phase 1)	Spring foundresses (Phase 1)	Summer queens (Phases 2 and 3)	Comparison between categories (Fisher's exact test)
Head	56% 5/9*	70% 7/10	50% 3/6	83% 10/12	100% 7/7	p = 0.17
Abdomen	30% 2/6	100% 11/11	25% 1/4	79% 11/14	77.8% 7/9	p = 0.005

* 0/2 newly eclosed gyne heads had detectable *vg* expression out of samples with detectable expression of all three reference genes

Table 3.5. The effect of breeding season phase (A.) and foraging behaviour (B.) on *vg* expression in worker heads and abdomens. As in Table 3.4, pg. 81, the proportion of samples with detectable *vg* expression are presented out of the number of samples with detectable mRNA levels. Breeding season phase had no effect on *vg* expression in worker heads or abdomens. A smaller proportion of workers collected while actively foraging (Foraging workers) had abdomen *vg* expression compared to workers collected from nest excavations (Not foraging; Nest workers)

A.	Body Part	Phase 2 workers	Phase 3 workers	Comparison between categories (Fisher's exact test)
	Head	44% 7/16	30% 3/10	p = 0.68
	Abdomen	56% 9/16	45% 5/11	p = 0.70

B.	Body Part	Nest workers	Foraging workers	Comparison between categories (Fisher's exact test)
	Head	33% 2/6	40% 8/20	p = 1
	Abdomen	83% 5/6	43% 9/21	p = 0.16

Table 3.6. The effect of caste on *vg* expression. In each category and for each body part, the proportion of samples with detectable *vg* expression levels are presented out of the number of samples with detectable mRNA levels. In heads and abdomens, a higher proportion of queens had detectable levels of *vg* expression than workers

Body Part	Queens (Spring foundresses and summer queens)	Workers (Phases 2 and 3)	Comparison between categories (Fisher's exact test)
Head	89% 17/19	38% 10/26	$p < 0.001$
Abdomen	78% 18/23	52% 14/27	$p = 0.08$

Table 3.7. Factors contributing to variation in *vg* expression levels in queen and worker heads (Head) and abdomens (Abdomens). Queen and worker heads and abdomens with high *vg* expression had high ovarian development scores, regardless of caste

Head		Model: Head <i>vg</i> expression ~ Head width + Ovarian development + Caste + Caste * Ovarian development				
Effects	<i>Coefficient</i>	<i>d.f.</i>	<i>F</i>	<i>p</i>	<i>Adjusted R²</i>	
Model		40	14.18	<0.001	0.55	
Head width	2.03	1	3.14	0.08		
Ovarian development	2.10	1	52.68	<0.001		
Caste		1	0.56	0.46		
OD * Caste		1	0.34	0.56		

Abdomen		Model: Abdomen <i>vg</i> expression ~ Head width + Ovarian development + Caste + Caste * Ovarian development				
Effects	<i>Coefficient</i>	<i>d.f.</i>	<i>F</i>	<i>p</i>	<i>Adjusted R²</i>	
Model		45	2.86	0.03	0.13	
Head width	-0.12	1	0.33	0.57		
Ovarian development	1.78	1	9.62	0.003		
Caste		1	1.35	0.25		
OD * Caste		1	0.14	0.71		

Table 3.8. Correct classifications of females to caste using a Linear Discriminant Analysis (LDA) with only physical traits and an LDA using only head and abdomen *vg* expression levels. Physical traits were head width, mandibular wear, and ovarian development scores. Females were only included if they had values for all measurements. A similar proportion of queens and workers were correctly assigned using the LDAs. Visual representation of each LDA and the corresponding Linear Discriminate values for each individual female are in Fig.3.9, pg. 95

Caste	Physical traits	<i>Vg</i> expression levels	
Queens (n=14)	11	8	
Workers (n=22)	19	18	
Correctly classified by LDA	83% 30/36	72% 26/36	Fisher's exact test p = 0.40

Table 3.9. Detectable *vg* expression in *L. laevissimum* female and male heads and abdomens. Males and females (gynes) likely eclosed in phase 3. Proportions of samples with detectable *vg* expression are out of the number of samples with detectable mRNA levels are shown

Body Part	Females (Gynes – Phases 3 and 4)	Males (Phase 3)	Comparison between categories (Fisher’s exact test)
Head	70% 7/10	0% 0/9	p = 0.003
Abdomen	100% 11/11	0% 0/11	p < 0.001

High *vg* expression level ↑
 Low *vg* expression level ↓

Phases 2 and 3

- Summer queens developing ovaries, *vg*↑
- Workers not developing ovaries, *vg*↓
- Workers developing ovaries, *vg*↑
- Newly eclosed females and males, *vg*↑

Phases 3 and 4

- Gynes prior to diapause, *vg*↑
- Adult males, *vg*↓ or *vg*↑

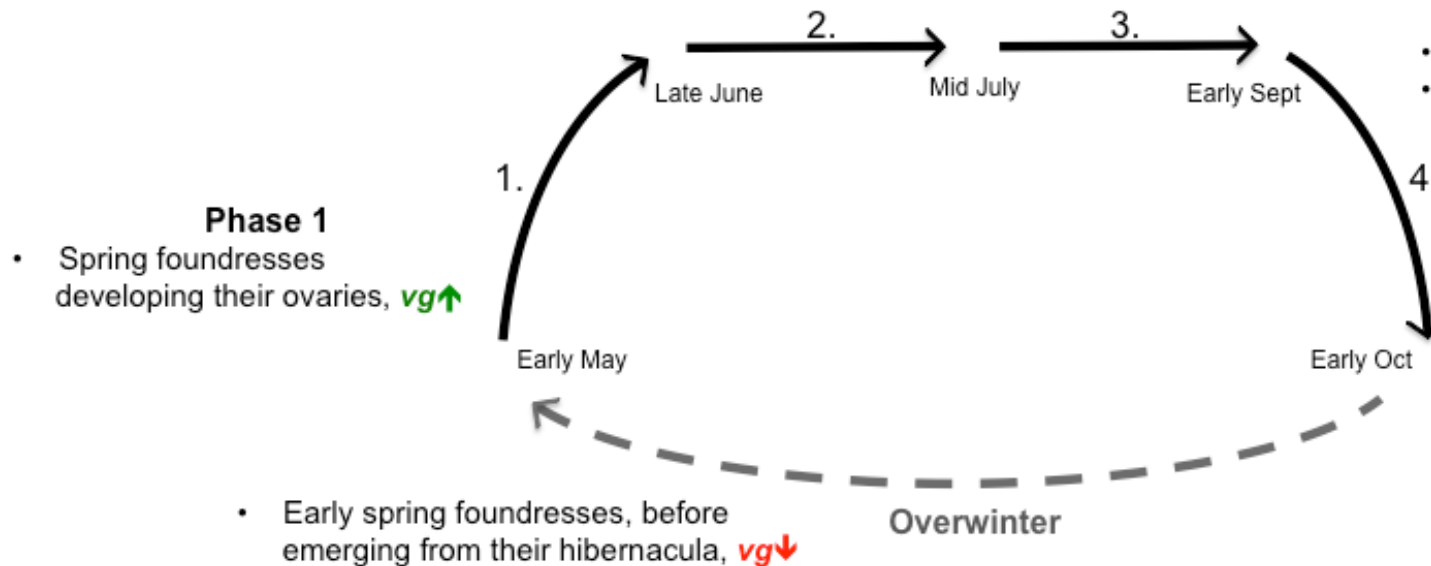


Figure 3.1. Predicted *vg* expression levels in *L. laevissimum* males and females at different points during the breeding season. For a detailed explanation of each phase of the *L. laevissimum* breeding season see Fig. 1.2, pg 13 in Chapter 1, and Chapter 2. Briefly, during phase 1, queens emerge from their hibernacula and provision their first brood (workers). In phases 2 and 3 queens remain in the nest laying eggs for a second brood that is provisioned by their workers. In phases 3 and 4 the second brood of males and future queens (gynes) eclose. Gynes mate, feed, overwinter, and initiate a nest the following spring, becoming new queens

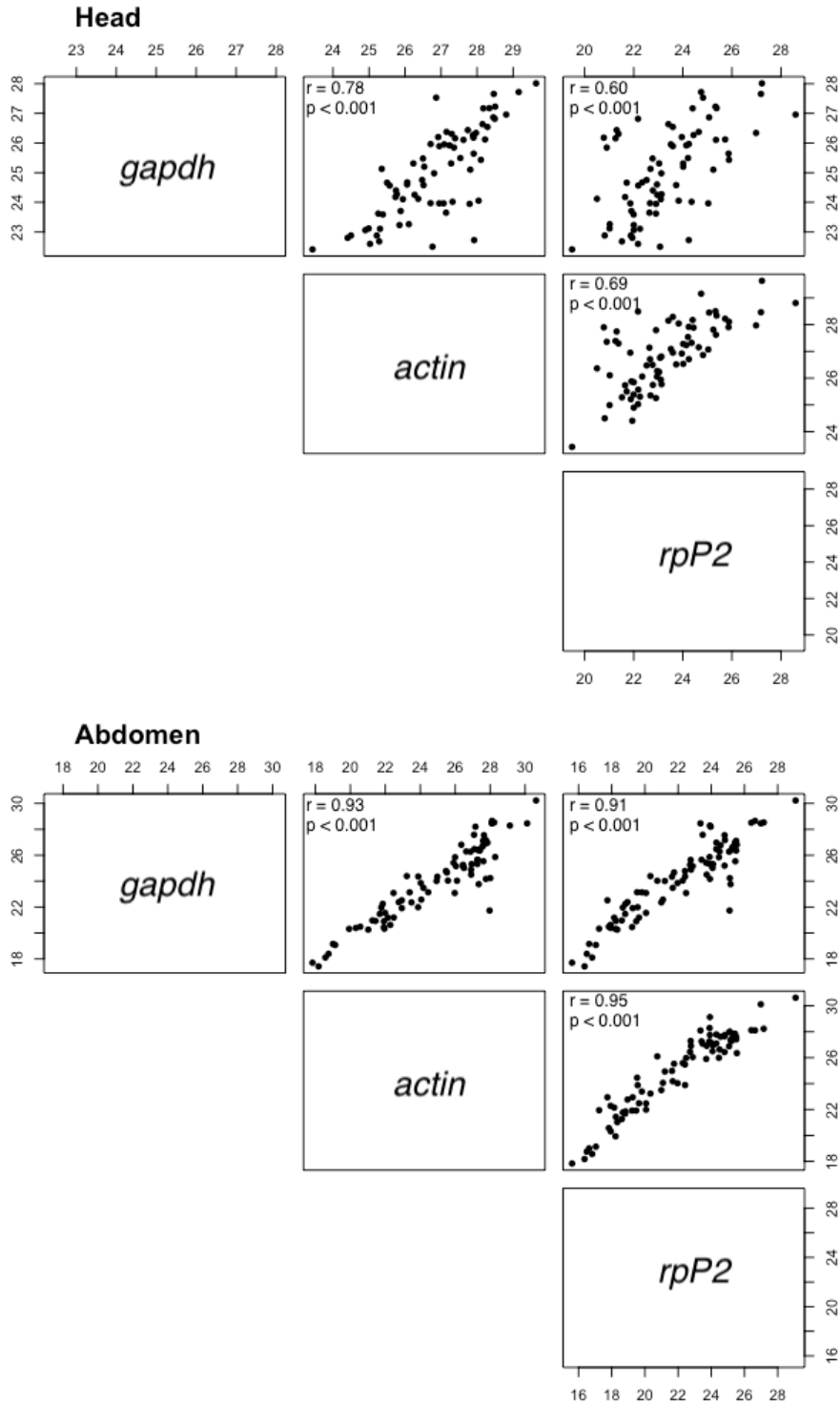


Figure 3.2. Validation of *gapdh*, *actin*, and *rpP2* of suitable reference genes for head and abdomen gene expression comparisons. Pair-wise correlations of the cycle number (C_q ; x and y-axes) of 3 reference genes for *L. laevissimum* individuals: *gapdh*, *actin*, and *rpP2*. Low C_q values represent high expression levels and high C_q values represent low expression levels. Pearson correlation coefficients and probabilities for each correlation are provided. Sample sizes of each sample category can be found in Table 3.2 (pg. 79), in total 72 head samples and 82 abdomen samples were used. Only head or abdomen samples that had detectable levels of all three genes were analyzed

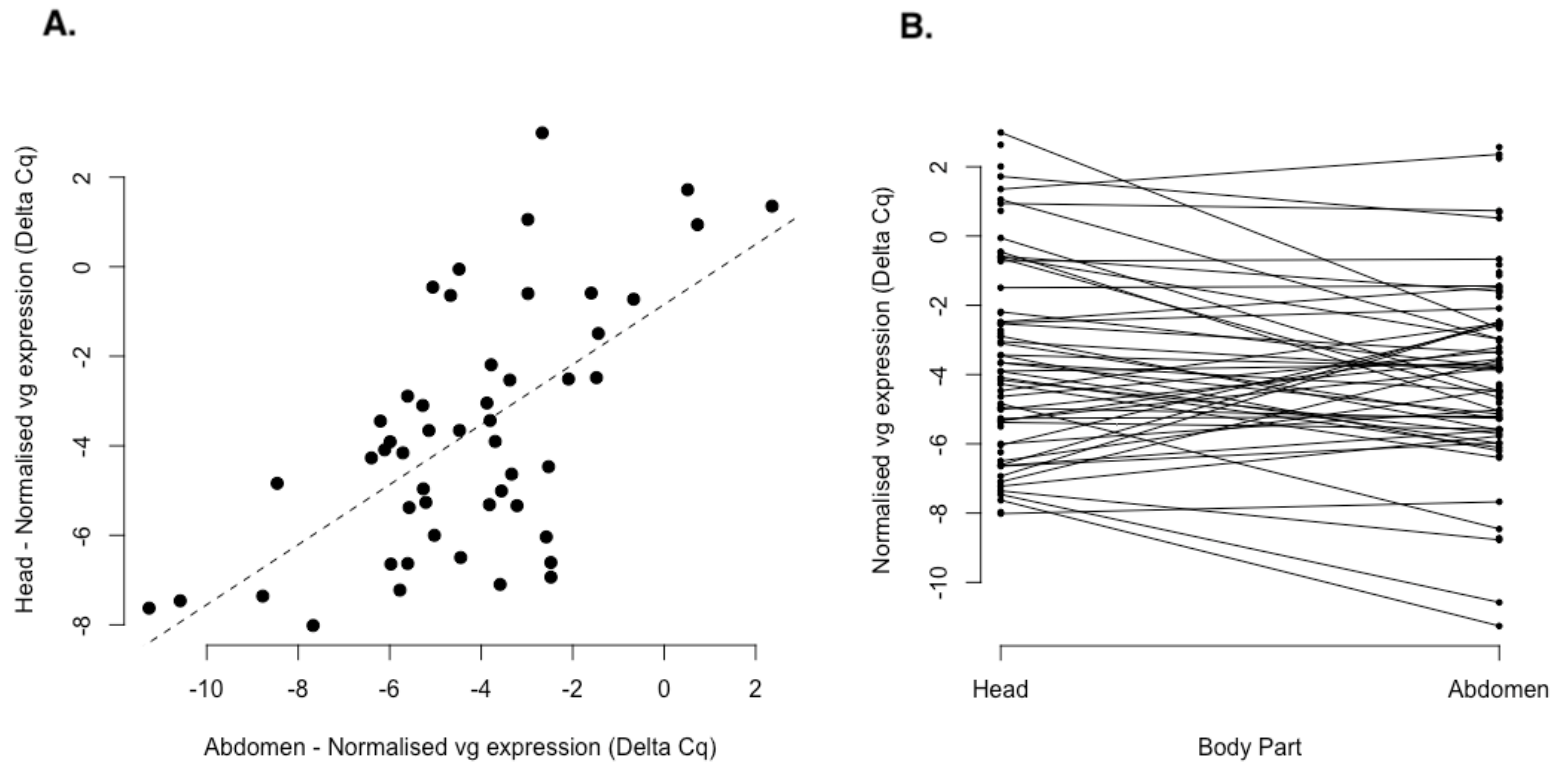


Figure 3.3. Intra-individual comparison of head and abdomen *vg* expression levels of *L. laevissimum* females. (A) *Vg* expression levels of individuals with measurements from both their head and abdomen. Females with high *vg* expression levels in their head had high *vg* expression levels in their abdomen. (B) *Vg* expression values of all female head and abdomen samples. Individuals with *vg* expression values from their head and their abdomen are connected by a line. Of the individuals with *vg* expression values for both body parts, *vg* expression levels were similar in their head and abdomen

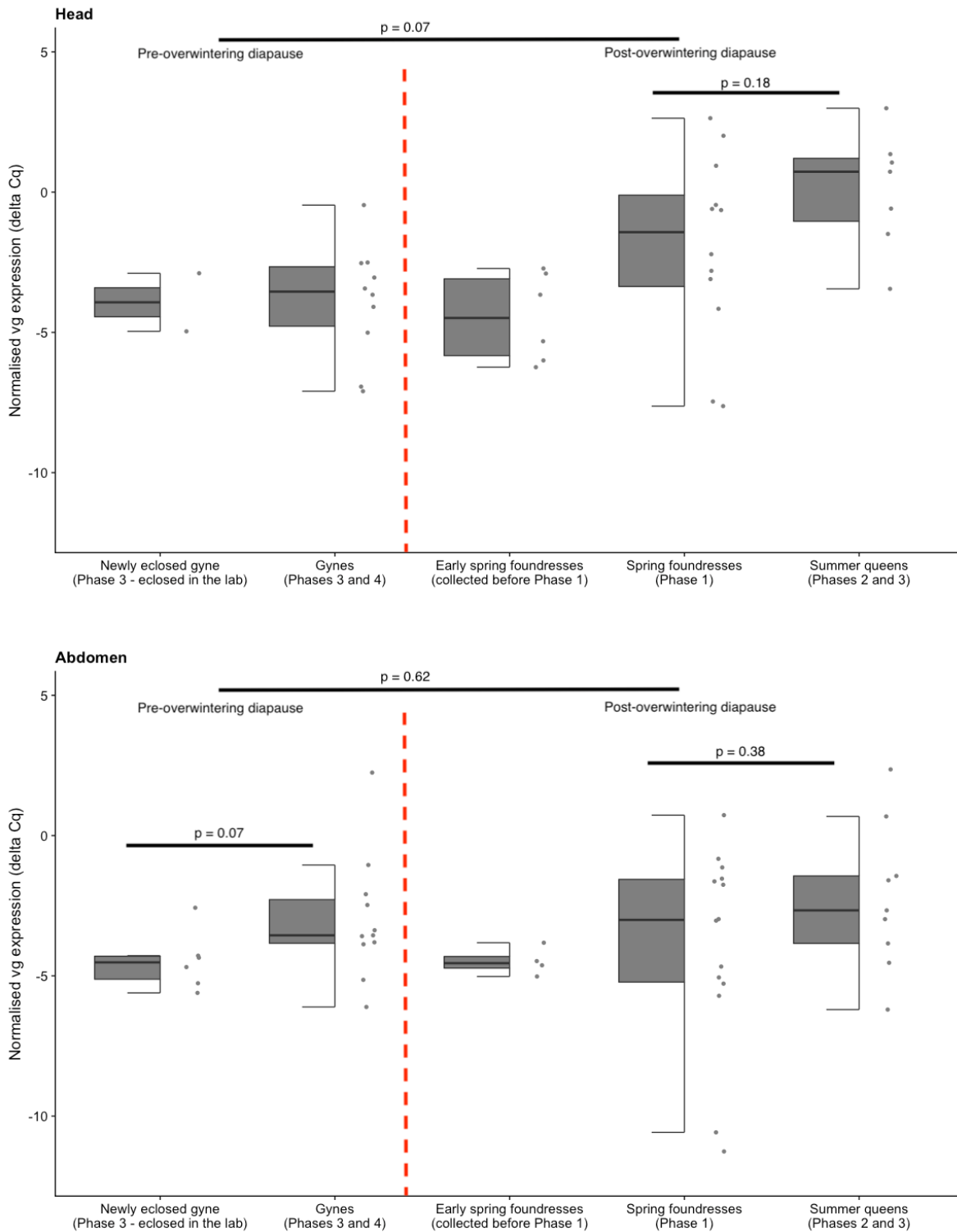


Figure 3.4. The effects of breeding season phenology, age, the timing of overwintering-diapause, and differential foraging behaviour on *vg* expression levels in the heads and abdomens of *L. laevissimum* gynes, foundresses, and queens. In heads (Head) and abdomens (Abdomen), *vg* expression levels were highest in spring foundresses and summer queens compared to the remaining categories

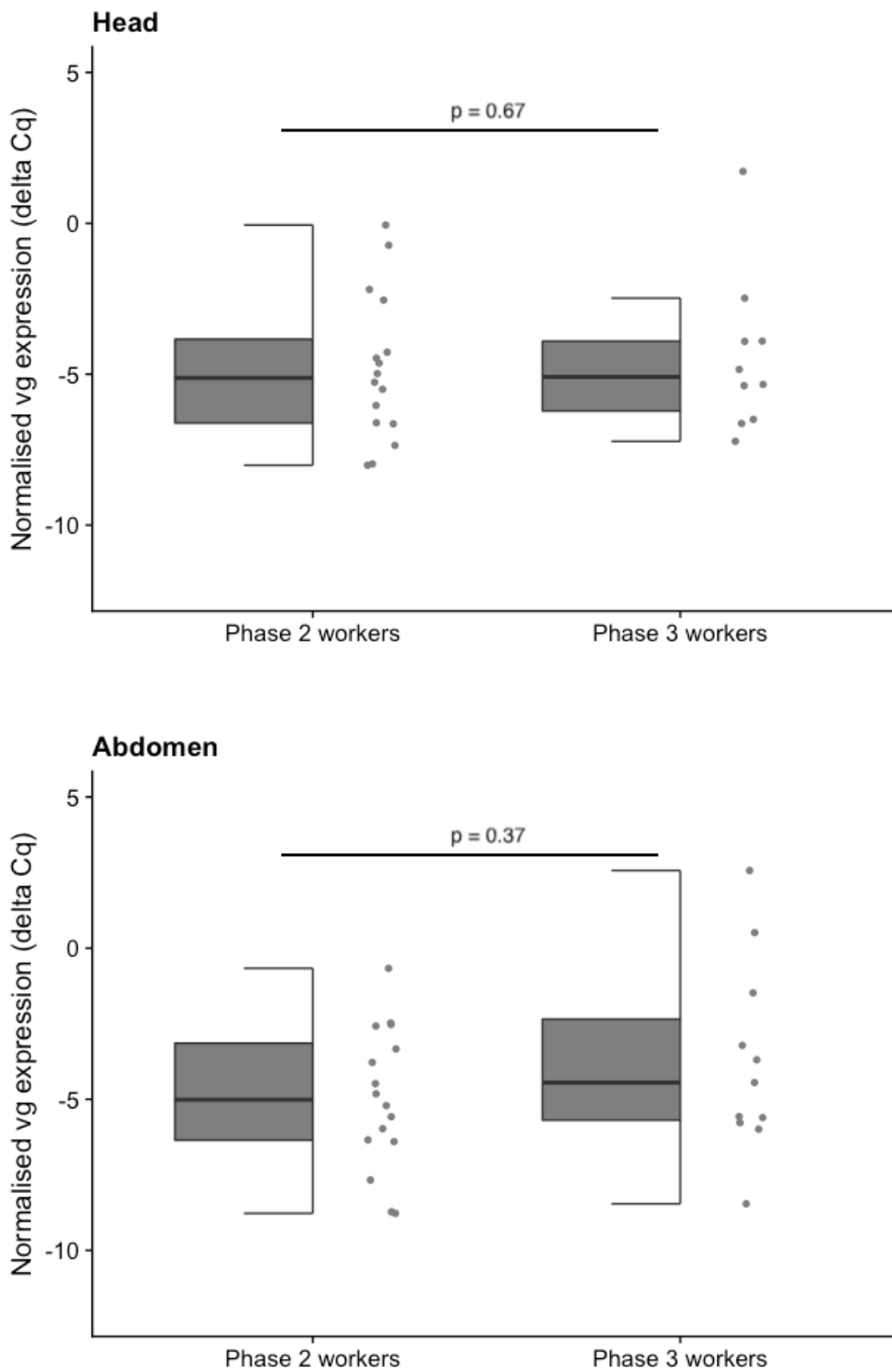


Figure 3.5. The effect of breeding season phase on vg expression levels in worker heads (Head) and abdomens (Abdomen). Phase 2 and phase 3 workers had similar head vg expression levels and similar abdomen vg expression levels

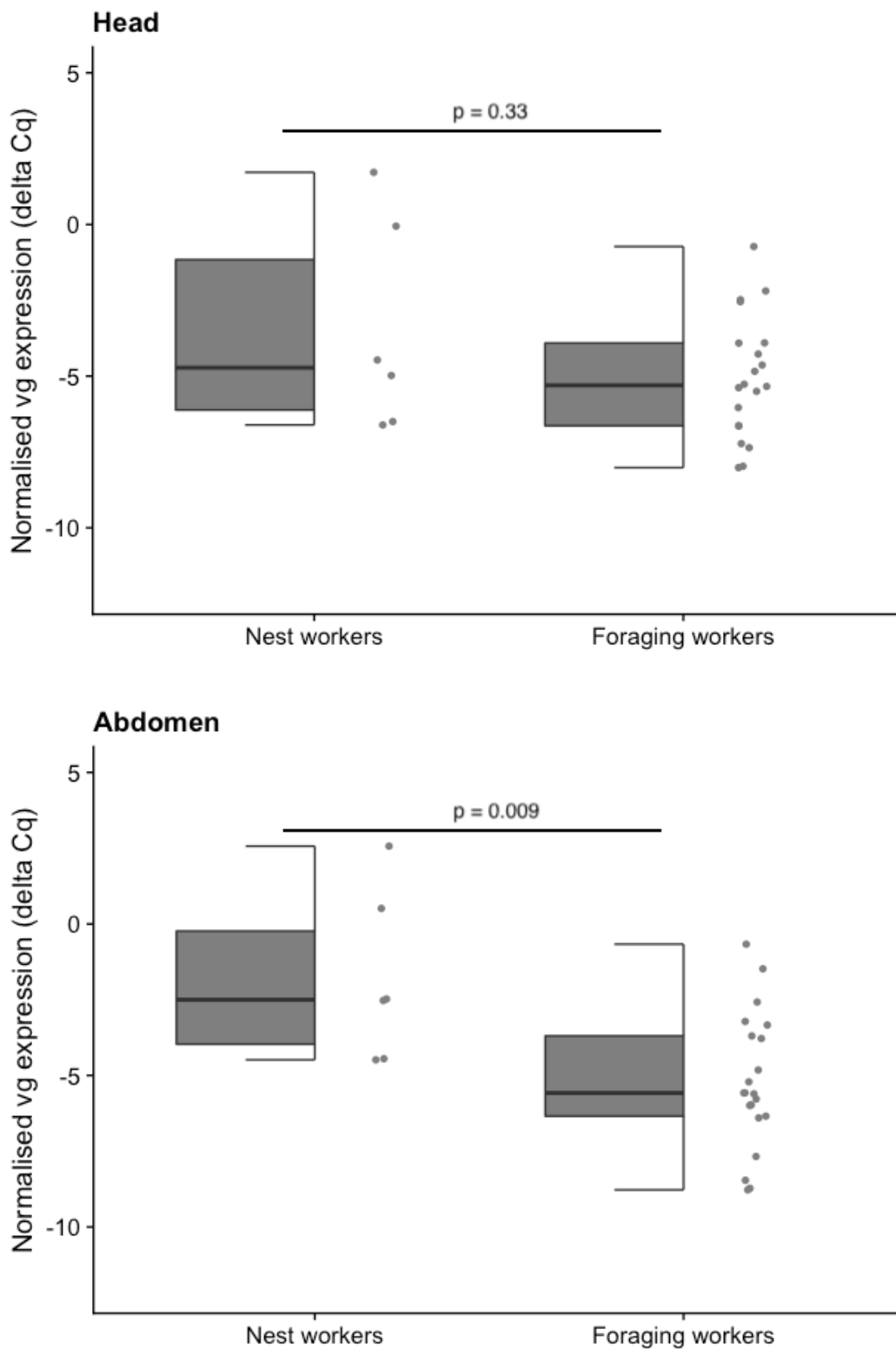


Figure 3.6. *Vitellogenin* expression levels in the heads (Head) and abdomens (Abdomens) of nest workers (collected from nest excavations) and foraging workers (caught on the wing while actively foraging). Workers had similar head *vg* expression, regardless of foraging behaviour). Nest workers had higher abdomen *vg* expression levels than foraging workers

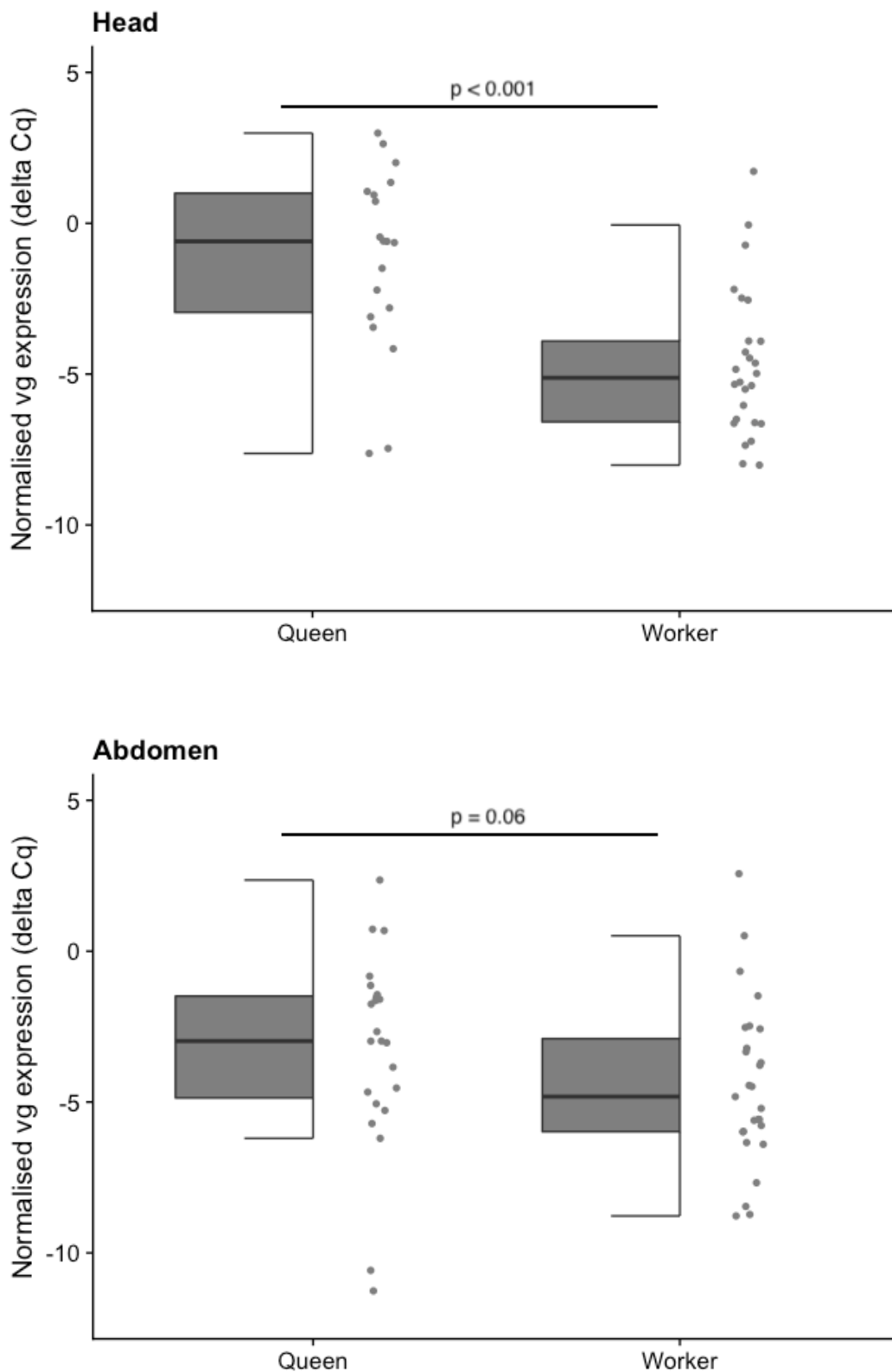


Figure 3.7. The effect of caste on head (Head) and abdomen (Abdomen) vg expression levels. Head and abdomen vg expression levels were higher in queens (spring foundresses and summer queens) than workers (phases 2 and 3).

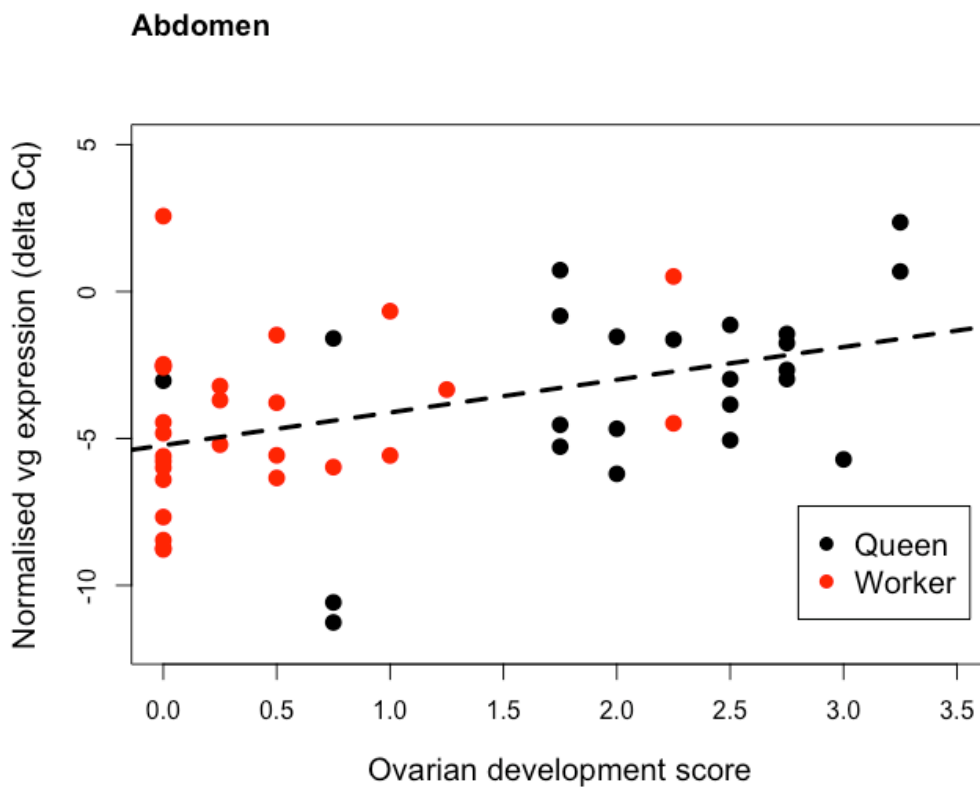
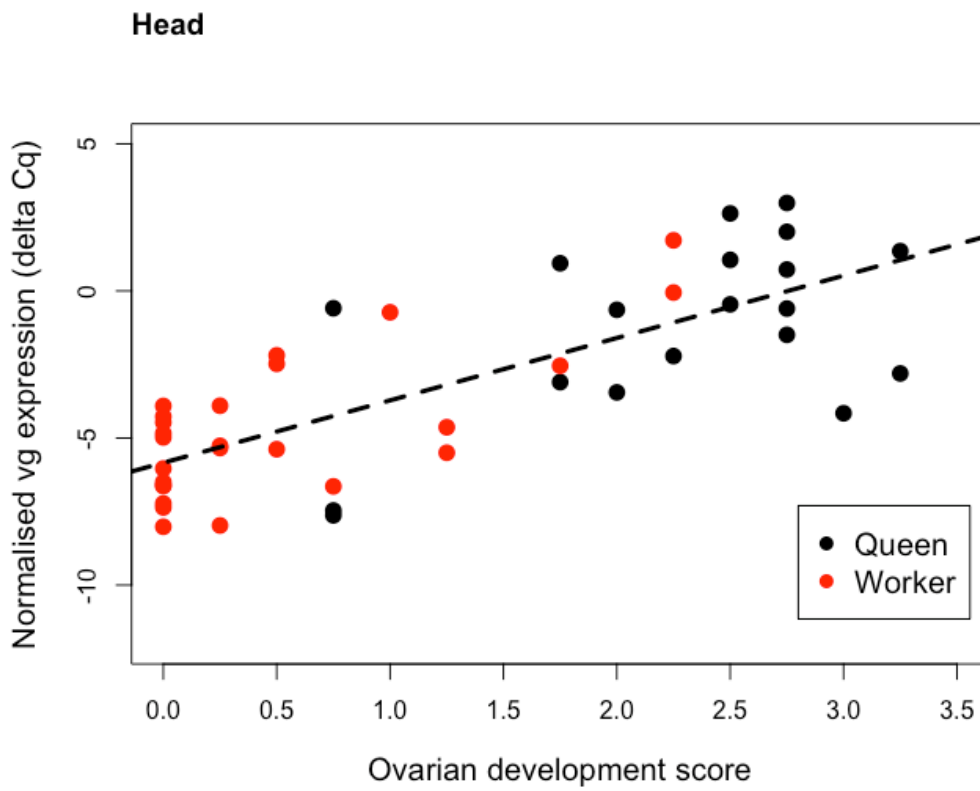
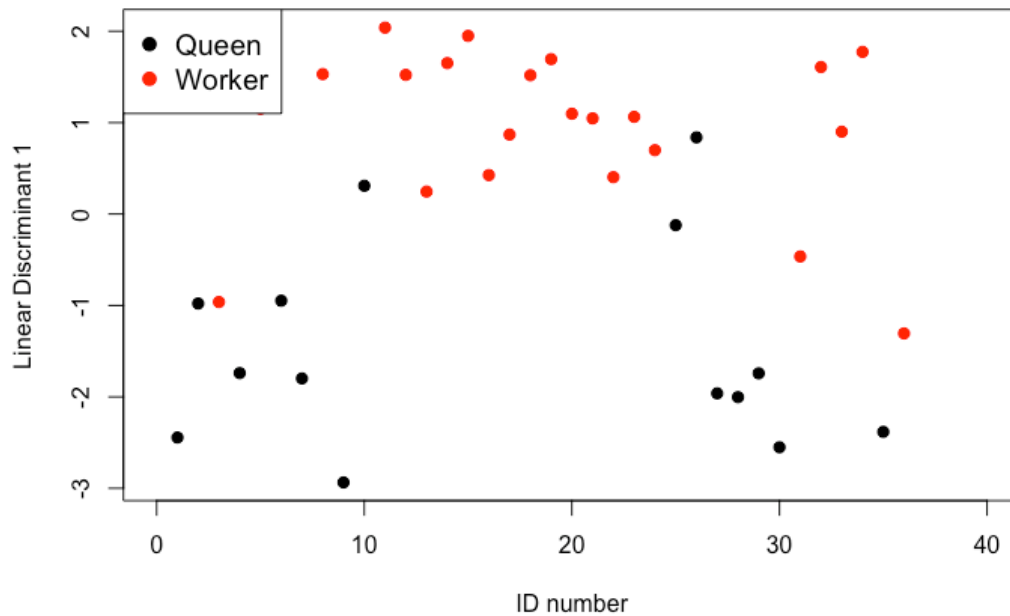


Figure 3.8. The effect of ovarian development scores and caste on *vg* expression levels in queen and worker heads (Head) and abdomens (Abdomen). Effect lines are drawn as black dashed lines. Variation in *vg* expression was best explained by variation in ovarian development score. Caste had no effect on *vg* expression after accounting for ovarian development (Table 3.7, pg. 84)

Physical Traits



Head and abdomen vg expression levels

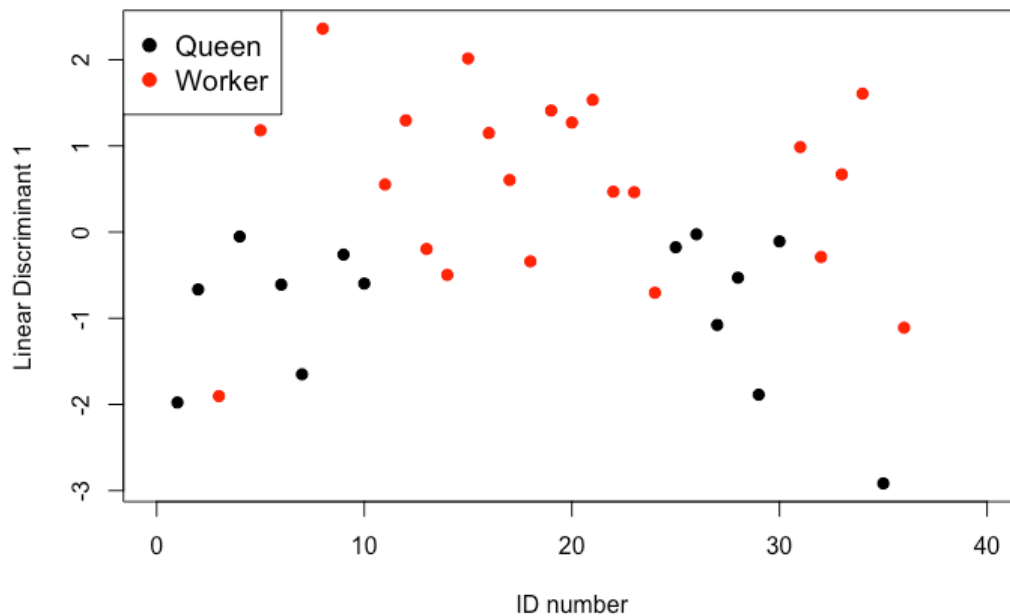


Figure 3.9. Linear Discriminant 1 score generated from a Linear Discriminant Analysis (LDA) with only physical traits (top) and an LDA using only *vg* expression levels (bottom) for each *L. laevissimum* female. Each individual female is colour-coded by their caste assignment using phenological and behavioural data, and is represented by an ID number, which is consistent between the top and bottom figure. Physical traits were head width, mandibular wear, and ovarian development scores. Females were only included if they had values for all measurements. The proportion of correct caste assignments generated from each LDA can be found in Table 3.8, pg. 85

Supplementary Table S3.1. Results and methods from studies that investigate *vg* expression or *Vg* titer levels during the life cycles of insect species. In some cases studies appear under multiple traits if they directly addressed multiple phenotypes

Trait and general pattern	Species	Samples	Body part	Method(s)	Expression Pattern	Reference
Reproductive status	<i>A. mellifera</i> (honeybee)	Queens and workers, larval stages, and males	Whole bodies and ovaries	Western, northern, and southern blot, semi quantitative RT-PCR (one reference gene)	Highest <i>vg</i> expression in ovaries of queens compared to workers	(Guidugli et al. 2005b)
<i>Vg</i> levels are higher in reproductive females	<i>A. mellifera</i> (honeybee)	Workers	Whole bodies	Microarray	Gene expression is higher in ovary-active workers than in ovary-inactive workers in one of two colonies	(Cardoen et al. 2011)
	<i>A. mellifera</i> (honeybee)	Workers and queens	Abdomens	Microarray, RT-qPCR (one reference gene)	Mixed results because of reference genes in RT-qPCR, array showed queens have higher <i>vg</i> expression	(Grozinger et al. 2007)
	<i>B. terrestris</i> (bumblebee)	Queens, workers, larvae, pupae, and males	Whole bodies	RNA-seq	Up-regulated in queens and reproductive workers compared to all other castes and developmental stages	(Harrison et al. 2015)
	<i>B. terrestris</i> (bumblebee)	Queens and workers	Heads and abdomens	RT-qPCR (one reference gene)	Highest expression in reproductive individuals, also high in aggressive individuals, before reproduction	(Lockett et al. 2016)
	<i>B. terrestris</i> (bumblebee)	Queen, gynes, and worker	Heads	Microarray	<i>Vg</i> expression level is associated with reproduction but not provisioning	(Woodard et al. 2014)
	<i>B. terrestris</i> (bumblebee)	Queens and workers	Heads	RT-qPCR (two reference genes)	<i>Vg</i> highest in fertile queens, then 10 day-old queenless fertile workers. Virgin queens (with inactivated ovaries)	(Amsalem et al. 2014)

<i>B. terrestris</i> (bumblebee)	Virgin queens, diapausing queens, reproducing queens, workers, and males	Brains, glands, ovaries, and fat bodies	RT-qPCR (two reference genes)	had higher <i>vg</i> than 4-day-old queenright sterile workers. But this effect seems to have to do more with aggression Highest expression in reproducing queens and workers followed by virgin queens, males, and then last was diapausing queens. Expressed mostly in fat bodies, but there was expression in the glands and flight muscles as well	(Jedlicka et al. 2016)
<i>B. hypocrita</i> (bumblebee)	Queens, workers, and drones at different stages	Abdomens with guts and ovaries removed	RT-qPCR (one reference gene)	<i>Vg</i> is similar in 1 day old queens, workers, and drones. Higher in 5 day old queens than workers and drones. After day 5 days workers have more <i>vg</i> than drones, queens not studied after 7days	(Li et al. 2010)
<i>M. genalis</i> (sweat bee)	Queens, workers, and reproductive solitary females,	Hemolymph	SDS-PAGE, Western blot	Queens had higher <i>Vg</i> titers than workers. Queens had similar <i>Vg</i> titers as solitary females, <i>Vg</i> titer predicted OD in solitary females	(Kapheim et al. 2012)
<i>P. metricus</i> (paper wasp)	Queens, gynes, and workers	Heads	RNA-seq, Microarray, RT-qPCR (one spike-in reference gene)	<i>Vg</i> expression was highest in queens, gynes, then foundresses and workers	(Toth et al. 2007, 2009, 2010)
<i>P. canadensis</i> (paper wasp)	Queens, workers, and young females	Whole bodies	Suppression subtractive hybridization (SS)	<i>Vg</i> expression was highest in queens, then workers, then newly eclosed females	(Sumner et al. 2006)

<i>E. tuberculatum</i> (ant)	Workers	Hemolymph and fat bodies	SDS-PAGE, Western blot, immunohistochemistry staining and fluorescence	Most Vg when workers are at there max OD potential	(Azevedo et al. 2011)
<i>P. barbatus</i> (ant)	Queen, nurse workers, and forager workers	Whole bodies	RT-qPCR (one reference gene)	Vg1 is highly expressed in queens compared to workers and in nurses compared to foragers. Pb_Vg2 was higher in foragers than in nurses and queens.	(Corona et al. 2013)
<i>S. invicta</i> (ant)	Queens and workers	Whole bodies	RT-qPCR (three reference genes)	Fire ants have 4 Vg genes, Vg1 and Vg4 have higher expression in workers compared to queens, Vg2 and Vg3 are higher in queens than workers.	(Tian et al. 2004; Wurm et al. 2011)
<i>T. longispinosus</i> (ant)	Queens and workers	Whole bodies	RNA-seq	Vg2 and Vg3 and Vg-receptor have higher expression in queens than foraging workers Vg1 is highly expressed in the foragers and infertile workers. Vg6 is highly expressed in the fertile workers, followed by the queens and then infertile workers and foragers.	(Feldmeyer et al. 2014)
<i>C. biroi</i> (ant)	Workers	Heads and abdomens	RNA-seq	Head and abdomen - Vgq had higher expression during the reproductive phase compared to the brood care phase and Vgw expression was higher during the brood care phase compared to the reproductive phase	(Oxley et al. 2014)

<i>L. niger</i> (ant)	Queens and workers	Whole bodies	RNA-seq	Vg was highly expressed in queens compared to workers	(Gräff et al. 2007)
<i>F. aquilonia</i> , <i>F. cinerea</i> , <i>F. exsecta</i> , <i>F. fusca</i> , <i>F. pratensis</i> , <i>F. pressilabris</i> , <i>F. truncorum</i> (ants)	Queens and workers	Whole bodies	RT-qPCR (three reference genes)	Vg expression was highest in queens compared to workers in <i>F. aquilonia</i> , <i>F. pressilabris</i> , and <i>F. truncorum</i>	(Morandin et al. 2014)
<i>C. cephalonica</i> (rice moth)	Males and females at multiple stages of development	Whole bodies	RNAi, RT-qPCR (one reference gene), SDS-PAGE	Vg expression was low in early larval stages but disappeared in later stages. In females, Vg was expressed early in pupal stage and throughout adult stage. In males, Vg expression was low in mated males but not virgin males. Vg RNAi in newly eclosed females caused abnormal ovaries.	(Veerana et al. 2014)
<i>D. melanogaster</i> (fruit fly)	Females	Fat bodies	GAL4/UAS system (gene switch, same idea as CRISPER-CAS9), RT-qPCR (one reference gene)	High expression of AmVg and DmCG31150 did not affect overall reproduction or age-specific reproduction	(Ren and Hughes 2014)
<i>M. separata</i> (army worm)	Adult females	whole bodies	RT-qPCR (two reference genes)	Vg expression was positively correlated with seasonal changes in ovarian development	(Xiao et al. 2016)
<i>N. orbicollis</i> (burying beetle)	Adult females	Hemolymph and fat bodies	RT-qPCR (total starting concentration as reference), SDS-PAGE	Vg titer, and vg expression were correlated with the reproductive cycle and parental care of the beetle	(Scott et al. 2005)
<i>N. vespilloides</i> (burying beetle)	Males and females at different points during parental	Brains	RT-qPCR (one reference gene)	Low vg expression during parental care, high expression	(Roy-Zokan et al. 2015)

		care			before and during ovary development – same time period for males	
Foraging / worker tasks	<i>A. mellifera</i> (honeybee)	Workers	Abdomens and hemolymph	RNAi, SDS-PAGE, RT-qPCR (one reference gene)	<i>Vg</i> knockdown results in more JH (forager worker)	(Guidugli et al. 2005b)
<i>Vg</i> is lower foragers compared to non-foragers in honeybees and ant species, but is similar between the same groups in bumblebees	<i>A. mellifera</i> (honeybee)	Workers	Hemolymph	RNAi, SDS-PAGE	<i>Vg</i> knockdown resulted in earlier worker foraging	(Nelson et al. 2007)
	<i>B. terrestris</i> (bumblebee)	Workers	Heads	RT-qPCR (two reference genes)	<i>Vg</i> expression was similar between age matched foragers and nurses	(Amsalem et al. 2014)
	<i>P. barbatus</i> (ant)	Queens, nurse workers, forager workers	Whole bodies	RT-qPCR (one reference gene)	<i>Vg1</i> is highly expressed in queens compared to workers and in nurses compared to foragers. <i>Pb_Vg2</i> was higher in foragers than in nurses and queens.	(Corona et al. 2013)
	<i>E. tuberculatum</i> (ant)	Workers	Hemolymph and fat bodies	SDS-PAGE, Western blot, immunohistochemistry staining and fluorescence	Highest expression at day 20, when workers are nurses, lowest when workers are foraging	(Azevedo et al. 2011)
Aging and development	<i>A. mellifera</i> (honeybee)	Workers	Hemolymph	RNAi, SDS-PAGE	<i>Vg</i> knockdown reduced worker lifespan, likely from susceptibility to oxidative stress (Seehuus et al. 2006)	(Nelson et al. 2007)
<i>Vg</i> is highest in young, caste matched individuals, and <i>vg</i> is higher in queens compared to workers	<i>A. mellifera</i> (honeybee)	Queens, workers, and drones at different ages	Heads, thoracies, and abdomens	RT-qPCR (two spiked-in control genes, <i>In Situ</i> hybridization and imaging, Western blot	<i>Vg</i> expression is highest in young caste matched bees. Old queens have higher expression than workers	(Corona et al. 2007)

Vg is expressed in male and female larvae, very low expression in pupae, and expression increases at eclosion	<i>A. mellifera</i> (honeybee)	Queens, workers, and males through development	Abdomens	Northern blot	vg expression in queen pupal stages, in workers pupal stages, and in freshly molted adult males	(Piulachs et al. 2003)
	<i>B. terrestris</i> (bumblebee)	Queens at different stages before during and after diapause	Fat bodies	RNA-Seq, RT-qPCR (two reference genes)	Vg expression levels showed no differences among mated, diapause and founder post-diapause queens	(Amsalem et al. 2015b)
	<i>B. hypocrita</i> (bumblebee)	Queens, workers, and drones at different developmental stages	Abdomens no guts no ovaries	RT-qPCR (one reference gene)	Vg increases through development and as individuals age, decreases for very old workers and males (queens not studied for long)	(Li et al. 2010)
	<i>O. cornifrons</i> (mason bee)	Females	Fat bodies	Northern blot	Low vg expression mid-diapause, increased in day 3 newly emerged adult, then declined	(Lee et al. 2015)
	<i>C. obscurior</i> (ant)	Queens	Whole bodies	RNA-seq	Vg expression is higher in 4-week-old queens vs. 18-week-old queens	(Von Wychetzkiet al. 2015)
	<i>D. melanogaster</i> (fruit fly)	Females	Fat bodies	GAL4/UAS system (gene switch, same idea as CRISPER-CAS9), RT-qPCR (one reference gene)	Overexpression of AmVg and CG31150 decreased lifespan	(Ren and Hughes 2014)
Aggression	<i>B. terrestris</i> (bumblebee)	Queenless workers	Heads and abdomens	RT-qPCR (two reference genes)	Aggressive bees have the highest vg expression	(Amsalem et al. 2014)
vg expression is highest in aggressive individuals compared to non-aggressive individuals	<i>P. dominula</i> (paper wasp)	Dominant foundresses, subordinate foundresses, and workers,	Brains and whole heads	RT-qPCR (two reference genes)	Expression followed the social rank of adult female wasps. Highest in single foundresses, then dominant foundresses, sub foundresses, and last workers, higher in	(Manfredini et al. 2018)

	head compared to whole brain					
Immunity	<i>A. mellifera</i> (honeybee)	Workers and queens	Hemolymph, fat bodies, and ovaries	Western blot, fluorescence microscopy	Vg as a carrier of immune-priming signals from mother to egg.	(Salmela et al. 2015)
	<i>A. mellifera</i> (honeybee)	Workers	Hemolymph	Immuno-electrophoresis assay	Hemolymph zinc levels was almost entirely explained by Vg titer (better health with more vg)	(Amdam et al. 2004)
<i>vg</i> can sequester ROS, Seehuus et al. (2006) showed that <i>vg</i> can carry immune signals						
Feeding	<i>A. mellifera</i> (honeybee)	Workers	Hemolymph	RNAi, SDS-PAGE	Decrease <i>vg</i> with increased sucrose response	(Amdam et al. 2006b)
	<i>A. mellifera</i> (honeybee)	Workers	Hemolymph and abdomens	SDS-PAGE, RT-qPCR (one reference gene)	Vg titer and transcription is higher in pollen-hoarding workers than nectar hoarding	(Amdam et al. 2004)
<i>vg</i> is lowest in females with a high sugar response						
Males	<i>A. mellifera</i> (honeybee)	Males	mucus glands, testes, rest of the reproductive tract, and fat bodies	SDS-PAGE, Western blot, RT-qPCR (one reference gene)	Decreased <i>vg</i> expression with age, Vg is expressed in reproductive tract and glands but not secreted to hemolymph	(Colonello-Frattini et al. 2010)
	<i>A. mellifera</i> (honeybee)	Queens, workers, and males through development,	Abdomens	Northern blot	<i>vg</i> expression in queen pupal stages, in workers pupal stages, and in freshly molted adult males	(Piulachs et al. 2003)
Males express <i>vg</i> after eclosion, in beetles males express <i>vg</i> through the breeding cycle						

	<i>B. hypocrita</i> (bumblebee)	Queens, workers, and drones at different ages,	Abdomens, no guts no ovaries	RT-qPCR (one reference gene)	<i>Vg</i> is similar in 1day old queens, workers, and drones. Higher in 5day old queens than workers and drones. After day 5 days workers have more <i>vg</i> than drones, queens not studied after 7 days (Li et al. 2010)
	<i>N. vespilloides</i> (burying beetle)	Males and females at different points during parental care	Brains	RT-qPCR (one reference gene)	Low <i>vg</i> expression during parental care, highest before and during ovary development – same time period for males (Roy-Zokan et al. 2015)
Diapause	<i>B. terrestris</i> (bumblebee)	Virgin queens, diapausing queens, reproducing queens, workers, and males	Brains, glands, ovaries, and fat bodies	RT-qPCR (two reference genes)	Highest expression in reproducing queens and workers followed by virgin queens, males, and then last was diapausing queens. Expressed mostly in fat bodies, but their was expression in the glands and flight muscles as well (Jedlicka et al. 2016)
<i>vg</i> is expressed in females preparing to enter diapause and decrease during diapause	<i>O. cornifrons</i> (mason bee)	Females	Fat bodies	Northern blot	Expression pattern: low mid-diapause, increased day 3 newly emerged adult stage, then declined (Lee et al. 2015)
	<i>Tetranychus urticae</i> (spider mite)	Females	Whole bodies	Northern blot	No <i>Vg</i> expression or ovarian development in diapausing adult females (Kawakami et al. 2009)

Chapter 4: *Foraging* expression in a primitively eusocial sweat bee, *Lasioglossum laevisimum*

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Author contributions: DNA, AS, and MHR designed the experiments. DNA and AS designed *L. laevisimum* gene specific primers. DNA collected, measured specimens, measured gene expression, and analyzed the data. AS and MHR provided equipment. DNA wrote the manuscript and AS and MHR edited the manuscript.

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INTRODUCTION

Eusocial organisation is characterized by cooperative brood care, overlapping generations, and a reproductive division of labour (Batra 1966; Wilson 1971; Michener 1974). In eusocial Hymenoptera, one or a few females are queens, which lay almost all the eggs in a colony, while the remaining majority of females are workers, which provide the necessary parental care to successfully rear the queen's offspring. Queens and workers exhibit a bias with respect to the undertaking of parental care tasks, which workers do more of than queens when both castes are present in a colony. One of these tasks is the provisioning of developing offspring (Wilson 1971; Michener 1974; Royle et al. 2012). Provisioning consists of foraging for food and bringing it back to the colony for developing offspring. Depending on the species, workers mass provision or progressively provision offspring (Michener 1974, 2000; Cowan 1991). Workers of mass-provisioning species collect and prepare the food needed for a larva to reach adulthood before an egg is ever laid, while workers of progressive provisioning species continually bring food to a larva as it grows and develops.

The ovarian ground plan hypothesis (OGPH) posits that the egg-laying and provisioning phenotypes exhibited by solitary ancestors underwent an evolutionary decoupling to be expressed separately in queens and workers of highly eusocial descendants (West-Eberhard, 1987; depicted in Chapter 1 Fig.1.1, pg. 7). In extant, highly eusocial taxa, queens and workers are morphologically distinct and each caste is suited to their social roles; i.e. egg-laying by queens and provisioning by workers (Wilson 1971; Michener 1974). Queens in these species express egg-laying phenotypes and rarely provision offspring, while workers provision offspring and very rarely lay eggs. The OGPH suggests that the expression of genes and molecular mechanisms associated with egg-laying and provisioning phenotypes in solitary ancestors

decoupled to be expressed separately in queens and workers (West-Eberhard 1987, 1996). Queens express genes associated with egg-laying, while workers express genes associated with provisioning. As mentioned in Chapters 1 and 3, the OGPB implies that the expression of genes associated with provisioning should be skewed towards workers in taxa whose social organization may represent an intermediate stage between solitary behaviour and highly eusocial castes, as in lineages such as Apinae (Chapter 1 Fig.1.1, pg. 7; Rehan and Toth 2015; Sumner et al. 2018).

Foraging gene expression influences foraging behaviour in insects

One major aspect of provisioning offspring is actively searching for food, i.e. foraging. Many studies have used solitary and social insect taxa to examine the relationship between specific gene expression patterns and foraging behaviour (Lockett et al. 2016; Fischer and O’Connell 2017; Weitekamp et al. 2017). The *foraging* gene (*for*) is one of the best studied genes with respect to insect foraging behaviour (Reaume and Sokolowski 2011; Fischer and O’Connell 2017; Weitekamp et al. 2017). Sokolowski (1980) was the first to show the link between the *for* gene and foraging phenotypes. In *Drosophila melanogaster*, “rover” larvae travel longer distances than “sitter” larvae while feeding. This difference in foraging behaviour is consistent in adult rover and sitter flies as well (Pereira and Sokolowski 1993). Differences in foraging behaviour between rovers and sitters are attributed to differences in *for* genotypes (de Belle and Sokolowski 1989), mRNA splicing (Anreiter et al. 2017), mRNA expression levels (Osborne et al. 1997; Anreiter et al. 2017), and epigenetic regulation of specific *for* promoters in adult brains and ovaries (Anreiter et al. 2017). The *for* gene itself encodes a cyclic guanosine monophosphate (cGMP) dependent protein kinase (PKG; Osborne et al. 1997). In general, increased *for* expression and the resulting increase in PKG activity results in increased foraging

behaviour, directly tying differential *for* expression to foraging phenotypes (Osborne et al. 1997; Allen et al. 2017). Furthermore, PKG activity may influence foraging phenotypes via interactions with metabolic and nutrition-related sensory pathways (Kaun et al. 2007; Hilliker et al. 2009; Kent et al. 2009; Allen et al. 2017).

The *for* gene also plays a role in regulating differences in foraging behaviour between individuals of social insect colonies (Table 4.1, pg. 128; partially summarized in Lockett et al. 2016). In social hymenopteran species, high *for* expression levels are associated with foraging phenotypes in some but not all species (summarized in Table 4.1, pg. 128, and partially in Lockett et al. 2016). In honeybees (*Apis mellifera*), at least two splice variants of the *for* transcript are expressed, *for α* and *for β* (Thamm and Scheiner 2014). The expression of *for α* is higher in foraging workers compared to non-foraging workers; conversely *for β* is expressed at similar levels in foraging and non-foraging workers (Ben-Shahar et al. 2002; Ben-Shahar 2003; Thamm and Scheiner 2014). To date, honeybees are the only social insect for which the expression of *for* splice variants have been investigated. Similar to *for α* expression patterns in *A. mellifera*, *for* expression levels are higher in foragers than non-foragers in *A. cerana*, a *Bombus terrestris* population, *Polistes metricus*, *Pogonomyrmex occidentalis*, and a subset of *P. barbatus* workers (Table 4.1, pg. 128). In contrast, *for* expression levels are higher in non-foragers compared to foragers in *B. ignites*, *Vespula vulgaris*, *Solenopsis invicta*, *Cardiocondyla obscurior*, and a different subset of *P. barbatus* workers (Table 4.1, pg. 128).

Taken together, results from distantly related social taxa indicate that *for* expression levels are correlated with differences in foraging behaviour, with only a couple exceptions in which no relationship was observed (Table 4.1, pg. 128). Interestingly, the direction of these correlations differs between species, and in some cases, within species (*B. terrestris* and *P.*

barbatus). An explanation for these conflicting results is that some of these expression assays are not well controlled since they use a single internal reference gene to normalize target gene expression, which can lead to inaccurate assessments of relative target gene expression levels (Vandesompele et al. 2002; Huggett et al. 2005; Bustin et al. 2009). If I tighten my criteria and consider only those studies in which microarray, transcriptome data, expression data normalised with >1 reference genes as per the MIQE guidelines (Bustin et al. 2009), or expression data with additional experiments or evidence are used then there is a much smaller collection of studies to consider. Foragers have higher *for* expression than non-foragers in *A. mellifera*, a *B. terrestris* population, and *P. metricus* (Table 4.1, pg. 128). However, non-foragers have higher *for* expression levels than foragers in *S. invicta* (Table 4.1, pg. 128). The correlation between *for* expression levels and foraging phenotypes is positive in some species of bees and *P. metricus*, and negative in *S. invicta*. These results suggest that there are unknown biological differences in the role and regulation of *for* expression between species (Sokolowski 2010). Specifically comparing *fora* expression levels or implementing a sampling effort that takes into account seasonal changes in behaviour through a breeding season may uncover some of these biological differences.

Variation in foraging behaviour in castes of eusocial sweat bees

It is widely understood that primitive eusociality may represent a transitional state between solitary and highly eusocial behaviour (Schwarz et al. 2007; Rehan and Toth 2015; Toth and Rehan 2017; Sumner et al. 2018; Taylor et al. 2018). In a primitively eusocial species, females are morphometrically similar and newly eclosed females are capable of exhibiting egg-laying and provisioning phenotypes (Yanega 1989; Schwarz et al. 2007). New females have the ability to found a nest, construct brood cells, forage for brood provisions, and lay eggs. In terms

of foraging behaviour, both queens and workers forage and collect brood provisions at some point during the breeding season (Fig.1.2, pg. 13 in Chapter 1). Whether or not a queen forages largely depends on the time of year, specifically the phase of the breeding season. Gynes (future queens) likely forage for their own metabolic needs during phases 2 and 3 before overwintering, foundresses forage and collect brood provisions while establishing their nests during phase 1, and queens no longer forage when their workers start foraging during phases 2 and 3 (Michener 1974, 1990; Richards and Packer 1998; Schwarz et al. 2007). On the other hand, most workers forage and provision the queen's brood several days after they eclose during phases 2 and 3, and continue foraging until they die in fall (Michener 1974). Therefore, workers are foragers most of their lives unlike queens, which forage in some, but not all phases of the breeding season. Sweat bee queens and workers are morphometrically similar, which means both castes are physically capable of undertaking similar tasks, including foraging (Yanega 1989; Schwarz et al. 2007). Whether or not an individual forages does not necessarily distinguish a queen from a worker. Instead of physical ability, whether or not a queen or worker forages is likely influenced by other environmental and genetic factors.

Two environmental factors that play a large role in whether or not a sweat bee female forages are weather conditions and the social environment of the nest. Daily and seasonal variation in weather conditions can have a dramatic influence as to whether or not a sweat bee forages. In general, sweat bees in Canada fly and collect provisions on clear days (no rain or severe wind) between April and September, when temperatures are $>14^{\circ}\text{C}$ (D.N. Awde pers. obs.; Packer et al. 1989; Richards 2004; Richards et al. 2015). Therefore, foraging activity can grind to a halt during periods of rainy or cool weather. Furthermore, weather conditions at specific periods during the breeding season can influence whether or not a female forages or opts

to enter diapause. Late in the breeding season, if weather conditions are favourable, second brood females may forage and collect provisions for an additional brood instead of entering diapause (Packer et al. 1989; Packer 1992; Awde and Richards 2018).

The social environment, specifically who else is in the nest, can have a large impact as to whether or not a eusocial sweat bee forages. During phase 1, nests are founded solitarily or by multiple females. In solitary nests, foundresses forage for brood provisions and for their own survival. In multi-foundress nests, one female becomes the dominant female and does not forage, while the subordinate female(s) takes on the foraging and provisioning tasks of the nest (Richards and Packer 1998). During phases 2 and 3, workers emerge and perform the foraging and provisioning tasks for solitarily founded nests, or workers collect provisions along with subordinate females from multi-foundress nests (Richards and Packer 1998). Solitary foundresses (queens) on the other hand, cease foraging and provisioning activity during phases 2 and 3 (Richards and Packer 1998). However, a queen may resume foraging activity to provision the next brood if she loses all of her workers or subordinates (Richards and Packer 1998). Whether or not a subordinate foundress or a worker forages is likely influenced by the queen's physical coercion. Dominant females have been observed physically ejecting subordinates from multi-foundress nests (Richards and Packer 1998), and queens are the most aggressive females in eusocial colonies (Michener and Brothers 1974).

The genetic mechanisms that influence foraging and provisioning behaviour in eusocial sweat bees are unknown. Furthermore, no studies have taken advantage of eusocial sweat bee castes to test the prediction that high *for* expression is positively correlated with foraging behaviour, as it is in other bee species (Table 4.1, pg. 128). Sweat bee castes exhibit an abundance of intra- and inter-caste variation in foraging behaviour through a breeding season,

which should provide valuable comparisons of *for* expression with respect to seasonal or social variation in foraging behaviour.

***Lasioglossum laevissimum* as a model to understand how *for* expression influences foraging and provisioning behaviour**

The main objective of this study was to investigate the relationship between *for* expression and whether or not individual sweat bees were foraging or provisioning when collected, while taking into account variables such as phase of the breeding season and caste. Field observations of a *Lasioglossum laevissimum* population in southern Ontario Canada have demonstrated the general intra- and inter-caste variation in foraging behaviour typical of eusocial sweat bee castes (Awde and Richards 2018). *Lasioglossum laevissimum* gynes emerge in late summer, overwinter, emerge from their hibernacula in spring, found and provision a nest, and then remain in the same nest through summer. Workers are only present during the summer, provision during the day then return to their nest in the evening. Variation in foraging and provisioning behaviour, and detailed observations of colony phenology make *L. laevissimum* an ideal model to investigate the relationship between *for* gene expression and whether or not an individual was foraging. *Lasioglossum laevissimum* queens collected at different phases of the breeding season can be used to describe changes in *for* that are associated with seasonal shifts in foraging and provisioning behaviour. Workers collected from nests in the morning and workers collected while actively provisioning mid-day can be used to describe *for* expression patterns stemming from a diurnal change in foraging activity. Finally, queens and workers can be compared to whether or not there is an underlying difference in *for* expression between eusocial castes.

Foraging expression is consistently associated with insect foraging behaviour (Table 4.1, pg. 128), which is a fundamental part of brood provisioning. In at least 2 bee species, *for* expression is highest in foragers compared to non-foragers. The OGPB suggests that genes associated with provisioning should have higher expression in workers than queens in a species, such as *L. laevissimum*, whose social organisation may represent a transitional stage between solitary behaviour and highly eusocial castes. Therefore, I hypothesized that in *L. laevissimum* individuals high *for* expression results in foraging behaviour, which is an essential part of provisioning. From this hypothesis I made specific predictions with respect to *for* expression patterns in *L. laevissimum* individuals. First, I predicted that spring foundresses (actively provisioning) and gynes (possible foragers) should have higher *for* expression levels than early spring foundresses (pre-provisioning) and summer queens (post-provisioning). Second, I predicted that workers collected while actively provisioning mid-day should have higher *for* expression levels than workers collected from nests in the morning before provisioning activity began. Finally, based on the OGPB and the hypothesized relationship between *for* expression and provisioning behaviour, I predicted that *L. laevissimum* workers should have higher *for* expression levels than queens.

METHODS

Lasioglossum laevisissimum collections and morphometrics

Descriptions of the collection, storage, and dissections of *L. laevisissimum* females and males are provided in Chapters 2 and 3. Briefly, samples were collected from an aggregation located at Brock University, Ontario, Canada. During phase 1, spring foundresses were collected on the wing while actively provisioning. During phases 2 and 3, summer queens, workers, and gynes were collected from nest excavations in the morning. Queens were classified as post-provisioning since they had ceased provisioning behaviour after their workers eclosed in phase 2. Workers collected from nests were classified as not provisioning since they were collected in the morning before foraging activity began. Gynes were classified as potential foragers since they likely forage for their own metabolic needs during the day. Additional workers and males and were collected on the wing. Workers that were collected on the wing were collected while actively provisioning and classified as such. Finally, I collected early spring foundresses still in their hibernacula ~2 weeks before phase 1 began by excavating 30x30x30cm soil cubes from areas in the aggregation that had nests the previous summer. These early spring foundresses were classified as pre-provisioning since they were only a few weeks from actively foraging and initiating their nests.

Adult females and males were measured and dissected under RNA preservative using a stereomicroscope as per Awde and Richards (2018). Size measurements and wear scores were used in part to assign each female to caste (see Chapter 2 for details), and to quantify their influence on normalised *for* expression in analyses described below. Briefly, head width (HW) was measured as the distance across the widest part of the head and wing wear (WW) was also scored from 0 (unworn wing margins) to 5 (damage along the entire wing margin). Mandibular

wear (MW) was also scored from 0 (pristine condition) to 5 (completely worn). After an individual was measured and dissected I removed the head and abdomen (gut removed) from the thorax and stored each body part (head, thorax, and abdomen) separately in preservative and in the freezer until RNA extraction. Samples sizes of each *L. laevissimum* category used in head and abdomen gene expression analyses can be found in Table 4.2, pg. 129.

RNA extractions and cDNA preparation

RNA extraction and cDNA preparation protocols can be found in Chapter 3. Briefly, RNA was extracted using a Total RNA Purification kit (Norgen Biotek Corp.) and eluted to a volume of 50 µl with water. RNA was converted into single-stranded cDNA, 20 µl final volume, using SuperScript III Reverse Transcriptase (Invitrogen), RNase Inhibitor (BioShop), dNTP mix (BioShop), and Oligo dTVN₂₀ primers (Sigma-Aldrich).

Primer design and PCR amplicon validation for RT-qPCR

I used gene specific primers of three reference genes for real-time quantitative PCR (RT-qPCR): *glyceraldehyde 3-phosphate dehydrogenase (gapdh)*, *actin*, and *acidic ribosomal protein P2 (rpP2)*, described in Chapter 3 (Table 3.3, pg. 80). Reference genes were used to control for technical and biological variation in the amount and quality of starting mRNA in each sample, and to normalize target gene expression. Using multiple reference genes provides a more robust method for producing accurate normalization of target gene expression compared to using a single reference gene (Vandesompele et al. 2002; Huggett et al. 2005; MIQE guidelines in Bustin et al. 2009).

I also designed and tested gene specific primers for the target gene, *for*. These primers specifically amplified the *forα* transcript (from exon 1 to exon 2) but not the *forβ* transcript (which, does not include exons 1 and 2) identified in honeybees (Thamm and Scheiner 2014). I

retrieved *for* mRNA and gDNA sequences of bee species from GenBank (*Apis sp.*, *Bombus sp.*, etc.). I also received a *L. albipes for* mRNA sequence through personal communication with Dr. Sarah D. Kocher (Princeton University). *Foraging* sequences were aligned using ClustalOmega (Sievers et al. 2011), and *for* primers were designed using the software program Primer-BLAST (Primer3; Ye et al. 2012). The annealing location of the *for* forward primer (5'-TCGCTGACAGTCGTCGATAA -3') was on exon 1 and the reverse primer (5'-AAACGATGGACCCGACATCT -3') annealed on exon 2. I used polyacrylamide gels to confirm the expected size of the *for* amplicon size (184 bp). I sequenced the PCR amplicon (Genome Québec) and verified its identity using BLAST, and the sequences will be uploaded to NCBI.

RT-qPCR protocol

Gene expression was measured with RT-qPCR, as in Chapter 3. Briefly, all 4 genes (*for* and the 3 reference genes) were measured for each cDNA sample on the same plate, with separate reactions, and no-template controls for each primer set. Reactions were performed in technical triplicates for each sample, with each set of gene-specific primers. I averaged the C_q values from the triplicate reactions to give a single value for each gene with each sample. The replicate furthest from the median was excluded if the standard deviation of the three replicates was $>0.5 C_q$. A melt-curve analysis (ramping from 65°C to 95°C in 0.5°C steps every 5 sec) was included to verify that amplification was not the result of primer dimerization or gDNA contamination.

Data analysis

I used the RT-qPCR reaction to determine whether *L. laevissimum* samples had detectable expression levels of each gene. If a C_q value for a given sample was not recorded

before the end of 31 cycles then expression was categorized as undetectable. Samples were only included in analyses if they had detectable levels of *gapdh*, *actin*, and *rpP2*. This represented my threshold for detectable levels of mRNA and therefore, the ability to detect *for* expression reliably. Of the samples with detectable mRNA, I compared the proportion that had detectable levels of *for* expression between *L. laevissimum* categories (males, gynes, summer queens, etc.) with Fisher's exact tests for each body part separately (Table 4.3, pg. 130). The proportion of samples with detectable *for* expression in their heads were similar between categories (Table 4.3, pg. 130; Fisher's exact test, $p = 0.19$), as was the proportion of samples with detectable *for* expression in their abdomens (Table 4.3, pg. 130; Fisher's exact test, $p = 0.88$). The proportion of samples with detectable levels of *for* was also similar between categories in a small subset of thorax samples (Fisher's exact test, $p = 0.14$). These preliminary analyses demonstrated that I was just as likely to detect *for* expression in each sample category, regardless of body part. I focused my attention on head and abdomen comparisons in subsequent analyses since these body parts are often the focus of gene expression studies using other social insects (Table 4.1, pg. 128). Therefore, analyses described below apply to head and abdomen samples only.

I validated *gapdh*, *actin*, and *rpP2* as suitable reference genes in head and abdomen samples that had detectable expression levels for all three genes, as in Chapter 3. Normalization of target gene expression by multiple, validated, reference genes provides a more accurate assessment of target gene expression than using a single reference gene (Vandesompele et al. 2002; Huggett et al. 2005; MIQE guidelines in Bustin et al. 2009). In *L. laevissimum*, *gapdh*, *actin*, and *rpP2* expression values had a significant positive correlation in pair-wise comparisons with each other using head and abdomen samples (Fig.4.1, pg. 132). Correlations between the three genes were tighter using abdomen samples (Pearson correlation coefficient range $r = 0.90 -$

0.96) compared to head samples (Pearson correlation coefficient range $r = 0.60 - 0.76$). All three genes were validated as suitable reference genes in head and abdomen samples separately using RefFinder.

Foraging expression was normalised to the geometric mean of the three reference genes [Normalized *for* expression (ΔC_q) = geometric mean of reference gene expression (C_q) – *for* expression (C_q)]. As in Chapter 3, if a body part had detectable levels of all three reference genes and no detectable *for* expression, I set the C_q value of *vg* to 31.

I used separate Kruskal-Wallis tests to investigate how normalised *for* expression varied between *L. laevissimum* sample categories. Male and female *L. laevissimum* were compared to describe *for* expression by sex. Early spring foundresses, spring foundresses, summer queens, and gynes were compared to understand how *for* expression varied by phase of the breeding season. Finally, workers collected from nests in the morning were compared to workers collected on the wing mid-day to understand how *for* expression varied with daily cycles in provisioning behaviour.

I quantified the influence of size, wear, and caste on normalised *for* expression (response variable) in each body part separately with two general linear models. Head width was included as the first predictor variable in each model to account for variation in *for* expression that may be the result of size; a trait used to differentiate a queen from her workers. Typically, the more a bee forages, the more wing wear it accumulates. However 80% of *L. laevissimum* females do not have wing wear (Awde and Richards 2018). Therefore, wing wear was left out of the model and mandibular wear, which is associated with the amount of nest construction a female does, was used instead. Finally, caste was included in the model after mandibular wear to investigate if there is a caste effect on *for* expression above and beyond differences in mandibular wear.

RESULTS

***Foraging* expression in heads and abdomens**

I first compared *for* expression levels between the heads and abdomens of *L. laevissimum* males and females that had values for both body parts. *Lasioglossum laevissimum* males and females with high *for* expression levels in their head had high expression levels in their abdomen (Fig.4.2A, pg. 133; Pearson correlation coefficient $r = 0.56$, $df = 31$, $p < 0.001$). Additionally, the same individuals had similar head and abdomen *for* expression levels (Fig.4.2B, pg. 133; Paired t-test; $t = 0.64$, $df = 32$, $p = 0.53$).

Inter-caste variation in *for* expression

Foundresses, Queens, and Gynes

I investigated variation in *for* expression at different points through the breeding season by comparing foundresses, queens, and gynes collected during different breeding season phases. Each sampling category represented a different provisioning / foraging phenotype (e.g. early spring foundresses were pre-provisioning). I predicted that spring foundresses (actively provisioning) and gynes (possible foragers) would have higher *for* expression levels than early spring foundresses (pre-provisioning) or summer queens (post-provisioning). Contrary to my prediction, early spring foundresses had the highest levels of *for* expression compared to spring foundresses, summer queens, and gynes (Fig.4.3, pg. 134; Heads - Kruskal-Wallis $\chi^2 = 13.40$, $df = 3$, $p = 0.004$; Abdomens - Kruskal-Wallis $\chi^2 = 12.84$, $df = 3$, $p = 0.005$). Females that were actively provisioning at the time of capture (spring foundresses) actually had the lowest levels of head and abdomen *for* expression. Interestingly, summer queens, which had ceased provisioning had higher levels of head and abdomen expression than spring foundresses (Fig.4.3, pg. 134;

Head - Kruskal-Wallis $\chi^2 = 2.7$, $df = 1$, $p = 0.10$; Abdomen - Kruskal-Wallis $\chi^2 = 5.36$, $df = 1$, $p = 0.02$), although, the difference in head *for* expression was not statistically significantly different.

Workers

Next, I compared normalised *for* expression levels of workers collected from nest excavations in the morning (not provisioning) to workers collected on the wing mid-day (actively provisioning) to see if *for* expression levels reflected a diurnal shift in provisioning behaviour. Head and abdomen samples generated similar results: workers collected on the wing had *for* expression levels similar to those of workers collected from nest excavations (Fig.4.4, pg. 135; Heads - Kruskal-Wallis $\chi^2 = 0.05$, $df = 1$, $p = 0.83$; Abdomens - Kruskal-Wallis $\chi^2 = 1.2$, $df = 1$, $p = 0.27$).

Foraging expression patterns in queens and workers

The OGPB suggests that the expression of genes associated with provisioning should be biased towards workers compared to queens. I predicted that workers should have higher levels of *for* expression than queens. Queens and foundresses differed in *for* expression levels depending on the time of year (Fig.4.3, pg. 134). Therefore, I used only summer queens as the representative queen group, since they no longer provision offspring and remain in the nest laying eggs. On the other hand, workers collected on the wing and workers collected from nests had similar *for* expression levels (Fig.4.4, pg. 135), therefore I used all workers, regardless of where or how they were collected, in the comparison to queens. I found that queens and workers had similar head and abdomen *for* expression levels (Fig.4.5, pg. 136; Head - Kruskal-Wallis $\chi^2 = 0.06$, $df = 1$, $p = 0.81$; Abdomen - Kruskal-Wallis $\chi^2 = 0.25$, $df = 1$, $p = 0.62$). Furthermore, head width, mandibular wear, and caste did not explain variation in *for* expression levels (Table 4.4, pg. 131; Head – Adjusted $R^2 = 0.07$; Abdomen – Adjusted $R^2 = -0.20$).

Foraging expression in L. laevissimum males

Finally, I investigated whether or not *for* expression varied by sex by comparing normalised *for* expression levels of males to females, regardless of age, caste, or when they were captured. Male heads had significantly higher *for* expression levels compared to female heads (Fig.4.6, pg. 137; Kruskal-Wallis $\chi^2 = 11.1$, $df = 1$, $p < 0.001$). Similarly, male abdomens had higher *for* expression levels compared to female abdomens, but this difference was not statistically significant (Fig.4.6, pg. 137; Kruskal-Wallis $\chi^2 = 3.53$, $df = 1$, $p = 0.06$). I produced similar results when I compared males to females that likely eclosed during the same phase of the breeding season (gynes), and when I compared males to females that were also captured on the wing during phase 3 (workers). In both cases males had significantly higher *for* expression levels than females in heads (Males vs. gynes - Kruskal-Wallis $\chi^2 = 6.82$, $df = 1$, $p = 0.01$; Males vs. worker - Kruskal-Wallis $\chi^2 = 7.50$, $df = 1$, $p = 0.006$), but not abdomens (Males vs. gynes - Kruskal-Wallis $\chi^2 = 2.13$, $df = 1$, $p = 0.14$; Males vs. worker - Kruskal-Wallis $\chi^2 = 0.64$, $df = 1$, $p = 0.42$).

DISCUSSION

Foraging expression in L. laevissimum queens and workers

Foraging expression is consistently associated with insect foraging behaviour (Table 4.1, pg. 128). Therefore, I hypothesized that in *L. laevissimum* individuals, high *for* expression results in foraging behaviour. The OGPB suggests that genes associated with provisioning, of which, foraging behaviour is an essential part, should be expressed more in workers than queens (West-Eberhard 1987, 1996; Rehan and Toth 2015). From these two hypotheses I made 3 specific predictions: 1. Workers should have higher *for* expression than queens. 2. Spring foundresses and gynes should have higher *for* expression levels than early spring foundresses and summer queens. 3. Workers collected while actively provisioning mid-day should have higher *for* expression levels than workers collected from nests in the morning before provisioning activity began. None of these predictions were borne out: workers and queens had similar *for* expression levels, early spring foundresses had higher *for* expression than spring foundresses and gynes, and workers caught on the wing and collected from nests had similar *for* expression levels. These results suggest a nuanced relationship between *for* expression levels and *L. laevissimum* foraging and provisioning behaviour, beyond simply whether or not a bee is foraging.

Foraging expression may be similar between *L. laevissimum* queens and workers because sweat bee females, regardless of caste, are foragers at some point during the breeding season (Fig.1.2, pg. 13 in Chapter 1; Schwarz et al. 2007). For this reason foraging behaviour is not a particularly useful trait on its own to differentiate sweat bee queens from workers. Furthermore, queens and workers are morphologically similar and physically capable of similar amounts of foraging behaviour (Yanega 1989; Schwarz et al. 2007). Therefore, variation in *for* expression

levels may not differentiate *L. laevissimum* workers from queens since foraging behaviour on its own doesn't necessarily indicate a female's caste.

Foraging expression levels have been compared between queens and workers in only two other hymenopteran species, *P. metricus* (Toth et al. 2007, 2010) and *B. terrestris* (Woodard et al. 2014). Similar to *L. laevissimum*, queens and workers of both of these species actively forage and provision offspring at some point during the breeding season. Fortunately, these studies report reliable gene expression results (Table 4.1, pg. 128). In *P. metricus*, *for* expression levels are higher in workers than queens (Toth et al. 2007, 2010). In contrast, *B. terrestris* workers and queens had similar *for* expression levels (Woodard et al. 2014). *Foraging* expression patterns of queens and workers in *L. laevissimum* are similar to *B. terrestris* but not *P. metricus*. In light of limited taxonomic sampling with respect to queen vs. worker comparisons, these results suggest that *Polistes* castes differ in *for* expression but bee castes do not (clade Anthophila in the superfamily Apoidea). Therefore, differences in *for* expression patterns between species may stem from lineage specific differences between wasps and bees, such as diet and nutritional requirements. The diets of *B. terrestris* and *L. laevissimum* are more similar to each other than they are to *P. metricus*. Most bee species eat pollen as their protein source while *P. metricus* is carnivorous and uses prey fluid as its protein source (Michener 2000; Wcislo and Fewell 2017). Furthermore, *for* genotypic and expression differences have been linked to food responsiveness and energy metabolism in *D. melanogaster* (Belay et al. 2007; Kaun et al. 2007; Kent et al. 2009). This means that beyond caste differences, *for* expression may vary with respect to other molecular pathways that influence differences in diet and metabolism between taxa.

The ovarian ground plan hypothesis (OGPH) suggests that gene expression associated with provisioning behaviour should be expressed more in workers than queens (West-Eberhard

1987, 1996; Rehan and Toth 2015). This was not the case with respect to *for* expression levels in *L. laevissimum*. An explanation for this result is that the *for* gene and its expression are not associated with provisioning behaviour in halictid bees in the first place. Therefore, *for* expression levels in *L. laevissimum* would not provide an appropriate test of predicted gene expression patterns between halictid queens and workers. This explanation cannot be completely ruled out without comparing *for* expression levels between provisioning phenotypes using other halictid species but this seems unlikely for two reasons. First, *for* expression levels did vary between provisioning phenotypes in *L. laevissimum* queens (discussed below), and therefore are associated with provisioning phenotypes in this species. Secondly, to date, only one study has demonstrated that *for* expression levels do not differ between foragers and non-foragers in a social hymenoptera species (studies with reliable data in Table 4.1, pg. 128). Furthermore, 5/6 of the studies showing that *for* expression levels differ between foraging phenotypes show that *for* expression levels are higher in foragers than non-foragers. Given these two points, it's likely that *for* expression is in fact associated with provisioning behaviour in hymenopteran species.

An alternative explanation for the results presented in this study, with respect to the OGPH, is that the expression of provisioning behaviour does not skew towards workers and away from queens through evolutionary time in eusocial halictid lineages. As mentioned above, all sweat bee females actively provision brood at some point during the breeding season. Although workers collectively undertake the provisioning tasks in summer, sweat bee queens provision brood alone in spring (Schwarz et al. 2007). The shared provisioning among workers in summer may mean that workers, on average, do less provisioning than queens. In fact, in Greek *L. malachurum* colonies, queens do about 3 times as much provisioning in their lifetime compared to each individual worker (Wyman and Richards 2003; Richards et al. 2005).

Therefore, provisioning behaviour and the molecular mechanisms underpinning it may not have become differentially expressed in sweat bee castes through evolutionary time since both queens and workers provision offspring at some point during their lifetime.

***Foraging* expression as a possible primer for foraging activity**

Although *L. laevissimum* castes have similar *for* expression levels, queens and workers do differ in *for* expression if we account for phases of the breeding season. In queens, *for* expression levels change through the breeding season, unlike workers, which only occur over a short period during the breeding season. *Foraging* expression levels were higher in early spring foundresses (pre-provisioning) compared to spring foundresses (actively provisioning), summer queens (post-provisioning), and gynes (possible foragers). Therefore, *for* expression levels may reflect the different life histories exhibited by queens and workers of temperate sweat bees instead of a simple inherent caste difference.

One explanation for the *for* expression patterns I found in queens is that higher *for* expression may act as “primer” as foraging or provisioning activity begins, as suggested by Heylen et al. (2008) and Oettler et al. (2015). This idea stems from the ‘sensory response threshold hypothesis’ as applied to honeybee workers, in which a phenotypic response is induced when a stimulus passes a particular threshold (Robinson 1992; Page and Erber 2002; Thamm et al. 2018). In the case of *L. laevissimum* queens, high *for* expression levels may prepare early spring foundresses to respond quickly to an external stimulus, such as an increase in soil temperature. This stimulus triggers a behavioural response; in this case, emerging from their hibernacula and initiating foraging behaviour. Because early spring foundresses have high *for* expression, they are “primed” for the external stimulus that triggers PKG activity, which can then influence downstream pathways, initiating foraging activity.

Data from *L. laevissimum* queens, honeybee workers (Heylen et al. 2008), and workers of two ant species, *C. obscurior* (Oettler et al. 2015) and *Pogonomyrmex barbatus* (Ingram et al. 2005), support the hypothesis that *for* expression may act as a “primer” for foraging behaviour. For example, as honeybee workers mature, they transition from brood-care behaviour (non-foraging) to foraging behaviour outside the hive when they are 21 days old (Seeley and Kolmes 1991). Overall, foraging workers have higher *for* expression levels compared to non-foraging workers (Ben-Shahar et al. 2002; Ben-Shahar 2003; Thamm and Scheiner 2014). Additionally, Heylen et al. (2008) showed that *for* expression was highest when workers were 18 - 22 days old, after which *for* expression decreased to levels comparable to 14 day old workers. In comparison, *L. laevissimum* queens display a peak in *for* expression before foraging activity. Early spring foundresses (pre-provisioning) had higher *for* expression compared to spring foundresses (actively provisioning). *Foraging* expression levels were higher in summer queens (post-foraging) than spring foundresses, but lower than early spring foundresses. It is important to note that the aforementioned honeybee (Heylen et al. 2008) and ant (Ingram et al. 2005; Oettler et al. 2015) studies draw their conclusions from gene expression data that was not well controlled (Table 4.1, pg. 128); however, these are the only studies in which individuals were sampled at several time points before foraging activity began. Furthermore, the association between *for* expression and foraging activity has been established several times in honeybee studies in which gene expression data was well controlled (Ben-Shahar et al. 2002; Ben-Shahar 2003; Thamm and Scheiner 2014).

In contrast to queen *for* expression patterns, there was no difference in *for* expression between *L. laevissimum* workers collected in the morning (before provisioning activity) and workers collected midday (actively provisioning). This means that the “priming” of high *for*

expression levels might occur at specific points during a bee's life, such as before the first bout of provisioning activity, but not consistently from day to day. In which case, I did not capture workers over a timescale that would allow for comparable categories of workers to that of queens at different phases of the breeding season. This would explain why queens vary in *for* expression at different phases of their lives while workers do not show differences in *for* expression from morning to mid-day. Therefore, workers may have had higher *for* expression levels earlier in their life, after eclosing and before their first foraging trip, compared to when I captured them.

***Lasioglossum laevisissimum* males have higher *for* expression than females**

As far as I can tell, this study is the first to test the prediction that *for* expression differs between sexes of a hymenopteran species, and the first study to find a *for* expression difference between sexes of an insect species. *Lasioglossum laevisissimum* males had higher *for* expression levels than all females, females of similar age, and females that were also caught on the wing during the same phase of the breeding season. Differences in *for* expression between *L. laevisissimum* sexes contrasts *D. melanogaster* studies in which *for* expression did not differ between sexes in transcriptome comparisons (Jin et al. 2001; Arbeitman 2002; Ranz et al. 2003; Catalán et al. 2012). The reason for this contrast could be that there are inherent differences in life histories between the sexes of fruit flies and eusocial sweat bees. For the most part, *D. melanogaster* sexes exhibit similar life histories in terms of foraging and food acquisition. On the other hand, *L. laevisissimum* males leave their natal nest shortly after eclosing, foraging for their own survival, and likely spend nights on flowers or other structures since adult males were not found during nest excavations (Awde and Richards 2018). Unlike *D. melanogaster* males and females, the life history of male *L. laevisissimum* is dramatically different than female *L.*

laevissimum, which eclose, forage, and return to their nest after foraging bouts and through the night. Therefore, the sex difference in *for* expression may be the result of the different life histories of *L. laevissimum* males and females.

Conclusions

Foraging expression levels of *L. laevissimum* females suggest a complex relationship between *for* expression and provisioning behaviour, beyond simply whether or not a bee is foraging. Queens and workers did not differ in *for* expression, which means that the expression of provisioning behaviour and its molecular mechanisms, as inferred by *for* expression, may not have become differentially expressed in queens and workers through evolutionary time in eusocial sweat bee lineages.

High levels of *for* expression may act as a “primer” before foraging activity begins in *L. laevissimum* foundresses, and possibly workers, which would explain the high levels of *for* expression in early spring foundresses, and the subsequent drop off in *for* expression in spring foundresses. This means that *for* may be expressed at a high level in preparation of an external stimulus that triggers the activity of PKG, which then activates down stream pathways, initiating the first foraging bout. This priming action occurs during a specific life stage rather than daily fluctuations, since workers collected from nests in the morning had similar *for* expression patterns as workers collected while actively foraging mid-day.

Finally, I found that *L. laevissimum* males had higher levels of *for* expression than females, which is the first sex difference in *for* expression observed using an insect. This may stem from the extreme difference in life histories of *L. laevissimum* males and females, and similar results are likely in other hymenopteran species in which males and females differ just as much.

Table 4.1. *Foraging (for)* expression patterns with respect to foraging phenotypes in social Hymenopteran species. Studies that used microarray data, transcriptome data, RT-qPCR expression data normalised with >1 reference genes as per the MIQE guidelines (Bustin et al. 2009), or RT-qPCR data with additional experiments are in bold

Species	Body part	Comparison	<i>For</i> expression levels are highest in ...	Reference
<i>Apis mellifera</i>	Brains	Forager vs. nurse workers	Foragers	(Ben-Shahar et al. 2002; Ben-Shahar 2003)
<i>Apis mellifera</i>	Brains	Workers transitioning from non-foragers to foragers	Foragers	(Heylen et al. 2008)
<i>Apis mellifera</i>	Brains, fat bodies, and flight muscles	Forager vs. nurse workers <i>fora</i> and <i>forβ</i> splice variants	Foragers (<i>fora</i> splice variant)	(Thamm and Scheiner 2014)
<i>Apis cerana</i>	Heads, thoraces, abdomens, legs, and antennae	Forager vs. nurse workers	Foragers	(Ma et al. 2018)
<i>Bombus ignites</i>	Heads	Forager vs. nurse workers	Non-foragers	(Kodaira et al. 2009)
<i>Bombus terrestris</i>	Heads	Forager vs. nurse workers	Foragers	(Tobback et al. 2011)
<i>Bombus terrestris</i>	Brains, fat bodies, and ovaries	Queens with workers (non-foragers) and without workers (foragers)	Foragers	(Lockett et al. 2016)
<i>Bombus terrestris</i>	Brains	Early (foragers) and late stage queens (non-foragers) with and without workers	Foragers	(Woodard et al. 2013)
<i>Bombus terrestris</i>	Brains	Foundresses, queens, workers, and gynes	No difference	(Woodard et al. 2014)
<i>Polistes metricus</i>	Brains	Foragers (foundresses and workers) and non-foragers (queens and gynes)	Foragers	(Toth et al. 2007, 2010)
<i>Polistes metricus</i>	Brains	Forager vs. non-forager worker	No difference	(Daugherty et al. 2011)
<i>Vespula vulgaris</i>	Brains	Forager vs. nurse workers	Non-foragers	(Tobback et al. 2008)
<i>Solenopsis invicta</i>	Heads	Foraging vs. nest workers (non-foragers)	Non-foragers	(Lucas et al. 2015)
<i>Cardiocondyla obscurior</i>	Heads	Forager vs. nest workers	Non-foragers (Highest in callows)	(Oettler et al. 2015)
<i>Pogonomyrmex occidentalis</i>	Brains	Forager vs. nest workers	Foragers (Collected mid-day)	(Ingram et al. 2011)
<i>Pogonomyrmex barbatus</i>	Brains	Forager vs. nest workers	Non-forager (Callows at dawn)	(Ingram et al. 2005)
<i>Pogonomyrmex barbatus</i>	Brains	Forager vs. nest workers	Foragers (Foragers mid-day)	(Ingram et al. 2016)

Table 4.2 *Lasioglossum laevisimum* categories used in head and abdomen *for* expression comparisons. The number of samples with detectable expression of all three reference genes are provided for each category. Head and abdomen measurements are not available for all individuals sampled

Category	Three reference genes with detectable expression levels				
	No. of individuals	Head	Thorax	Abdomen	Both Head and Abdomen
Early spring foundresses (before Phase 1)	7	6	2	4	3
Spring foundresses (Phase 1)	8	6	3	8	6
Summer queens (Phase 2 and 3)	7	5		7	5
Workers – not foraging (Phases 2 and 3)	5	4		5	4
Workers - foraging (Phase 3)	6	6	4	6	6
Gynes (Phases 3 and 4)	6	5		5	4
Males (Phase 3)	6	5		6	5
Total	45	37	9	41	33

Table 4.3. The effect of breeding season phase, caste, sex, and collection method on *for* expression in *L. laevissimum* heads, thoraces, and abdomens. Proportions of samples with detectable *for* expression out of the number of samples with detectable mRNA levels are shown, as well as the fractional values below

Body Part	Early spring foundresses (before Phase 1)	Spring foundresses (Phase 1)	Summer queens (Phases 2 and 3)	Workers - not foraging (Phases 2 and 3 - from nests)	Workers - foraging (Phase 3 - on the wing)	Gynes (Phases 3 and 4)	Males (Phase 3)	Comparison between categories (Fisher's exact test)
Head	100% 6/6	100% 6/6	80% 4/5	50% 2/4	50% 3/6	80% 4/5	60% 3/5	p = 0.19
Abdomen	100% 4/4	88% 7/8	100% 7/7	80% 4/5	83% 5/6	100% 5/5	83% 5/6	p = 0.88
Thorax	100% 2/2	100% 3/3			25% 1/4			p = 0.14

Table 4.4. Factors contributing to *for* expression levels in queen and worker heads (Head) and abdomens (Abdomens). Mandibular wear and caste had no effect on *for* expression in head or abdomen samples

Head		Model: Head <i>for</i> expression ~ Head width + Mandibular wear + Caste				
Effects	<i>Coefficient</i>	<i>d.f.</i>	<i>F</i>	<i>p</i>	<i>Adjusted R²</i>	
Model		11	1.34	0.31	0.07	
Head width	2.62	1	2.76	0.12		
Mandibular wear	-0.11	1	1.03	0.33		
Caste		1	0.23	0.64		

Abdomen		Model: Head <i>for</i> expression ~ Head width + Mandibular wear + Caste				
Effects	<i>Coefficient</i>	<i>d.f.</i>	<i>F</i>	<i>p</i>	<i>Adjusted R²</i>	
Model		15	0.04	0.99	-0.20	
Head width	-0.69	1	0.10	0.76		
Mandibular wear	0.02	1	0.01	0.94		
Caste		1	0.02	0.88		

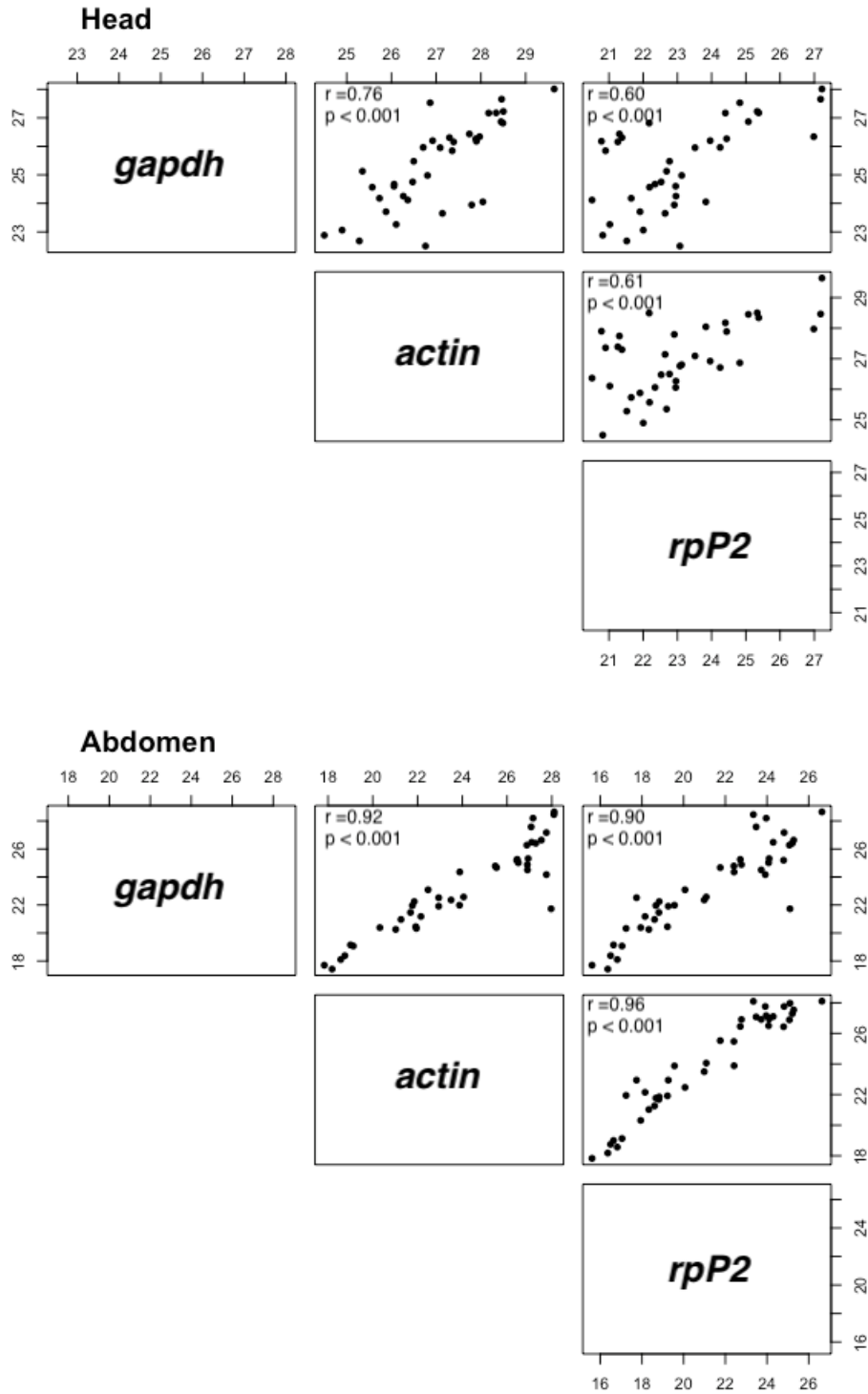


Figure 4.1. Validation of three reference genes used in *L. laevissimum* for expression comparisons. Correlations of cycle number (C_q ; x and y-axes) of 3 reference genes for *L. laevissimum* samples: *gapdh*, *actin*, and *rpP2*. Low C_q values represent high expression levels and high C_q values represent low expression levels. Pearson correlation coefficients (r) and probabilities (p) for each correlation are provided. A. Head samples of 37 *L. laevissimum* individuals B. Abdomen samples of 41 *L. laevissimum* individuals. Sample sizes for each *L. laevissimum* category are in Table 4.2, pg. 129

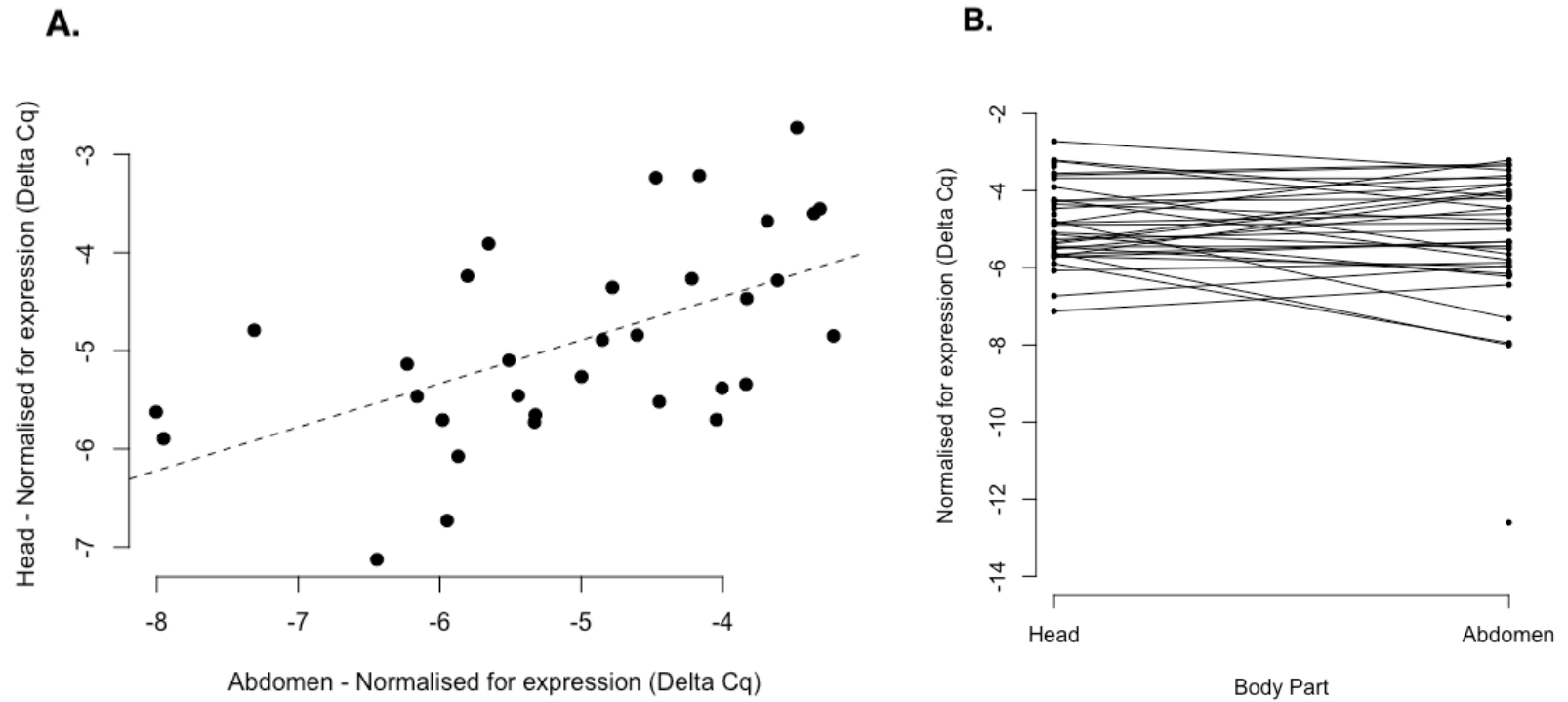


Figure 4.2. Comparisons of head and abdomen *for* expression levels. (A) *Lasioglossum laevisssimum* samples that had *for* expression measurements in their head and abdomen. *Foraging* expression levels in the head were positively correlated with *for* expression values in the abdomen (B) *Foraging* expression levels in the head and abdomen of all *L. laevisssimum* samples. Dots connected from a line are from the same individuals. Head and abdomen samples had similar *for* expression levels

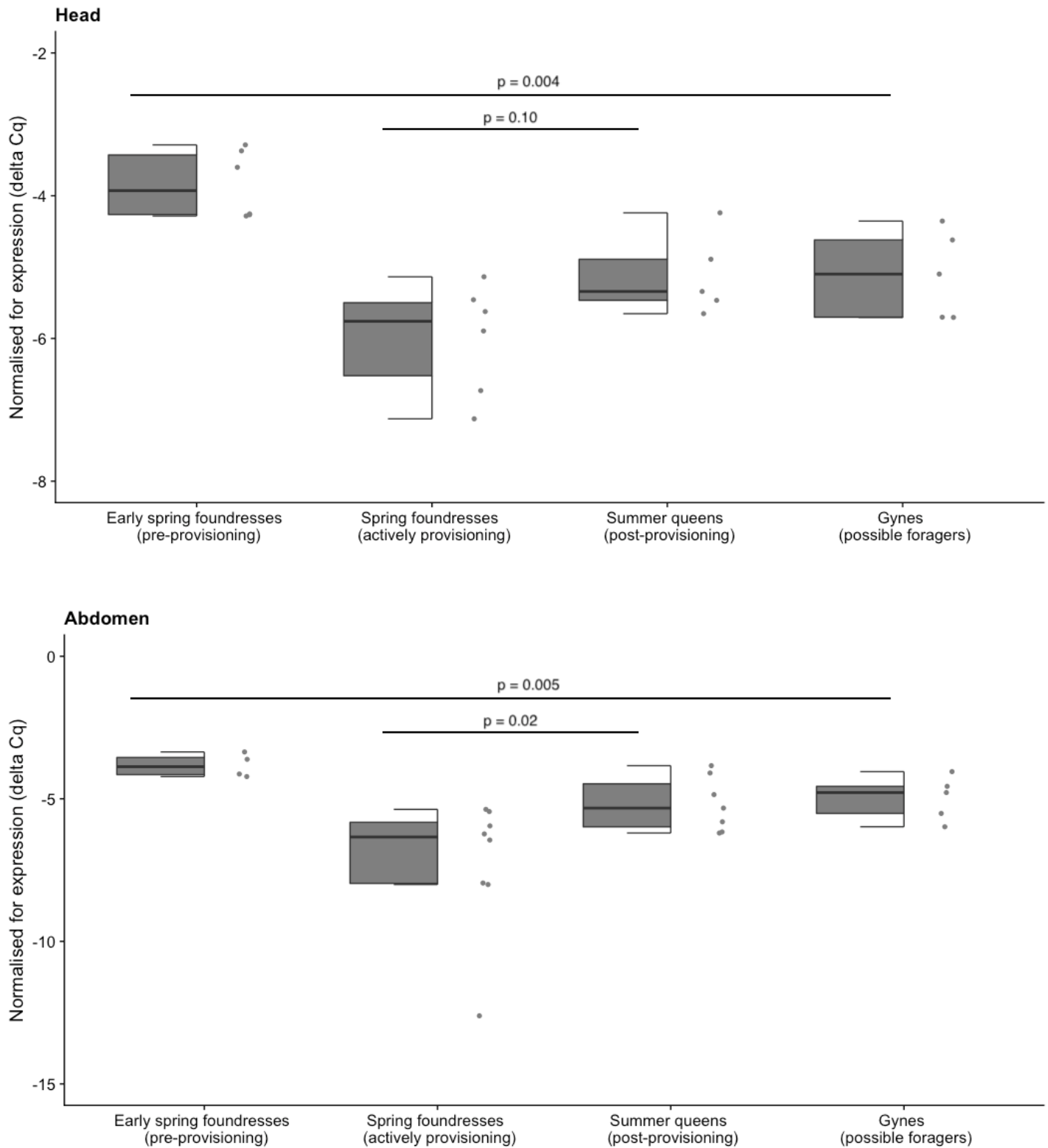


Figure 4.3. The effect of breeding season phase on normalized *for* expression levels in foundresses, queens, and gynes. Head (Head) and abdomen (Abdomen) *for* expression levels were highest in early spring foundresses, which were collected ~ 2 weeks before they emerged from their hibernacula and started provisioning

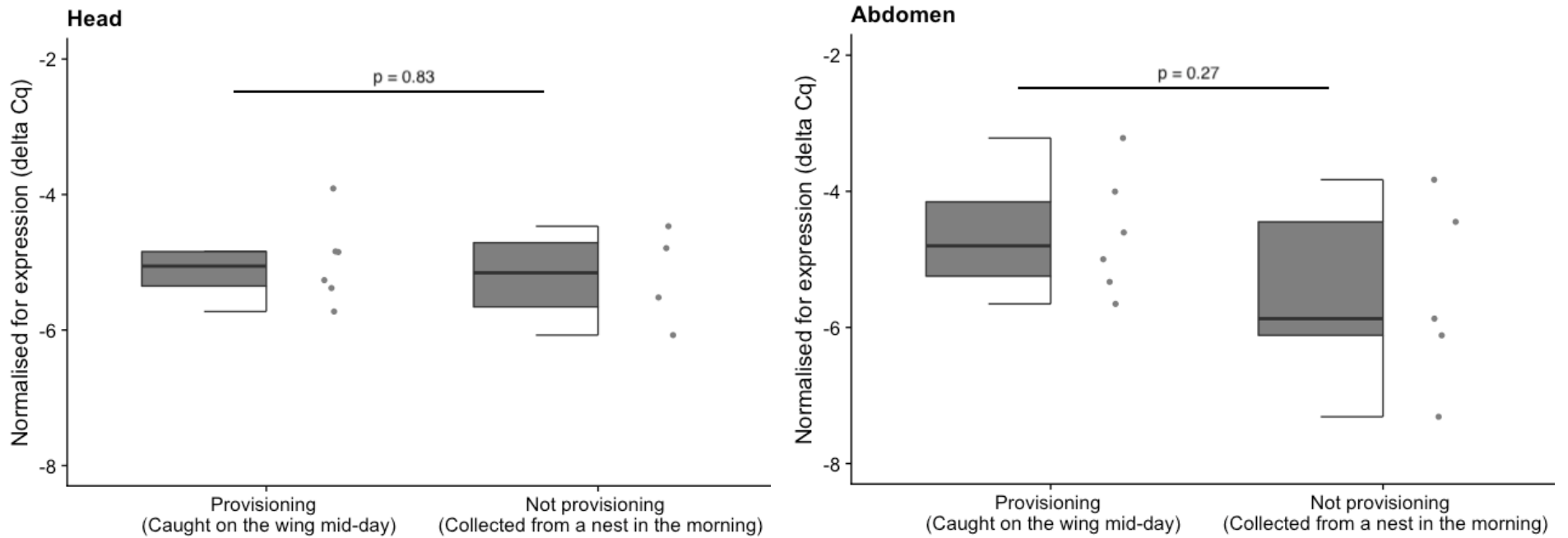


Figure 4.4. Normalized *for* expression levels with respect to the provisioning behaviour of workers collected mid-day compared to workers collected from nests in the morning. Workers had similar head (Head) and abdomen (Abdomen) *for* expression levels regardless of the time of day during which they were captured

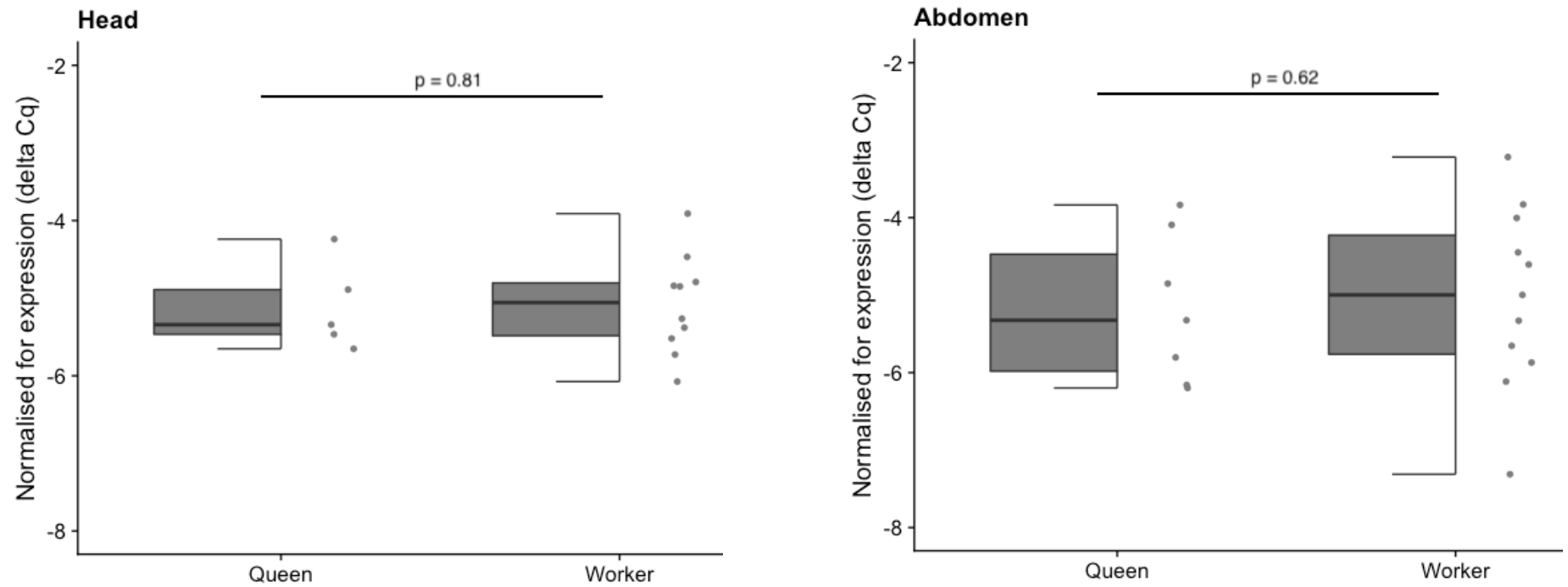


Figure 4.5. The effect of caste on *for* expression levels. Queens had similar head (Head) and abdomen (Abdomen) *for* expression levels as workers

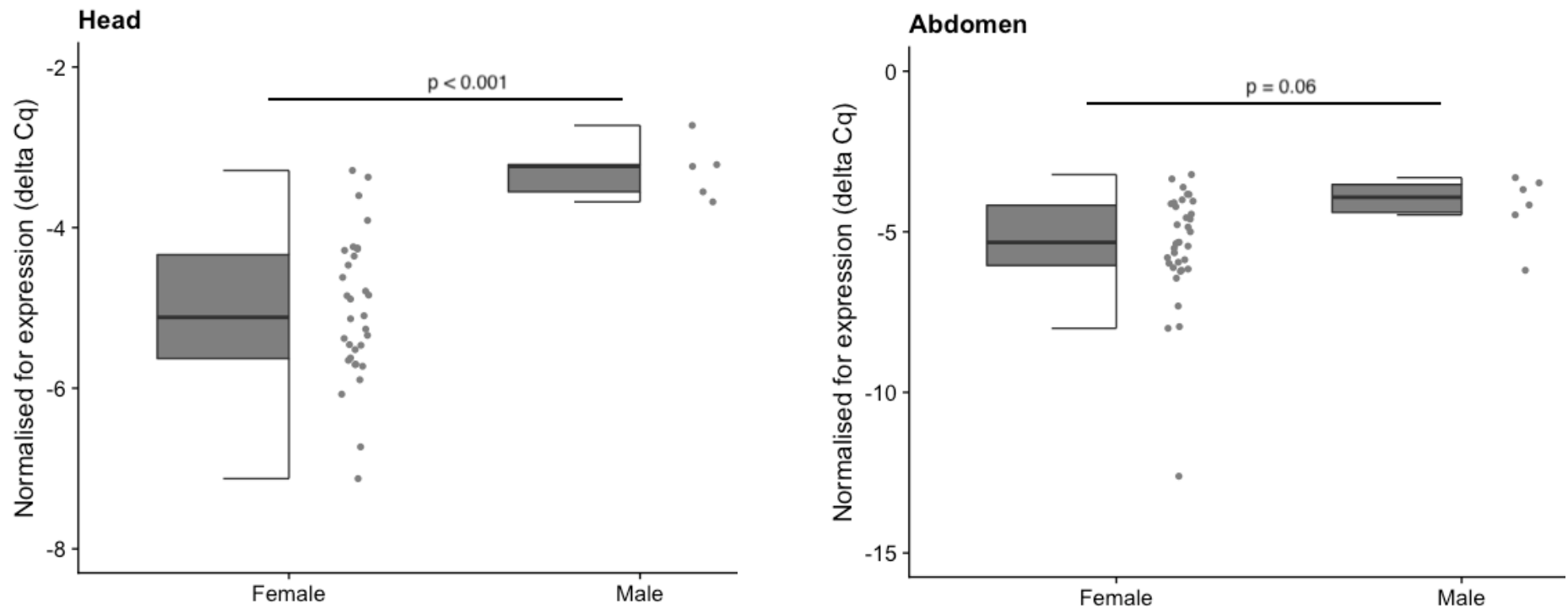


Figure 4.6. The effect of sex on normalized *for* expression levels. Early spring foundresses, spring foundresses, summer queens, workers, and gynes are included in the female category. Male heads and abdomens had higher *for* expression levels than female heads and abdomens

Chapter 5: General Discussion

The question as to why some individuals in eusocial groups behave as queens while others behave as workers has been a long-standing interest for many evolutionary biologists and ethologists. The aim of this thesis was to describe proximate mechanisms, environmental and genetic, that influence variation in specific caste phenotypes of a eusocial sweat bee, *L. laevissimum*. Because the castes of eusocial sweat bee species are morphologically and developmentally similar, and newly eclosed females are behaviourally totipotent, I was able to describe proximate mechanisms of caste phenotypes that stem from environmental and gene expression influences, rather than developmental differences. In general, halictid castes show incredible inter- and intra-caste variation in important caste phenotypes such as egg-laying and provisioning, therefore I was able to use *L. laevissimum* to assess the influence that the expression levels of specific genes have on caste traits, above and beyond simple caste designations. These descriptions of proximate mechanisms that influence caste traits are important for evaluating and developing hypotheses that describe the evolutionary mechanism by which highly eusocial descendants evolved from solitary ancestors.

The influence of the social environment on worker reproduction

In chapter two I described the social characteristics and individual morphometric data of a *L. laevissimum* population located at Brock University. I took advantage of a natural experiment, the occurrence of queenright and queenless nests, to investigate the influence that the social environment had on worker egg-laying behaviour. I predicted that if *L. laevissimum* queens manipulate worker behaviour through continuous aggression then queenright workers should have less ovarian development than queenless workers. However, this was not the case;

queenright workers were just as likely to have well-developed ovaries as queenless workers, which is similar to two other sweat bee species, *L. imitatum* and *L. malachurum* (Michener and Wille 1961; Soro et al. 2009). This suggests that sweat bee queens may exert influence on workers soon after they eclose, and this influence has long-lasting effects on worker behaviour. It is important to recognize that in *L. laevissimum* and other sweat bee colonies a proportion of workers develop their ovaries and likely lay eggs in queenright nests (Kukuk and May 1991; Richards and Packer 1995, 1996; Wyman and Richards 2003; Richards et al. 2015). This means that queens do not prevent every worker from developing their ovaries, which suggests that some workers eventually reproduce and act as supplement egg-layers in the nest. Sweat bee queens likely have a maximum egg-laying capacity, which may be outstripped by the ability of workers to provision brood cells (Kukuk and May 1991). In large nests with many workers, queens should favour some amount of worker egg-laying so that provisions the queen cannot use will be used by her daughters, and therefore provide indirect fitness for the queen.

In Ontario *L. laevissimum*, a small proportion of workers had well-developed ovaries (17%), which was smaller than the proportion of workers with well-developed ovaries in an Alberta *L. laevissimum* population (63%). Interestingly, even though a high proportion of Alberta workers had developed ovaries, genetic analyses suggest that workers rarely produce offspring (Packer and Owen 1994). Therefore, in both Ontario and Alberta queens are the primary producers of males and gynes in the second brood. Comparisons of these two *L. laevissimum* populations suggest that queens can limit worker reproduction differently. Ontario queens are more effective than Alberta queens at preventing worker ovarian development in the first place, while Alberta queens still limit worker reproduction by successfully preventing worker egg-laying, even if their workers have developed ovaries.

***Vitellogenin* and *for* expression levels reflect behavioural and life history differences between queens and workers**

In chapters three and four I compared *vg* and *for* expression levels of *L. laevissimum* queens and workers. In insects, *vg* and its protein product, Vg, have several roles, one of which one includes supplying developing ovaries with vitellin (Sappington and Raikhel 1998; Tufail and Takeda 2008). In *L. laevissimum*, *vg* expression was higher in queens than workers, and *vg* expression was strongly correlated with ovarian development in both castes. I also found that *vg* was expressed in females preparing to overwinter. These females had no ovarian development. This suggests that *vg* expression, and likely Vg proteins, play an important role in pre-diapause fat storage needed to survive the inactive period pre- and post-diapause. This is important with respect to sweat bee castes since gynes (future queens) overwinter but workers very rarely do. Therefore, workers may express *vg* for ovarian development, but in the absence of ovarian development, workers might suppress *vg* expression, which prevents them from preparing for overwintering diapause.

In chapter four, I describe the relationship between *for* gene expression and foraging activity, an important aspect of brood provisioning. *Foraging* expression levels were not associated with caste in *L. laevissimum*. *Foraging* expression is often associated with foraging phenotypes in bees (summarized in Table 4.1, pg. 128; and partially summarized in Lockett et al. 2016). Queens, which had ceased foraging activity for the summer, had *for* expression levels similar to workers. Early spring foundresses collected from their hibernacula (pre-provisioning) had higher *for* expression levels than actively provisioning spring foundresses. These results point to a nuanced relationship between *for* expression and *L. laevissimum* foraging behaviour.

In chapter four I suggest that *for* expression might act as a “primer” before foraging activity. High levels of the *for* transcript prepare individuals for an external stimulus, such as temperature, that initiates the activity of PKG, which activates downstream pathways that initiate foraging activity. This may occur in other species such as honeybees and harvester ants as well (Heylen et al. 2008; Oettler et al. 2015). In *L. laevissimum*, queen *for* expression peaks before foraging activity, while *for* expression is at its lowest when foraging in spring. Interestingly, workers collected from inside nests, which were not foraging, had *for* expression levels similar to workers collected on the wing while actively provisioning. An explanation for the difference between queen and worker results might be that the “priming” action of *for* occurs at specific points during a bee’s life, but not from day to day. Therefore, in workers, *for* expression levels may have peaked soon after eclosion before the first foraging bout, and then stabilized before I collected them.

The decoupling of ancestral egg-laying and brood provisioning phenotypes in bees

The ovarian ground plan hypothesis (OGPH) suggested that the expression of the egg-laying and provisioning phenotypes of an ancestral solitary wasp’s life cycle decoupled to be expressed separately in queens and workers of highly eusocial descendants (West-Eberhard 1987, 1996; depicted in Fig.1.1, pg 7). Furthermore, gene expression associated with egg-laying and provisioning behaviours also decoupled to be expressed differently in queens and workers of highly eusocial descendants. In eusocial descendants, queens more highly express genes associated with egg-laying and workers more highly express genes associated with provisioning. Therefore, in primitively eusocial taxa, such as *L. laevissimum*, which may represent a transitional stage between solitary behaviour and highly eusocial organisation, egg-laying genes should be expressed more in queens than workers and provisioning genes should be expressed

more in workers than queens (West-Eberhard 1987, 1996; Rehan and Toth 2015; Toth and Rehan 2017; Sumner et al. 2018; Taylor et al. 2018). This was only partially the case in *L. laevissimum*. The expression levels of a gene associated with egg-laying behaviour, *vg*, skewed towards queens compared to workers. However, the expression levels of a gene associated with provisioning behaviour, *for*, were similar in queens and workers. Based on the OGP, females that have high expression levels of egg-laying genes should have low expression levels of provisioning genes, and females that have high expression levels of provisioning genes should have low expression levels of egg-laying genes. Therefore, *vg* and *for* expression levels should be negatively correlated in queens and workers. However, in *L. laevissimum*, *vg* and *for* expression levels of queens and workers were not correlated in heads (Fig.5.1, pg. 143; Pearson correlation coefficient $r = 0.31$, $df = 19$, $p = 0.18$) or abdomens (Fig.5.1, pg. 143; Pearson correlation coefficient $r = -0.13$, $df = 24$, $p = 0.50$). Therefore, as originally stated, the OGP may not accurately describe the proximate mechanism by which eusociality evolved in bees.

The solitary ground plan from which eusocial queens and workers evolved is likely different for bees than it is for wasps. The behaviour and life history of extant solitary bee species likely represent an ancestral solitary ground plan from which eusocial bee species evolved (Linksvayer and Wade 2005; Rehan and Toth 2015; Toth and Rehan 2017). Solitary bees, for the most part, mass-provision their offspring one at a time (Michener 1974). A solitary female develops her ovaries and forages for brood provisions at the same time. She then lays an egg on the completed provision mass (Michener 1974). After oviposition, the mother moves on to the next brood cell, repeating the process and continuing her concurring egg-laying and provisioning behaviour. This is in contrast to the solitary ground plan that West-Eberhard (1987) suggested eusocial wasp descendants evolved from. In a solitary, progressive-provisioning wasp,

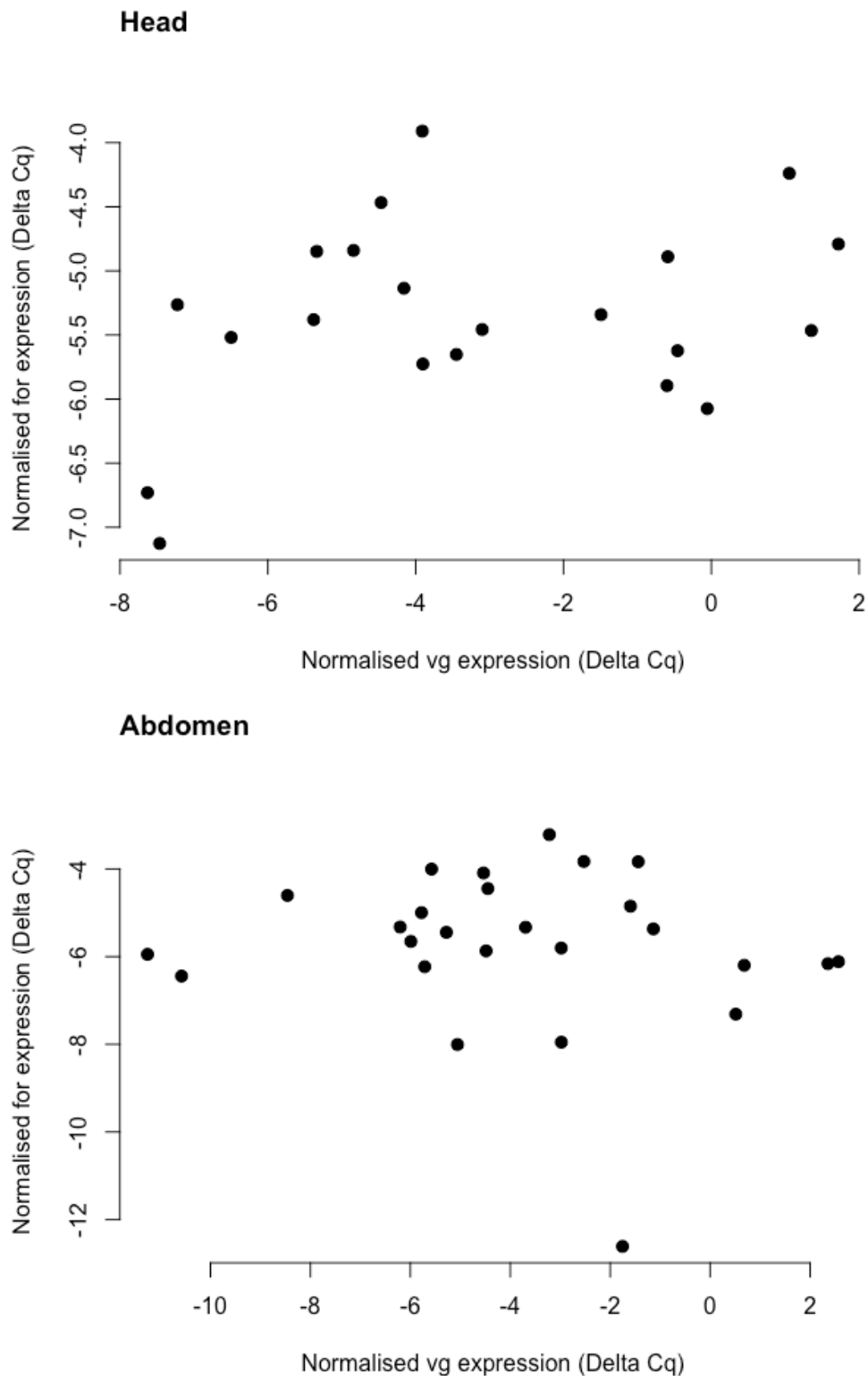


Figure. 5.1. The relationship between *vg* expression and *for* expression levels in *L. laevissimum* queen and worker heads and abdomens. *Vitellogenin* and *for* expression levels are not correlated head (Pearson correlation coefficient $r = 0.31$, $df = 19$, $p = 0.18$) or abdomen samples (Pearson correlation coefficient $r = -0.13$, $df = 24$, $p = 0.50$). Queens ($n = 11$ heads; $n = 12$ abdomens) are made up of spring foundress ($n = 6$ heads; $n = 8$ abdomens) and summer queens ($n = 5$ heads; $n = 7$ abdomens). Workers ($n = 10$ heads; $n = 11$ abdomens) are made up of phase 2 workers ($n = 2$ heads; $n = 2$ abdomens) and phase 3 workers ($n = 8$ heads; $n = 9$ abdomens)

a female develops her ovaries while she constructs a brood cell; she then lays eggs in brood cells and ceases ovarian development. After the egg-laying phase, the mother transitions to a provisioning phase, actively provisioning and defending her developing brood. When her offspring eclose, the transition from egg-laying to provisioning behaviour is repeated. Since the ancestral solitary ground plans from which eusocial bee and wasp descendants likely evolved are different, I suggest a modification to the OGP, the *Anthophila ground plan hypothesis*, which applies specifically to eusocial evolution in bee lineages (Described below; Fig.5.2, pg. 145).

The Anthophila ground plan hypothesis

The Anthophila ground plan hypothesis refers to the ancestral solitary ground plan from which eusocial bee lineages may have evolved. From this ground plan, I describe how the biasing of egg-laying and provisioning behaviours, and their molecular mechanisms, may have occurred through evolutionary time. I do this by describing the expression of these phenotypes, and the molecular mechanisms that underlie them, in taxa whose sociality may represent transitional stages between solitary and eusocial behaviour (Fig.5.2, pg. 145).

The expression of egg-laying and provisioning behaviour of a solitary bee ancestor likely resembled that of extant, mass-provisioning, solitary bee species (Linksvayer and Wade 2005; Rehan and Toth 2015; Toth and Rehan 2017). Females of solitary bee species express both egg-laying and provisioning during the breeding season, as described above. This is the case in solitary taxa with either one or two broods per breeding season. The latter likely represents a transitional state between solitary species that produce one brood and eusocial species with behavioural castes, which produce two broods (Seger 1983; Plateaux-Quénu et al. 1989; Hunt 2012; Kocher et al. 2014).

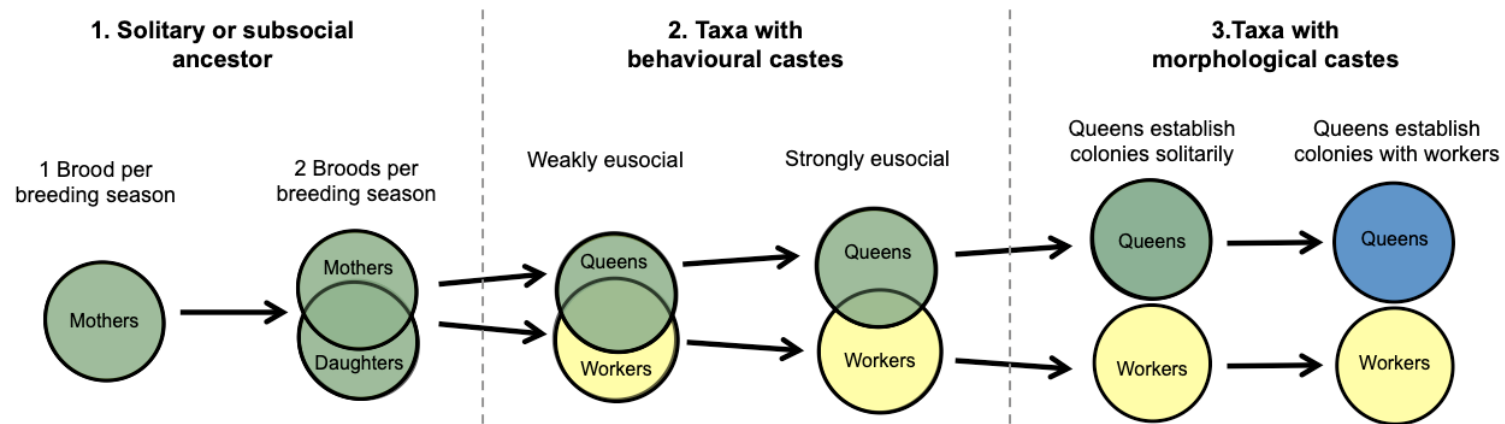
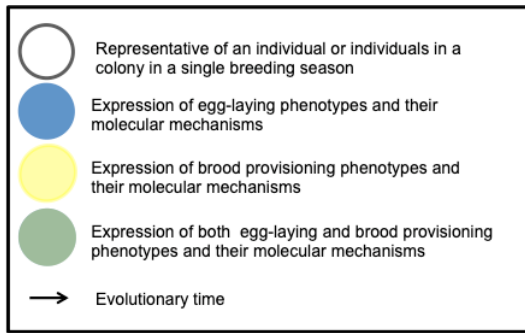


Figure 5.2. The Anthophila ground plan hypothesis. Outlines the proximate mechanism by which queens and workers evolved in eusocial bee lineages. Ancestral solitary egg-laying and provisioning behaviours and the molecular mechanisms underpinning them became differentially expressed in queens and workers of highly eusocial descendants in which queens establish colonies with workers. Outlined circles indicate a representative individual or individuals in a colony for a given taxa in a single breeding season. The shaded colour of each circle indicates the expression of egg-laying (blue), brood provisioning (yellow), or both (green) behaviours, and the underlying expression of genes associated with those behaviours for the representative individual(s). 1. In a solitary or subsocial ancestor both behaviours / molecular mechanisms are expressed in each individual female. This is the case in solitary taxa with one brood per breeding season, or two broods per breeding season. 2. In eusocial species with behavioural castes, each female is capable of expressing both behaviours. Queens express more egg-laying behaviours / molecular mechanisms than workers, which express more brood provisioning behaviours / molecular mechanisms than queens when both castes are in a colony. In weakly eusocial species, queens have weak control over worker reproduction, therefore a large proportion of worker express queen-like egg-laying behaviour / molecular mechanisms. In strongly eusocial species, queens exhibit strong control over worker reproduction and a small proportion of workers express queen-like egg-laying behaviours / molecular mechanisms. 3. In eusocial species with morphological castes, egg-laying behaviours / molecular mechanisms are no longer expressed in workers. Queens express provisioning behaviours / molecular mechanisms in species in which queens establish colonies solitarily. However, provisioning behaviours / molecular mechanisms are expressed only in workers and are not expressed in queens of species in which queens establish new colonies with the aid of worker

Eusocial taxa with behavioural castes, such as *L. laevissimum*, may represent a transitional stage between solitary behaviour and advanced eusocial organisation (Rehan and Toth 2015; Toth and Rehan 2017; Sumner et al. 2018; Taylor et al. 2018). In *L. laevissimum*, queens and workers actively provision brood at some point during the breeding season. Therefore, provisioning behaviours and the molecular mechanisms underpinning them are expressed in both castes. On the other hand, egg-laying behaviours and the molecular mechanisms underpinning them are expressed more in queens than workers. Eusocial species with behavioural castes, such as eusocial halictids, vary from weakly to strongly eusocial, depending on how well queens control worker reproduction (Breed 1976; Packer and Knerer 1985; Wyman and Richards 2003; Peso and Richards 2010). Therefore, in weakly eusocial species, queens and a large proportion of workers express egg-laying behaviours and their molecular mechanisms. In contrast, in strongly eusocial species, queens and a small proportion of workers express egg-laying behaviours and molecular mechanisms.

Eusocial species with developmental castes, in which queens initiate colonies solitarily, such as *Bombus sp.*, may represent a transitional state between eusocial organisation with behavioural castes and eusocial organisation with morphologically distinct queens and workers (Rehan and Toth 2015; Toth and Rehan 2017; Sumner et al. 2018; Taylor et al. 2018). In eusocial *Bombus*, queens initiate colonies solitarily and express both egg-laying and provisioning behaviours. Workers on the other hand, are developmentally different than queens (Cnaani et al. 1997), rarely get to lay eggs, and genes associated with egg-laying in queens are associated with different phenotypes in workers (Amsalem et al. 2014). Therefore, workers express provisioning behaviours and their molecular mechanisms, but rarely express egg-laying behaviours and molecular mechanisms.

The final transition in the evolution of queens and workers may have occurred when eusocial taxa with developmental castes evolved a life history trait in which both workers and queens are required to initiate new colonies, as seen in *Apis sp.* (Wcislo and Fewell 2017). In *Apis*, queens lay eggs and do not provision offspring, even when colonies are initiated. Therefore, queens express only egg-laying behaviours and their molecular mechanisms, and do not express provisioning behaviours and molecular mechanisms.

In conclusion, the *Anthophila ground plan hypothesis* is similar to the OGP in that both hypotheses posit that the expression of egg-laying and provisioning phenotypes, and their molecular mechanisms, decoupled to be expressed separately in queens and workers of species that exhibit advanced eusociality. However, in contrast to the OGP, which suggests that the ground plan from which eusocial wasps evolved was a progressive-provisioning solitary ancestor, the *Anthophila ground plan hypothesis* suggests that the ground plan from which eusocial bee lineages evolved was a solitary, mass-provisioning solitary ancestor. Moreover, from this solitary ground plan I detail the process in which egg-laying and provisioning behaviours, and their molecular mechanisms, may have decoupled through several evolutionary transitions to be expressed separately in queens and workers of highly eusocial bee species.

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