From control to constraint: a study of reproduction in the eusocial honey bee and the solitary red mason bee

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The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Jointly-authored publication:

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Contributorship (following CRediT: Allen *et al.*, 2019):

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Elizabeth J. Duncan: Conceptualization, Resources, Writing - review & editing, Visualization, Funding acquisition, Supervision, Project administration.

II

Never underestimate the simplicity,
inherent to complexity

Acknowledgements

Believe it or not, the University actually provides a template for the acknowledgements section.

> *'This research has been carried out by a team which has included (name the individuals).*
> *My own contributions, fully and explicitly indicated in the thesis, have been.....(please specify)*
> *The other members of the group and their contributions have been as follows: (please specify).'*

We can only guess at whether these words were carefully crafted by a dispassionate bureaucrat, some cold-hearted and bitter academic, or by Hades himself who subsequently whispered them into the ears of an unsuspecting and innocent mortal. But now that I have displayed those words here, I hope to be in line with whatever University policy may or may not outline them. So now we can safely commit them to the void that is amnesia, where such unfeeling things belong. Indeed any and all acknowledgements I have ever read, both within and outside this University, have been cheesy without fail. As well they should be. So I will endeavour to do the same here. For the Odyssean nature of doing a PhD, has left me enlisting, recruiting, conscripting and consigning all of the varying modes and degrees of support known to mankind. And I am grateful to them all.

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Finally, I'd like to thank my mother, for always having pushed me, for better or for worse. And my dad, for providing the counterweight, telling me he's proud of me no matter what.

♦ Sadly at the time of writing, I remain unconvinced of this. Time will tell.

Abstract

The major evolutionary transition from solitary to eusocial living is hallmarked by the reproductive division of labour. I investigated mechanisms underlying *reproductive control* in a solitary bee (*Osmia bicornis*), with the aim of informing how and why such mechanisms were co-opted into *reproductively constraining* workers in a eusocial species (*Apis mellifera*, mrca 95 mya; Peters *et al.*, 2017). I start out by introducing the problems of reproductive constraint and the evolution of eusociality (Chapter 1).

In order to test functional links and perform manipulation, it is imperative to establish a reference species within the laboratory. To address the lack of a temperate European solitary model species, I attempted to establish *O. bicornis* in a laboratory environment (Chapter 3). Preliminary erratic successes of nesting and egg-laying behaviour were achieved, and future recommendations were laid out. To further facilitate *O. bicornis* as a model species; microsatellite markers were mined, designed, tested and validated in collaboration with the NERC Biomolecular analysis facility in Sheffield (Chapter 4). The broad applicability of these markers is discussed.

The capricious nature of laboratory egg-laying necessitated appraising *reproductive control* directly. To enable assaying oogenesis, I performed the first microstructural study of the *O. bicornis* ovary (Chapter 5). Since mating plays an important role in the ovary activation of eusocial queens and other insect species, I concurrently examined the effect of mating status on the ovary of *O. bicornis* (Chapter 5) — with special reference to the potential role of mating status in reproductive constraint. Finally, I investigated how a known mechanism of *reproductive constraint* (Duncan *et al.*, 2016) operates in the related solitary bee, to ascertain its ancestral role (Chapter 6).The mechanism was found to be reversed in *O. bicornis*.

Chapter 7 places the overall findings within their wider context, and outlines future avenues of research.

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Abbreviations

anti-DIG AP —fragments from polyclonal anti-digoxigenin antibodies, conjugated to alkaline phosphatase

AP — alkaline phosphatase

BCIP — 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt

CBF — complete bee food

cDNA — complementary DNA

csd — complementary sex determiner locus

DAPI — 4′,6-diamidino-2-phenylindole

ddH2O — double distilled water

DEPC — 0.1% diethylpyrocarbonate

DMSO — dimethyl sulfoxide

DNA — deoxyribonucleic acid

DoL — division of labour

EDF — extended depth of focus

EtOH — ethanol

HWE — Hardy-Weinberg equilibrium

IHC — immunohistochemistry

IIS — insulin/insulin-like signalling

ISH — *in situ* hybridisation

JH — juvenile hormone

LB — lysogeny broth (Luria broth, Lennox broth, or Luria-Bertani medium)

LD — linkage or gametic disequilibrium

MeOH — methanol

MIP — maximum intensity projection

mrca — most recent common ancestor

MRJP1 — major royal jelly protein 1 precursor

my — million years

mya — million years ago

NBAF — NERC Biomolecular analysis facility

NBT — nitro-blue tetrazolium chloride

NERC — Natural Environment Research Council

PBS — phosphate buffered saline

PMT — photon multiplier tube

pHH3 — phospho histone H3

QLW — queenless worker

QMP — queen mandibular pheromone

QRW — queenright worker

QTL — quantitative trait locus

RA — royal actin

RCF — relative centrifugal force (with: g or xg ; 'gravity' or 'times gravity' respectively)

RGPH — reproductive ground plan hypothesis

RJ — royal jelly

RNA — ribonucleic acid

RPGH — reproductive ground plan hypothesis

Rpm — rotations per minute

RT — room temperature•

RT — reverse transciptase•

SOB — super optimal broth

SOC — super optimal broth with catabolite repression

SSC — saline-sodium citrate

ssDNA — single-stranded DNA

Tor — target of rapamycin

tRNA — transfer RNA

Vg — vitellogenin

• Apparent from context.

Chapter 1 General introduction

> *'I will not here enter on these several cases, but will confine myself to one special difficulty, which at first appeared to me insuperable, and actually fatal to my whole theory.' — Darwin (1859)*

The evolution of eusociality, poses a problem within the general theory of evolution by natural selection, due to its defining feature of reproductive altruism (Darwin, 1859). The problem the so called 'neuter' insects pose to Darwin's theory of evolution by natural selection is self-evident. How can an adaption arise through variation, inheritance and selection when the adaptation itself precludes inheritance? Eusocial evolution has therefore drawn substantial attention and interest over the past 161 years, and is considered a major transition in evolution (Smith and Szathmary, 1995). Consequently, understanding this phenomenon is implicitly important with regard to our general understanding of evolution.

Eusociality is traditionally defined by several characteristics. Individuals must share a common nest site, must cooperate in caring for young, reproductive division of labour (castes) must be present and an overlap of generations must occur (Wilson, 1971)[1]. This higher level of social organisation is found mainly within the Hymenoptera (Figure 1.1), where there may have been up to nine independent origins of eusociality (see Hughes *et al.*, 2008 and references therein). Eusociality is also sparsely found outside of the Hymenoptera, namely: in the Isoptera (Wilson, 1975), the Hemiptera (Stern, 1994), the Coleoptera (Smith *et al.*, 2009), the Thysanoptera (Gadagkar, 1993; Kranz *et al.*, 1999), Decapods (Duffy, 1996) and even farther removed from the Insecta; in the naked mole-rat, a vertebrate species (Jarvis, 1981).

[1] While the exact definitions of *eusociality* and *superorganismality* have both narrowed and expanded over time; both terminology and etymology fall outside the remit of the current work, and the reader is referred to the comprehensive work by Boomsma and Gawne (2018).

Accordingly, with their eleven independent origins (Wilson and Holldobler, 2005), the eusocial insects remain highly studied. This level of study follows in large part from the abundance and key roles of eusocial insects in ecosystems. Many bee species are pollinators and have an impact on the conservation of plant species on a landscape scale, in addition to providing a pivotal ecosystem service (Klein *et al.*, 2007). Other eusocial insects, predominantly ants and termites, act as bioturbators providing soil turnover (Debruyn and Conacher, 1990), and serve as ecosystem engineers (Fox-Dobbs *et al.*, 2010). Indeed, the eusocial insects[2] are known to be both ecologically successful and dominant (Wilson, 1990; or see Parr *et al.*, 2016 for a functional example).

[2] The terms *social* and *eusocial* are used interchangeably throughout this thesis. Concordantly; *solitary* is taken to mean non-eusocial.

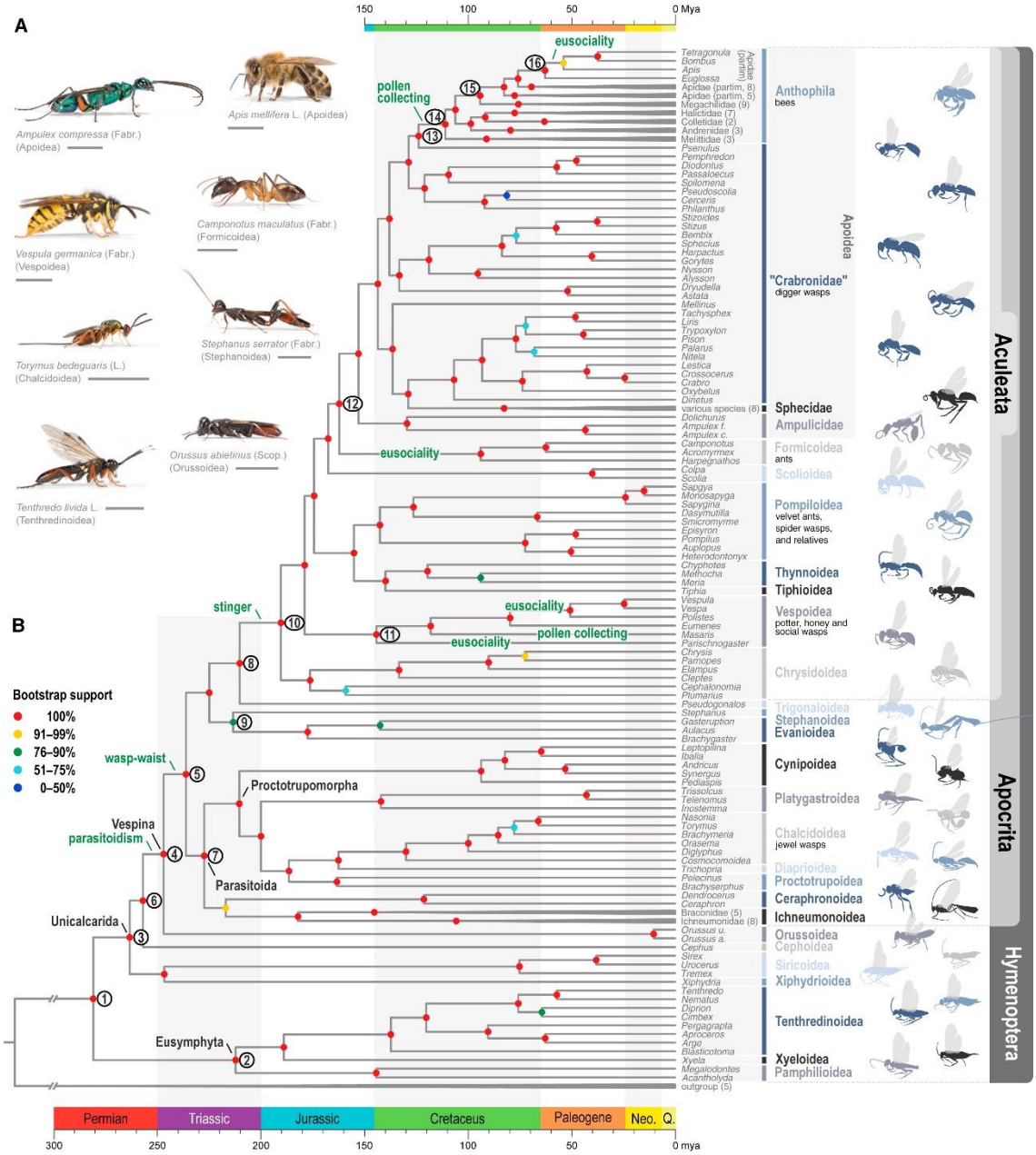

Figure 1.1: Phylogenetic relationships within the Hymenoptera.. Reproduced with permission from Peters *et al.* (2017). A) Hymenopteran representatives. B) Phylogenetic relationships and divergence times within Hymenoptera. Note that only major eusocial lineages were considered in Peters *et al.* (2017). Four independent origins of eusociality are indicated on the tree. For full details, see Peters *et al.* (2017). The subjects of this thesis (*Osmia bicornis*, Megachilidae; and *Apis mellifera*, Apidae) are to be found within the aculeate Antophila, and share their most recent common ancestor (mrca) some 95 million years ago (mya; Cretaceus).

1.1 Historic overview: outlining the underlying problem

The problem that eusociality poses, broadly relates to altruism in nature. An altruistic behaviour is specifically defined as being beneficial to the recipient and costly to the actor in terms of fitness (Table 1.1). In social insects, the neuter or altruistic worker essentially forfeits its own reproduction to the benefit of the reproductive (queen).

Table 1.1: Categorisation of (pairwise) social behaviours in nature (Gardner and West, 2010).

	Actor	Recipient
Cooperation[a]	+	+
Altruism	-	+
Selfishness	+	-
Spite	-	-

[a] i.e.: mutual benefits, direct benefits and 'weak altruism' *(Lehmann and Keller, 2006)*.

Debates on social evolution reached the forefront in the 1960s. Several prominent biologists, among which V.C. Wynne-Edwards and K. Lorenz, had suggested that organisms evolved to regulate their population size and avoid overexploiting resources (Wilson and Wilson, 2007). Such ideas were criticised by G.C. G.C. Williams (1966) and J. Maynard Smith (haystack model, 1964), arguing that selection acts at the level of the individual rather than the group. Group selection was subsequently considered naïve (Wilson and Wilson, 2007). The main issue being that such a system could easily be invaded by cheaters. The group selection of the 1960s does not form an evolutionary stable strategy (ESS: Smith and Price, 1973). Group selectionism was offset by the work of W.D. Hamilton (1964a) on neighbour modulated fitness and inclusive fitness, two concepts of social fitness which are formally equivalent. Of the two concepts, inclusive fitness is generally considered the more workable (West and Gardner, 2013; Birch, 2016). Inclusive fitness constitutes a fundamental maximizing property of Darwinian fitness (West and Gardner, 2013) and takes into account an individual's own reproductive success alongside the propagation of identical copies of its genes present in other individuals. In its simplest form, this can be represented by '*greenbeards*' that share a pleiotropic gene, which recognises other (unrelated) carriers of said gene and invokes altruistic behaviour towards them (Hamilton, 1964a; Dawkins, 1976) — thereby aiding their propagation indirectly. Greenbeard existence has been doubted, since they are likely to act against the interests of the rest of the genome and

are prone to evolutionary invasion by '*falsebeards*', yet some examples do exist (see: Gardner and West, 2010; Madgwick *et al.*, 2019).

Generally, inclusive fitness refers to related individuals. Hamilton (1964a) made use of Wright's coefficient of relationship (r; Wright, 1922; Orlove and Wood, 1978; Bennett, 1987; Pamilo, 1990) to quantify the probability of a replica gene occurring in a related individual. This r is generally referred to as relatedness, and individuals are argued to maximise their inclusive fitness following Hamilton's rule: C < r B, where C equals the cost of the behaviour to the actor, and B equals the benefit to the recipient (Hamilton, 1964a)[3,4]. Altruism then occurs when the costs of helping a relative are outweighed by the benefits accrued by helping related genes propagate. A train of thought that was already adopted by R.A. Fisher (1930), J.B.S. Haldane (Dugatkin, 2007) and arguably C.R. Darwin (1859; p. 237) himself, but it was succinctly formalized by Hamilton. The specific case of helping relatives at one's personal expense was further coined kin selection (Maynard Smith and Wynne-Edwards, 1964), and is embedded within the broader inclusive fitness theory.

Hamilton (1964b) further advanced the specific case of the Hymenoptera, in what is known as the '*haplodiploid hypothesis'*. In essence: relatedness among sisters is inflated (Figure 1.2) within the haplodiploid sex determining system (assuming monogamy). Hamilton himself called the idea in itself *'thoroughly naïve'* (Hamilton, 1964b, p. 29), as further skews in sex ratios would be required (Trivers and Hare, 1976). Yet, the multiple origins of eusociality within the Hymenoptera hint at the importance of haplodiploidy (Gardner *et al.*, 2012; Gardner and Ross, 2013). The Hymenopteran sex determining system has further implications to for instance conservation genetics as well. This since it can exacerbate population bottlenecks where increased inbreeding leads to the production of generally sterile or subfertile diploid males, increasing extinction proneness (Packer and Owen, 2001; Zayed and Packer, 2005; Zayed, 2009). With regard to the overall importance of relatedness to Hymenopteran evolution and conservation, Chapter 4 outlines the development of microsatellite markers for *O. bicornis*. These allow for estimating relatedness in the absence of pedigree information (Queller and Goodnight, 1989).

---

[3] The original formulation in Hamilton (1964a) being:
- K > 1/r,
where K can be equated to -B/C.

[4] Altruism between unrelated individuals was later explained through the use of game theoretic models (e.g. reciprocal altruism; Trivers, 1971).

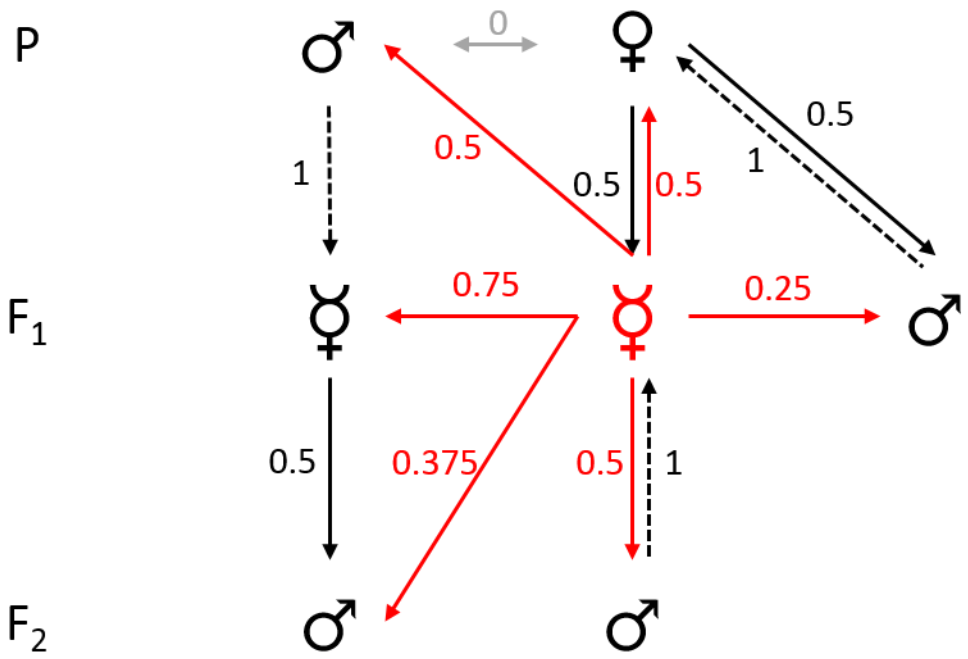

Figure 1.2: Relatedness skew within Hymenoptera. Male individuals develop from unfertilised eggs, whereas females develop from fertilised eggs. Relatedness coefficients by pedigree (r; Wright, 1922) for family members of a haplodiploid species following a single monogamous mating in the parental generation (P) are displayed. The direction of the arrow indicates the direction of relationship the relatedness coefficient refers to. Arrows and coefficients in red indicate relatedness from the point of view of a focal daughter (e.g. honey bee worker), showing a clear skew in relatedness towards sisters (r = 0.75). The grey two way arrow indicates mating between unrelated individuals. Black dotted arrows indicate male (haploid) points of view, black full arrows indicate the remaining viewpoints. (F_1 = first generation, F_2 = second generation, ♂ = male, ♀ = female, and ☿ = virgin female/worker)

Meanwhile, group selection has seen some revival under the denomination '*multi-level selection*' (Wilson and Wilson, 2007). It posits that altruism (including eusociality) has evolved in those instances where between-group selection was greater than within-group selection. Just as inclusive fitness theory partitions selection into direct and indirect components, so does multi-level selection partition it into within and between group components (Marshall, 2011). Both concepts have received considerable attention in mathematical modelling (for an overview of models, see Lehmann and Keller, 2006).

The debate surrounding inclusive fitness and multilevel selection has been re-ignited for some time, in a push for the multi-level selection perspective predominantly led by E.O. Wilson (Wilson, 2005; Wilson and Holldobler, 2005;

Fletcher *et al.*, 2006; Foster *et al.*, 2006a; Foster *et al.*, 2006b; Wilson and Wilson, 2007; Wilson, 2008; Nowak *et al.*, 2010; Abbot *et al.*, 2011; Boomsma *et al.*, 2011; Strassmann *et al.*, 2011; Ferriere and Michod, 2011; Herre and Wcislo, 2011; Nowak *et al.*, 2011; Bourke, 2011). Yet, it has been well established that inclusive fitness and multi-level selection are formally equivalent (Grafen *et al.*, 1984; Lehmann *et al.*, 2007; Marshall, 2011; Lehtonen, 2016; Rubin, 2018), with only a single notable opponent (van Veelen *et al.*, 2012). Both perspectives are not mutually exclusive, and ultimately, eusocial insects constitute *groups* of closely related *kin*. Nonetheless, the inclusive fitness approach offers a more tangible design principle or maximand (West and Gardner, 2013; Pernu and Helantera, 2019). This leaves inclusive fitness as more applicable, as it is both causal and generates readily testable hypotheses (Abbot *et al.*, 2011; West and Gardner, 2013; Marshall, 2016; Pernu and Helantera, 2019).

Nowak *et al.* (2010) in particular, caused substantial controversy within the field. However, they did correctly emphasise the need for certain pre-adaptations and requirements necessary for the evolution of eusociality. Such preadaptations include: ancestral monogamy (Hughes *et al.*, 2008; Boomsma, 2009), population viscosity (Hamilton, 1964a, b), kinship (Hamilton, 1964a, b; Trivers and Hare, 1976), nest construction and defence (Wilson and Holldobler, 2005; Nowak *et al.*, 2010), overlapping generations (Hunt and Amdam, 2005; Santos *et al.*, 2019), and parental care (e.g. progressive provisioning; Queller, 1994; Wilson and Holldobler, 2005; Nowak *et al.*, 2010)[5].

This historic overview demonstrates a breadth and depth of theoretical work. Yet, this theoretical work is mainly a paradigm of genes, whereas frameworks of how these are linked to *form* are largely underdeveloped (Pigliucci, 2007) — with the exception of M. J. West- Eberhard, whom has created a framework for the synthesis of genes, phenotype and development (West-Eberhard, 2003). Current empirical work however, might help bridge this gap.

[5] The evolution of maternal care is also thought to be more likely to evolve under the haplodiploid system (Wade, 2001).

1.2 Genes underlying altruism

Development of theory (section 1.1) makes profuse use of mathematical models which serve as '*proofs-of-concept'* (Servedio *et al.*, 2014). However, the actual genes and pathways underlying eusociality have yet to be identified. Hence, Thompson *et al.* (2013) suggested certain intuitive criteria for genes underlying (reproductive) altruism. They should satisfy Hamilton's rule (i), be environmentally sensitive (ii), increase in both number and complexity (iii), should co-evolve or be dependent on genes underlying recognition (iv), may reside in regions of low-recombination (v), be partially additive (vi), and exhibit strong pleiotropy (vii) (Thompson *et al.*, 2013). Nevertheless, '*uncovering genes underlying eusociality*' in itself may be a somewhat misleading statement. It is the sort of wording that implies outdated conceptualisations: that the evolution of eusociality progressed in a sequential and stepwise manner towards an hypothetical '*eusocial end goal*', that the evolution of eusociality proceeded in a single and fixed manner in all lineages, and that there is a single or that there are several linked genes underlying eusociality. Not only is the concept of a *goal* inherently at odds with the theory of evolution by means of natural selection (Darwin, 1859), but eusociality also exists along multiple traits that are themselves part of a continuum/spectrum (Sherman *et al.*, 1995; Linksvayer and Johnson, 2019). Consequently, the evolution of eusociality can be considered to be anything but a *'social ladder'* (Linksvayer and Johnson, 2019).

1.2.1 The reproductive groundplan hypothesis (RGPH)

In spite of these considerations, convergent evolution does appear to exist with regard to eusociality. Many conserved pathways consistently reappear, and regulate the same or similar underlying processes of eusociality (Woodard *et al.*, 2011; Berens *et al.*, 2015; Warners *et al.*, 2019; Linksvayer and Johnson, 2019). The repeated use of similar processes along independent origins, indicate that a suite of reproductive and behavioural traits were likely linked and co-opted into the reproductive division of labour. Hence, built upon the foundational conceptualisation and work by M.J. West-Eberhard on heterochrony and heterotopy[6] (West-Eberhard, 1987, 2003; West-Eberhard, 2005), the importance of an underlying reproductive groundplan (RGPH) has been stressed and become a central paradigm in the field (Amdam *et al.*, 2003; Linksvayer and Wade, 2005; Amdam *et al.*, 2006; Page *et al.*, 2009; Kapheim, 2016; Warners *et al.*, 2019). Under the RGPH, reproductive traits and traits

[6] Heterochrony = a difference in timing and/or duration of a developmental stage or process over evolutionary time. Heterotopy = a spatial change of developmental processes during evolutionary time.

underlying maternal care (e.g. provisioning) are thought to have been de-coupled. While not all work supports this, this may be down methodology and a neglect of complexity (e.g. non-tissue and timing specific comparisons: Kapheim, 2016). Naturally, the differences in ancestral life history traits and ecological niches across the multiple origins of eusociality complicate things further, as they have resulted in different eusocial 'routes' being taken and underscore the importance of lineage specific differences (Kapheim, 2016; Linksvayer and Johnson, 2019).

1.2.1.1 Hormonal signalling cascades

Cogent explanations for convergent evolution and the RGPH specifically, could reasonably be found within the broader aspects of insect oogenesis and reproduction. In female insect oogenesis and reproduction are controlled through a combination of nutrient-signalling pathways (insulin signalling and target of rapamycin signalling as systemic nutrient sensing pathways), hormones (juvenile hormone and 20-hydroxyecdysone), and peptides (male accessory gland secretions or sex peptides, adipokinetic hormone) and neuropeptides (neuroparsins, neuropeptide F)[7] (Roy *et al.*, 2018; Lenaerts *et al.*, 2019b). Of these, historically, juvenile hormone (JH) and 20-hydroxyecdysone (ecdysterone or 20E) have been considered the main regulators of insect reproduction and oogenesis (Riddiford, 2012). Canonically, JH and 20E are thought to have antagonistic roles (e.g.: Masner and Hangartner, 1973; Masner *et al.*, 1975; Lezzi and Wyss, 1976; Liu *et al.*, 2018). This is due to — as their respective names indicate — JH maintaining larval (or juvenile) stages during development, up to the critical weight of the larva, after which a spike of 20E causes moulting (Belles and Piulachs, 2015; Jindra *et al.*, 2015).

[7] Note that these denominations are somewhat arbitrary, particularly the distinction between '*peptide'* and '*hormone*', as insulin and adipokinetic hormone are both considered '*peptide hormones*' for instance.

Some work has been carried out on JH within the Hymenoptera. This because, there seems to have been a rewiring of the endocrine network in eusocial insects (Robinson and Vargo, 1997), or a shift in the timing of hormonal spikes (Page *et al.*, 2009).However, as Robinson and Vargo (1997) put it:

> *'Because of the pervasive role of JH in insect reproduction, social insect biologists have looked to this hormone to provide clues to the physiology of reproductive division of labor. Rather than trying to elucidate hormone regulation in social insects for its own sake, the primary goal of this research has been to use endocrine approaches to gain insight into the behavioral and/or pheromonal mechanisms by which the queen's status as dominant reproductive is established and maintained. Otherwise, the methods used to study the gonadotropic role of JH in the social Hymenoptera are the same as those used to study endocrine regulation of reproduction in other insect orders: studies correlating ovary development with either JH titers or rates of biosynthesis, and studies showing the effect of exogenous hormonal treatment on ovary development and egg-laying behavior.'*

Fortunately, recent progress has been made outside the field of eusocial research. The receptor for JH has been discovered through a strain of *Drosophila melanogaster* that were resistant to the JH analog methoprene (Jindra *et al.*, 2015). The JH receptor, named Methoprene-resistant (Met), has been characterised (Charles *et al.*, 2011), and consequently the JH pathway has become fairly well understood (Jindra *et al.*, 2015). The synthesis of JH occurs in the corpus allatum and correlates to input of nutritional signals, mediated by insulin signalling (ISS). JH will subsequently be transported by the haemolymph to all tissues, and carry out its signalling function by entering cells. It possibly does so through a suspected but currently unknown transporter. Heat shock protein 83 (Hsp83) will then chaperone JH into the nucleus where it will form a complex with Met and Taiman (a transcriptional co-activator). This complex will then promote transcription of target genes. For instance *Krüppel-homolog 1* (*Kr-h1*) and *early-trypsin*. (Jindra *et al.*, 2015)

Likewise, 20E has become more well-studied. 20E forms a complex with the Ecdysone receptor (EcR) and Ultraspiracle (Usp) in the nucleus (Hodin and Riddiford, 1998; Roy *et al.*, 2018), similar to JH. While the roles for JH and 20E in development are relatively straightforward across the insect phylogenetic spectrum, their specific roles in reproduction and oogenesis tend to vary slightly. JH is the main hormone regulating female reproduction in most hemi- and holometabolous insects, whereas 20E is known to be the main regulator of female reproduction in lepidopterans, most dipterans, and some hymenopterans. (Roy *et al.*, 2018)

The advances made for JH and 20E, elucidating not only their mechanisms, but also their cross-talk with the insulin signalling pathway and others (Roy *et al.*, 2018) — might facilitate further investigation of their role in the RGPH (Page *et al.*, 2009). For instance, JH is difficult to measure directly, yet our current knowledge allows us to investigate differences in the levels of transcription of JH associated elements of biosynthesis as a proxy (e.g.: *Met* and *Kr-h1* as in, Lenaerts *et al.*, 2019a; Lenaerts *et al.*, 2019b). JH possesses a gonadotropic function in both solitary bees as well as primitively eusocial species, yet seems to have lost this role in more advanced social species (Roy *et al.*, 2018). Vitellogenin production has seemingly uncoupled from JH, and the regulatory hormonal role may have been taken over by 20E in these species (Roy *et al.*, 2018). This situation seems to mirror the evolutionary transition from dynamic and aggressive reproductive conflict in smaller colonies, towards the pheromonal control of reproduction in larger colonies (Robinson and Vargo, 1997). These advances in insect hormonal signalling merit an in depth re-visitation of the earlier work.

1.2.2 Sociogenomics

Whereas direct investigations of hormonal signalling waned, the advent of more widespread genomic tools and resources, has led to an increase in differential gene expression studies. For instance, in the honey bee alone, differential gene expression studies in relation to caste differentiation (Evans and Wheeler, 1999, 2001; Lago *et al.*, 2016), worker egg laying (Thompson *et al.*, 2006), and reproductive division of labour (Grozinger *et al.*, 2007) have been carried out. Such studies emphasise the importance of changes in gene regulation (Kapheim, 2016). Changes in gene regulation in both time (heterochrony) and space (heterotropy) can allow for phenotypic novelty — referred to as evolutionary co-option — to arise, as it is not always maladaptive (West-Eberhard, 2003). Overall, there has been an increase in genomic resources and tools available, and these have powered a surge in *sociogenomics* research (1.2.2; Kapheim, 2016). In the remainder of this chapter, I lay out contemporary efforts in uncovering genes underlying altruism, with special reference to *A. mellifera* as it is the most best-studied species to date, and is the subject of comparison in this thesis.

A traditional approach to researching the evolution of eusociality, is the use of phylogenetic analysis (e.g. ancestral monogamy in eusocial lineages: Hughes *et al.*, 2008). Phylogenetic studies are still used, but tend to be combined with genomic and bioinformatic tools (phylogenomics) to further the study on the origin and evolution of eusociality. *A. mellifera*, being a model

organism already, entrenched its role further in this respect when it was the first eusocial species to have its genome sequenced (Weinstock *et al.*, 2006). Two notable phylogenetic studies making use of genomic resources are outlined here (Woodard *et al.*, 2011; Kapheim *et al.*, 2015b), since they both include *A. mellifera*.

Woodard *et al.* (2011), sequenced ten transcriptomes (pooled tissues and stages) spanning three independent origins of eusociality. Orthologue sequence alignments were produced, and differences in the rate of evolution[8] were characterised between eusocial and non-eusocial species. Additionally, they performed two separate tests, with '*primitively'* and '*highly'* eusocial species each being tested versus the remaining pool of species. Subsequently, gene ontologies (GO) were constructed for genes with signatures of accelerated rates of evolution. They found that genes relating to gland development (secretions in hive, pheromones, etc.), signal transduction (changes in behaviour) and carbohydrate metabolism (processed honeys) evolved rapidly in eusocial lineages. They also discovered a signature of accelerated evolution in brain-related GOs for primitively eusocial species, but not highly eusocial species. Woodard *et al.* (2011) argued that primitively eusocial species might face greater socio-cognitive challenges than highly eusocial species (e.g. learning of signature CHC mixes, as in the model for queen pheromone evolution proposed by Smith and Liebig, 2017).

In a similar study, Kapheim *et al.* (2015b) sequenced the genomes of ten related species. With a phylogeny spanning two independent origins of eusociality and two independent '*elaborations'* of eusociality (i.e. increases in social complexity, typically caste polymorphism and colony size; Bourke, 1999). In their comparison, they found that with increasing social complexity there was an increase in the capacity for gene regulation. This was evidenced by: more *cis* transcription factor binding sites (scanned from known *D. melanogaster* binding sites), more DNA methylation (lower $CpG_{o/e}$), and enriched GO terms for gene regulation (making use of dN/dS as in Woodard *et al.*, 2011). This was offset by the constrained evolution of neural and endocrine-related genes (lower dN/dS). When regarding both origins of eusociality, genes showed common patterns but had lineage specific differences (i.e. different genes/pathways, but with similar GO enrichment). For instance, concurrent with Woodard *et al.* (2011), they found that signal transduction was important. Finally, next to no shared enriched GO terms were found for the eusocial elaborations. Indicating that eusocial elaborations '*do not necessarily involve common molecular*

[8] Non-synonymous to synonymous nucleotide substitutions; dN/dS.

pathways' (Kapheim *et al.*, 2015b), possibly due to lineage specific differences in ecology and social organisation being exacerbated.While these types of studies (Weinstock *et al.*, 2006; Woodard *et al.*, 2011; Kapheim *et al.*, 2015b) offer a plethora of information, they should be treated with care. They are explorative in their disposition. For instance, many of the differential gene expression studies mentioned (at the start of section 1.2) largely ignored: castes (reproductive vs. non-reproductive), developmental stage (larvae vs. adult) and age class (Kapheim, 2016). Likewise, the above studies (Woodard *et al.*, 2011; Kapheim *et al.*, 2015b) represent great data-mining techniques, and suggest new avenues of research. Yet, trends in the transitions to eusociality will prove inadequate on their own, and experimental work with functional verification remain pivotal to our understanding of its evolution.

1.2.3 Organisation through caste development

In contrast to sociogenomic studies, differential gene expression studies provide a higher resolution albeit on a smaller scale. Larval developmental stages between queen and worker destined larvae (Barchuk *et al.*, 2007; Cameron *et al.*, 2013a, b) are well studied for instance.

Adult *A. mellifera* queens and workers differ markedly in their morphology (as in many advanced eusocial species). Queens are larger, possess notched mandibulae, lack corbiculae (pollen baskets), have unbarbed stingers, and have more ovarioles (an average range of 5-12 in a worker's ovaries vs. 150-180 in a queen's ovary; Winston, 1991). Queen destined larvae (QDL) also have: a higher metabolism, a shorter developmental time, and with the resultant queens also have a longer lifespan (1-3 years; Winston, 1991). These differences reflect their role inside the hive, with queens serving as egg-laying machines whereas workers forage (pollen basket or corbicula) and need to mould wax (flat mandibulae) among other tasks. These differences are established in early larval development through differential feeding of a substance called royal jelly (RJ). Differences in gene expression as early as six hours after hatching have been found (with RJ being presented to queen destined larvae within this time; Cameron *et al.*, 2013a). Such changes will then lead on to, for instance, a pulse in juvenile hormone (JH) in queen-destined larvae and increased developmental cell-death in the ovary anlagen of worker destined larvae (Hartfelder and Steinbruck, 1997; Reginato and Cruz-Landim, 2001; Reginato and Da Cruz-Landim, 2002; Tanaka *et al.*, 2006; Dallacqua and Bitondi, 2014), giving rise to the diminished worker ovaries (Winston, 1991).

JH is pivotal to caste differentiation. Topical application of JH can make larvae acquire queen fate or '*rescue*' knockouts (e.g.: Mutti *et al.*, 2011). JH

titres are known to increase from 3th instar larvae onwards, peaking at the 4th instar, when extensive growth takes place (de Azevedo and Hartfelder, 2008). The role of JH also ties into the RGPH. West-Eberhard (1996) suggested that JH not only underlay physiological maturation, but also directed the behavioural changes in solitary progressively provisioning ancestors. JH is then thought to have become uncoupled in queens and workers during social evolution (e.g. no longer matures worker ovaries due to nutritional status), but further retained its role in controlling behavioural changes (i.e. age polyethism; Robinson and Vargo, 1997).

The uncoupled action of JH might for instance be regulated through epigenetic changes. DNA methylation, for instance, is known to lead to differential gene expression across *A. mellifera* castes (Elango *et al.*, 2009). Kucharski *et al.* (2008) further found that silencing DNA methyltransferase (*Dnmt3*; using siRNA) resulted in queen phenotypes in worker destined larvae. Hence, diphenic caste development (in *A. mellifera*) is currently thought to be the complex product of nutritional and hormonal signalling, in conjunction with epigenetic changes.

1.2.4 Reversible adult reproductive constraint

The reproductive division of labour is not exclusively regulated through developmental mechanisms and caste however. Reproductive constraint may constitute any process — from behaviour, to physiology and morphology — that reduces the ability of the non-reproductive caste to reproduce (Khila and Abouheif, 2010). Behavioural traits are generally considered more phenotypically plastic than are morphological or physiological traits. From the perspective of phyletic gradualism, behaviour is also more inclined to have preceded (or even led to) evolutionary novelties in morphology and physiology (Price *et al.*, 2003; West-Eberhard, 2005). A common behavioural constraint is for instance worker policing (e.g. Wenseleers and Ratnieks, 2006) in the presence of a queen. Physiological reproductive constraints are typically initiated developmentally however. Khila and Abouheif (2010) classified developmental reproductive constraints for ant species (Table 1.2). The reproductive constraints characterised (Khila and Abouheif, 2010; Table 1.2) indicate that they can be both non-absolute[9] and reversible, as in many species workers can start reproducing in the absence of the queen. For instance, the first and second constraint allow for the production of trophic eggs by queenright workers in *Aphaenogaster rudis*, while queenless workers may consequently produce more viable eggs (Khila and Abouheif, 2008). The

[9] I.e. lead to subfertility as opposed to complete sterility.

combinations of these constraints may signify different trade-offs in the ecology of these species.

The honey bee worker possesses at least two of the reproductive constraints characterised by Khila and Abouheif (2010). The absence of a spermatheca in honey bee workers (constraint 3 - Table 1.2; Winston, 1991; Gotoh *et al.*, 2013; with the exception of certain *Apis mellifera capensis* females) and a reduction in ovariole number (constraint 4 - Table 1.2; Winston, 1991; Tanaka *et al.*, 2006) are both evident. Non-reproductives often lack spermatheca. Due to the relatedness skew in Hymenopterans (Figure 1.2), worker policing of female offspring is predicted to occur more frequently than is policing of male offspring (Bourke, 1999). The greater selective pressure levelled against fertilised worker offspring therefore likely explains the frequent absence of spermatheca in non-reproductives across eusocial evolution (Bourke, 1999). The possible role of mechanisms controlling oogenesis with regard to mating are discussed in further detail in Chapter 5. Constraints 1 and 2 may also exist in *A. mellifera*, given that: the maternal determinant *Nanos* is not localised in vitellogenic oocytes of (queenless) workers (constraint 1 - Table 1.2; Dearden, 2006— i.e. the vitellarium, see Figure 1.3)[10], and that queen ovarioles are substantially larger than those of workers (constraint 2 - Table 1.2; Winston, 1991, p. 42). Yet these remain to be tested explicitly in *A. mellifera*.

Table 1.2: Reproductive constraints as characterised by Khila and Abouheif (2010). Both reversible (1-2), and irreversible (3-5) constraints are known to be present in ant species. Note that constraint 5 is the only absolute form of reproductive constraint, and is incidentally quite rare (9 out of 283 ant genera: Khila and Abouheif, 2010).

Reproductive constraint	Mechanism
1	Mis-localisation mRNA[a]
2	Quantitative activity of ovaries
3	Loss of spermatheca
4	Reduction of ovariole number
5	Complete loss of genetalia

[a] *Khila and Abouheif (2008)*

Reproductive constraint is incomplete and retains elements that are reversible in honey bee workers. As is the case for non-reproductives of many social species, honey bee workers practice physiological self-restraint (Hoover *et al.*, 2003), in addition to mutual policing (Wenseleers and Ratnieks, 2006). In

[10] *A. mellifera Vasa* expression also differed between worker and queen ovarioles, but in the terminal filament and germarial regions (ovariole regions are indicated in Figure 1.3).

other words: worker ovaries are kept in an inactivated state under queenright[11] conditions, through queen mandibular pheromone (QMP) and brood pheromone (Jay, 1972; Mohammedi *et al.*, 1998; Hoover *et al.*, 2003; Tanaka and Hartfelder, 2004). Oocyte development is supressed at an early stage in these queenright honey bee workers (Tanaka and Hartfelder, 2004; Duncan *et al.*, 2016). Specifically, cystocyte clusters do not progress beyond the germarium (region indicated in Figure 1.3), where oocytes and nurse cells are specified (Tanaka and Hartfelder, 2004; Ronai *et al.*, 2015; Duncan *et al.*, 2016). Curiously, germarial disorganisation is similar to the situation seen in queens of *A. mellifera* kept virgin for an extended duration (Berger and Abdalla, 2005). This, in conjunction with the absence of spermatheca in *A. mellifera* workers (constraint 3 - Table 1.2; Winston, 1991). This raises the interesting possibility of adult reproductive constraint being evolutionary ancestral to mating status — a hypothesis which is further explored in Chapter 5.

Duncan *et al.* (2016) showed that active Notch signalling in the germarium (Figure 1.3) is associated with inactive ovaries in queenright workers. Notch is known to form and maintain the germline stem cell niche in the *D. melanogaster* germarium (Song *et al.*, 2007). Through the use of a Notch inhibitor, Duncan *et al.* (2016) managed to increase ovary activation in both the presence and absence of QMP — categorically demonstrating that germarial Notch signalling regulates QMP mediated adult reproductive constraint.

Another, possibly related aspect of this mechanism, has been germarial apoptosis (Tanaka and Hartfelder, 2004; Ronai *et al.*, 2015). Since programmed cell death is already associated with several checkpoints in the ovary of *D. melanogaster* (McCall, 2004; Pritchett *et al.*, 2009), and since oocyte abortion inherently needs to be '*cleaned up*', this makes intuitive sense. The suggested germarial checkpoint (Ronai *et al.*, 2015) is responsive to starvation in *D. melanogaster* at least (Drummond-Barbosa and Spradling, 2001). Notch signalling is likewise responsive to starvation in *D. melanogaster* (Bonfini *et al.*, 2015), and Notch is generally known as an '*arbiter of differentiation and death*' (Miele and Osborne, 1999). Hence it may be that Notch amalgamates multiple identified pathways within its signalling function.

[11] Queenright = under queen presence; queenless = under queen absence.

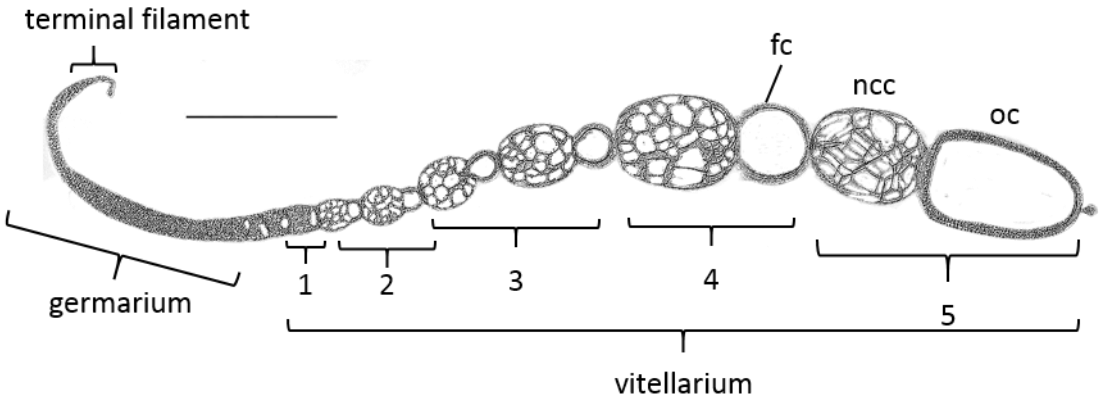

Figure 1.3: Schematic of a queenless honey bee worker ovariole. Regions of the ovariole are anterior to posterior: the terminal filament which may contain putative germline stem cells (Tanaka and Hartfelder, 2004), the germarium where oocytes and nurse cells are specified, and the vitellarium where vitellogenesis takes place. Numbers denote oocyte stages following Wilson *et al.* (2011) (fc = follicle cells, ncc = nurse cell chamber, oc = oocyte, with scalebar = 500 µm).

Honey bee adult reproductive constraint, is signalled by the queen, through the use of priming pheromones (Winston and Slessor, 1998; Hoover *et al.*, 2003). QMP is made up of four major components (Slessor *et al.*, 1988; Jarriault and Mercer, 2012), is highly derived and specific to *A. mellifera* (Van Oystaeyen *et al.*, 2014), and it is known to repress ovary activation in a range of species (unspecified prawn species: Carlisle and Butler, 1956; *Kalotermes flavicollis*: Hrdý *et al.*, 1960; *Musca domestica*: Nayar, 1963; *D. melanogaster*: Sannasi, 1969; Camiletti *et al.*, 2013; Lovegrove *et al.*, 2019; Princen *et al.*, 2019b). Since non-eusocial species are susceptible to the anti-ovarian properties of QMP, it follows that QMP likely targets a conserved pathway (Lovegrove *et al.*, 2019). Especially considering the fact that both individual queen pheromones of different species, and blends thereof, did not replicate a similar phylogenetically broad repressive effect (Lovegrove *et al.*, 2019). Complicating matters further, in honey bees, a functional redundancy in worker ovary repression was found (Princen *et al.*, 2019a). Not only do the two main QMP components [Figure 1.4; (2*E*)-9-oxodec-2-enoic acid and (2E)-9-hydroxydec-2-enoic acid] work independently of each other (i.e. non-synergistically), but other compounds produced by honey bee queens were found to similarly repress ovary activation in workers (Princen *et al.*, 2019a). In any case, Lovegrove *et al.* (2019) convincingly argue that QMP-activity is novel,

and that it likely specifically targets conserved mechanisms (e.g. Notch signalling: Duncan *et al.*, 2016)[12].

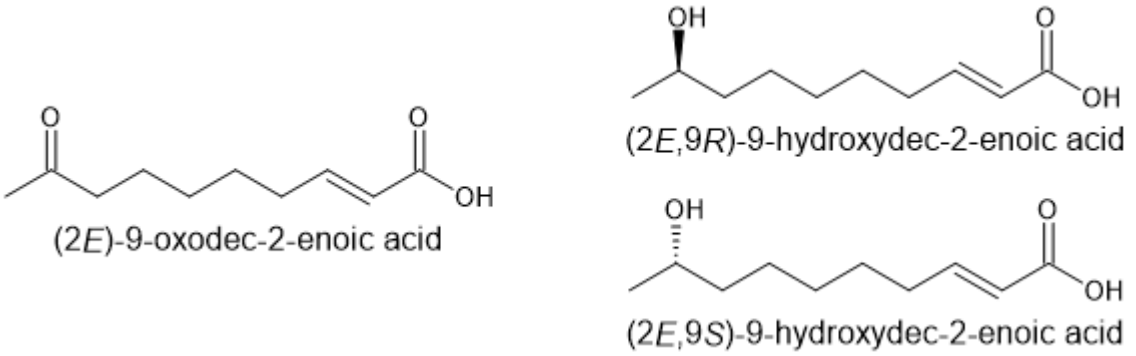

Figure 1.4: Two main components of QMP. 9-ODA [(2*E*)-9-oxodec-2-enoic acid] and 9-HDA [(2*E*)-9-hydroxydec-2-enoic acid]. The two stereo-isomers of 9-HDA are presented (top right = -9-HDA; and bottom right = +9-HDA). These compounds make up the majority of QMP's five major components, with one '*queen equivalent'* containing 150µg 9-ODA and 55µg 9-HDA (71% R-(-) and 29% S-(+)) on average, yet these and the other major components act in synergy (Slessor *et al.*, 1988). This synergistic action also applies to the individual stereo-isomers of 9-HDA (Slessor *et al.*, 1988).

Broadly speaking, queen pheromones have likely evolved as honest signals of queen fecundity (Keller and Nonacs, 1993; Van Oystaeyen *et al.*, 2014; Oi *et al.*, 2015). They have likely been derived from cuticular hydrocarbons (CHCs), which serve pleiotropic roles in: desiccation avoidance, species recognition, kin recognition, and mating status and fecundity (Van Oystaeyen *et al.*, 2014; Smith and Liebig, 2017; Holman, 2018). The latter three traits in particular will have facilitated CHCs to gradually transition towards functioning as a queen fertility signal (Smith and Liebig, 2017). In a striking example of convergent evolution, the predominating components in queen pheromones across social lineages are the non-volatile saturated hydrocarbons (particularly long-chained linear alkanes; Van Oystaeyen *et al.*, 2014). CHCs functioning as honest fecundity signals in social and eusocial context, may have been derived directly as by-products of: ovary development, sex pheromones,

[12] Given the contrasting situation of both pheromonal signalling redundancy within *A. mellifera* itself (Princen *et al.*, 2019a) and a broad phylogenetic spectrum effect of *A. mellifera* QMP (Lovegrove *et al.*, 2019) — it may also be prudent to remind ourselves of the at least 9000 year old domestication of *A. mellifera* (Crane, 1983; Crane, 1999; Bloch *et al.*, 2010). This domestication was presumably accompanied by a loss of certain selective pressures and trade-offs, and the addition of other (artificial) selective pressures.

oviposition deterring pheromones, or a combination thereof (Oi *et al.*, 2015). This point will be expounded on in Chapter 5.

1.2.5 Swindling bees

As stated earlier (1.1), altruistic systems can be susceptible to invasion by cheaters (Maynard Smith and Wynne-Edwards, 1964). Cheating phenotypes have been identified within *A. mellifera*, and present interesting opportunities to study '*genes underlying altruism*'.

1.2.5.1 *Anarchy*

Oldroyd *et al.* (1994) found drone comb above a queen excluder; implying worker laid drones under queenright conditions. Further paternity analysis (microsatellite markers) showed that the drones were laid by three to four patrilineal workers (Oldroyd *et al.*, 1994). Workers of this anarchistic strain of honey bees showed increased rates of ovary development, increased tolerance to QMP, and increased survival rates of worker laid eggs (Oldroyd and Osborne, 1999; Oldroyd and Ratnieks, 2000). Using backcrosses, Oxley *et al.* (2008) tried to identify specific genes underlying the phenomenon, and managed to find four quantitative trait loci (QTLs; explaining only 25% of variance) associated with the phenotype. Ronai *et al.* (2016a) further investigated one QTL regions, and focussed on a candidate gene dubbed *Anarchy* (PMP34; a peroxisomal ATP transporter: Visser *et al.*, 2002) which was the best predictor of ovary state among four shortlisted candidate genes. They further found it to be associated with *Buffy,* a mitochondrial inducer of apoptosis (Tanner *et al.*, 2011; Dallacqua and Bitondi, 2014). *Anarchy* mRNA localised to degenerating oocytes and nurse cells in queenright worker ovaries, localised around the germinal vesicle of maturing oocytes in queenless worker ovaries, and was differentially expressed between queenless and queenright workers (Ronai *et al.*, 2016a). Whether and how PMP34 is regulated by QMP, and how it functions to regulate oogenesis are all questions that remain to be addressed.

1.2.5.2 *Thelytoky*

Onions (1912) discovered that some workers of *Apis mellifera capensis* (South African Cape honey bee) can produce diploid females through parthenogenesis (thelytoky), due to an abnormal meiosis (spindle rotation failure; Lattorff and Moritz, 2013). These thelytokous workers activate their ovaries in the presence of the queen, develop queen-like traits and produce queen-like QMP (Lattorff and Moritz, 2013). These workers can function as social parasites to the related *Apis mellifera scutellata* (East African lowland

honey bee) by killing their queen and exploiting the colony (the '*capensis* calamity'; Lattorff *et al.*, 2007; Lattorff and Moritz, 2013 and references therein).

Lattorff *et al.* (2005) found a recessive inheritance pattern of thelytoky in worker offspring when backcrossing with both *Apis mellifera carnica* (Carniolan honey bee) and *A. mellifera capensis*. The subsequently uncovered recessive allele *thelytoky* (*th*), was shown to increase 9-ODA synthesis and social parasitism (Lattorff *et al.*, 2007). Jarosch *et al.* (2011) traced *th* to a single locus homologous to *gemini* (*gem*)[13] in *D. melanogaster* (Hoskins *et al.*, 2007). Jarosch *et al.* (2011) proposed a model where alternative splicing of two exon cassettes caused the phenotype. This was later contested in a study using backcrosses with *Apis mellifera scutellata* (Chapman *et al.*, 2015), but may be inconclusive. Thelytokous workers inherently skew reproduction by being queenlike (inherently inhibiting the egg-laying of regular workers: Lattorff *et al.*, 2007), thereby making it hard to quantify arrhenotokous laying to compare with in backcrosses. Additionally, only ten microsatellite markers were used in Chapman *et al.* (2015; five of which were linked); which could be considered doubly inadequate given the extremely high recombination rate of *A. mellifera* (Weinstock *et al.*, 2006; Beye *et al.*, 2006). To date, the debate surrounding the genetic underpinnings of thelytoky remains unsettled (Wallberg *et al.*, 2016; Aumer *et al.*, 2017; Aumer *et al.*, 2019; Christmas *et al.*, 2019), and further research is still required.

1.3 Study species

In order to study the evolution of adult reproductive constraint, I made use of two species. By comparing a highly eusocial and well-studied species to a related solitary species using molecular techniques, I aimed to gain insights into the mechanisms underlying the evolution adult reproductive constraint.

1.3.1 The eusocial honey bee, *A. mellifera*

A. mellifera is very widespread and relatively easy to maintain, making them a model system (Dearden *et al.*, 2009b). The reproductive division of labour of the honey bee consists of a single multiply mated queen, functionally sterile female workers and fertile males (drones). The queen is essentially an egg laying machine, living longer than both workers and drones. Workers care for eggs laid by the queen, feed larvae, clean and maintain the nest, attend the queen, collect and store pollen, collect and process nectar, and guard the nest. These (summer) tasks are roughly associated with age (i.e. age polyethism), with tasks further away from both queen and hive carried out by older workers.

[13] Gemini = ‘genitalia missing’.

Drones are usually only born in spring, during the swarming season, when an old queen and the aerial part of the workforce[14] look for a new place to nest and found a new colony (i.e. colony reproduction; when the old colony has grown large enough)[15]. During the swarming season, new queens will mate multiply (with thirteen males on average; Estoup *et al.*, 1994) during a mating flight. (Winston, 1991)

A honey bees' role within the colony thus depends on both its sex as well as its division into either the reproductive caste (queen) or the non-reproductive caste (worker). The former is genetically modulated through the *complementary sex determiner locus* (*csd*; Gempe and Beye, 2009) — with heterozygotes becoming females and hemizygotes resulting in male offspring. Meanwhile caste differentiation is phenotypically plastic (discussed in section 1.2.3). Exactly when a new queen is raised, depends mainly on current queen presence or absence. Alternately, queen destined larvae may also be raised in order to replace the old queen (supersedure). Queen presence is signalled through QMP which is produced in her mandibular gland (see section 1.2.4; Naumann *et al.*, 1991). It is licked off of her by queen retinue workers, and subsequently spread throughout the colony via trophallaxis and incorporation into the secreted wax nesting material (Naumann *et al.*, 1991).

1.3.2 The solitary red mason bee, *Osmia bicornis*

Studies on facultative eusocial bees exist (*Ceratina japonica*; Maeta *et al.*, 1993; Sakagami *et al.*, 1993). Since group living is artificially inducible in such species, they seem ideal to test hypotheses like the RGPH. However, such species are only secondarily solitary, and the reproductive plasticity they exhibit is likely of a vestigial nature (Kapheim, 2019). Recently, direct tests of the RGPH using solitary bees have become more numerous (*Euodynerus foraminatus* — N-American wasp: Tibbetts *et al.*, 2013; *Nomia melanderi* — arid N-American bee: Kapheim and Johnson, 2017b; Kapheim and Johnson, 2017a; *Synagris cornuta* — tropical African wasp: Kelstrup *et al.*, 2018; *Euglossa dilemma* — C-American bee: Saleh and Ramirez, 2019). However, the species used in these studies, are almost exclusively American and African. Indeed, the most suitable species tend to be tropical, which is no coincidence. The lack of seasonal winter in tropical climates precludes the need for hibernation, with implications for diapause. Subsequently, a tropical climate allows for

[14] Young nurse bees are yet incapable of flight.

[15] While swarming behaviour and mechanisms are fairly well-studied, the exact '*releaser conditions*' for both rearing new queens, as well as swarming by the old queen remain unknown (Visscher and Camazine, 1999; Seeley and Buhrman, 1999).

progressive provisioning, multivoltinism and facultative diapause, communal nesting and nest construction to evolve. These traits are considered pre-adaptations to eusociality, and are typically lacking in temperate solitary bees (e.g. *O. bicornis*; Raw, 1972). It is unlikely to be a coincidence that highly eusocial species are likewise predominantly tropical and subtropical (Roubik, 1992, p. 380-389).

Consequently, there is a distinct lack of a European and temperate model solitary bee species to compare the well-studied '*model social insect species*' *A. mellifera* and *B. terrestris* to. Hence, in order to conduct a cross-species comparison of the reproductive biology of the highly eusocial honey bee, we elected to use the solitary red mason bee, *Osmia bicornis* (syn. *Osmia rufa*, L. 1758; Hymenoptera, Megachilidae). This species was favoured over species more closely related to *A. mellifera* (i.e. favoured over other solitary Apidae more closely related to *A. mellifera*; Peters *et al.*, 2017) due to its commercial availability as a supplementary pollinator (Dr Schubert plant breeding; Landsberg, Germany), the fact that it is a well-studied species (for a full overview, see Chapter 3), and its potential to be reared in the laboratory (Sandrock *et al.*, 2014; see Chapter 3 for a full discussion). Furthermore, crucial to molecular studies, *O. bicornis* has also recently had an annotated genome published (Beadle *et al.*, 2019), as well as having global DNA methylation data available (Strachecka *et al.*, 2017).

Not only is *O. bicornis* a common solitary bee in the UK (Falk, 2015), it also possesses at least several traits considered pre-adaptations to the evolution of eusociality. These are: suspected monogamy (Seidelmann, 2014a, 2015), population viscosity (gregarious nesting: O'Toole, 2000; degree of nest-site fidelity: Steffan-Dewenter and Schiele, 2004), the potential for kin recognition (Raw, 1992), nest construction and defence (Seidelmann, 2006, 1999a), and maternal care (Seidelmann, 2006; Ivanov, 2006).

Additionally, *O. bicornis* retains adult (pupal) diapause, rather than a developmental (larval) diapause (Raw, 1972). This is also significant towards evolving eusociality, for a less obvious and more complicated reason. Adult diapause is prerequisite towards evolving mated female hibernation, which in turn is required to facilitate (partial) bivoltinism (Quinones and Pen, 2017). Within the sex determining system of the Hymenoptera, bivoltinism leads to temporally biased sex-ratios (Seger, 1983). I.e. spring generations are male-biased and summer generations are generally female-biased (Seger, 1983; Quinones and Pen, 2017). Hence, in a bivoltine Hymenopteran, where sex is biased towards females in the summer generation, female helping is promoted in that generation, since helpers can capitalise on the relatedness asymmetry

outlined in Figure 1.2 (Trivers and Hare, 1976)[16]. Using ancestral state reconstruction, it has been shown that adult diapause is ancestral to lineages of bees that have evolved eusociality (Santos *et al.*, 2019).

For all the reasons outlined above, *O. bicornis* lends itself to be established as a European solitary model species. Furthermore, the caveats initially outlined are not insurmountable(e.g. bivoltinism and diapause refer to hypotheses in wasps specifically; Hunt and Amdam, 2005; Hunt *et al.*, 2007). I would argue that the very nature of investigating *conserved mechanisms or genetic toolkits* (Kapheim, 2016), such as the *conserved Notch signalling pathway* (Duncan and Dearden, 2010), should be inherently amenable to study using a more distantly related cousin species. Particularly when studying QMP mediated adult reproductive constraint in *A. mellifera*, with its phylogenetically broad repressive effects (Lovegrove *et al.*, 2019).

The full life-history of *O. bicornis* will be further discussed in Chapter 3, alongside attempts of establishing this species in a laboratory setting.

[16] Under equal sex-ratios, the inclusive fitness gained by rearing female siblings that are more related (r = 3/4) is cancelled out through the rearing of male brood (brothers; r = 1/4). Consequently, the capitalising on the relatedness asymmetry requires the preferential rearing of sisters over brothers, biased sex-ratios (spatially or temporally), or both (Trivers and Hare, 1976).

1.4 Aims

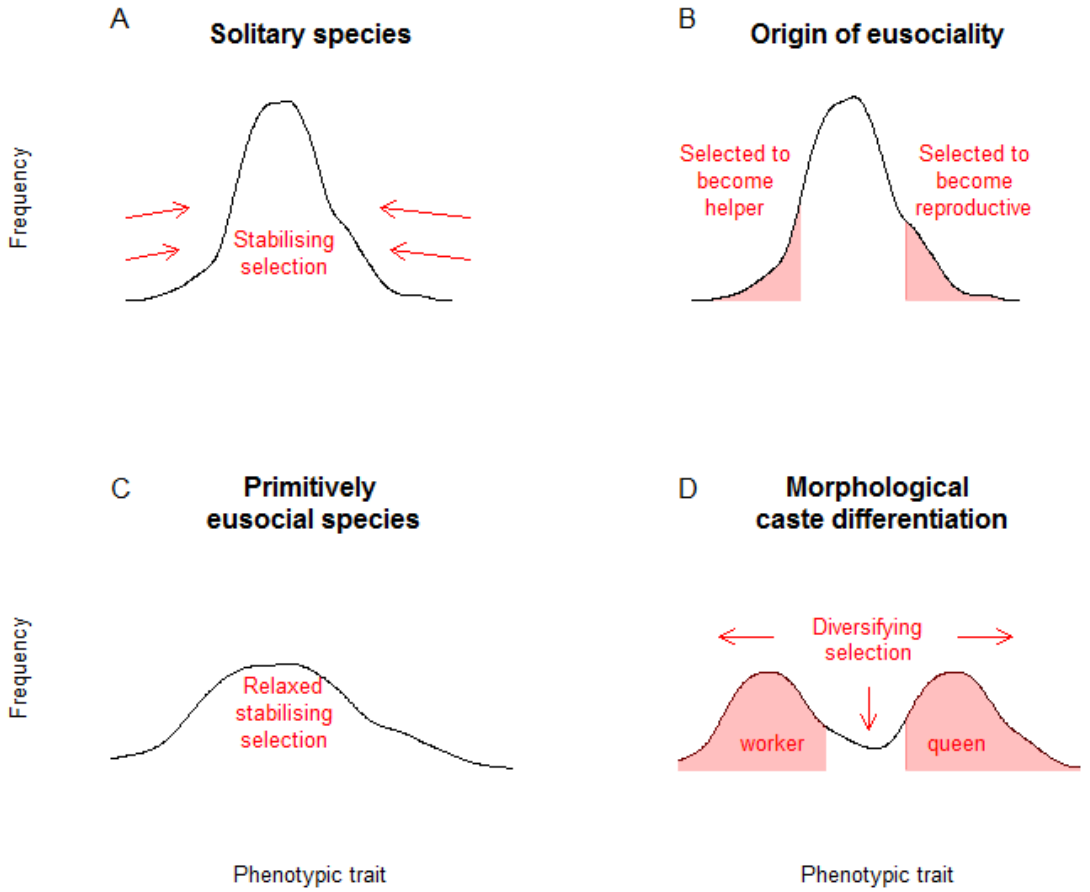

Figure 1.5: Abridged Gadagkar (1996) model. Model proposed by Gadagkar (1996) on the origin and evolution of eusociality through selection for developmental plasticity. A) In the ancestral solitary state there is limited developmental plasticity in a reproductive phenotypic trait, due to stabilising selection. B) Yet, at the incipience of eusocial living. individuals at the extreme ends of the phenotypic trait space would be well adapted to fill the ecological niches of either helper or dominant reproductive. C) With the increasing reliance on inclusive fitness and co-operative broodcare, stabilising selection is relaxed on reproductive traits, making them more phenotypically plastic. D) Selection against intermediate phenotypes that are both suboptimal workers as well as suboptimal reproductives starts occuring (diversifying/disruptive selection) — and further directional selection towards extremes gives rise to caste differentiation. Figure reproduced from Gadagkar (1996).

Figure 1.5 (A through to D) outlines a model by Gadagkar (1996). Building upon the accumulated work of West-Eberhard (see references in Gadagkar, 1996), he elegantly displayed how behaviour could help initiate and select for developmental plasticity. The ‘phenotypic trait’ depicted in Figure 1.5 could represent ‘time taken to reproductive maturity’, ‘ovary size’, ‘mandible

size', or any other caste related trait. In solitary species, less developmental phenotypic plasticity is thought to exist, but it is still present (Figure 1.5A). Changes in behaviour, in conjunction with inclusive fitness benefits, subsequently drive selection (Price *et al.*, 2003) for individuals at the extremes. Individuals at the extremes are those best suited to be either helpers or reproductives (Figure 1.5B-C). Ultimately — stemming from selection for increased developmental plasticity (Figure 1.5C) — specialised diphenic castes arise from the same genome (Figure 1.5D).

In this work, in essence, I will attempt to compare plastic control of reproduction in a solitary bee (Figure 1.5A), to that of a eusocial worker (Figure 1.5D). While Figure 1.5 (Gadagkar, 1996) specifically refers to developmental plasticity, I will be focusing on reversible mechanisms of adult reproductive control (solitary), and constraint (eusocial worker; such as constraint 1 and 2 in Table 1.2). This because under the RGPH, mechanisms of adult reproductive constraint are predicted to be rooted in conserved and environmentally controlled pathways (sections 1.2.3 and 1.2.4).

1.5 Outline of thesis

The general goal of this thesis was thus to analyse the molecular and physiological basis of these constraints on worker reproduction, to attempt to directly link them to the environmental factors that originally controlled them (e.g. nutrition or mating); to elucidate what factors and mechanisms may have been at play during the evolution of honey bee eusociality. Additionally, I aimed to establish *O. bicornis* as a model species for the future study of bee social evolution.

In Chapter 3 I discuss attempts to get *O. bicornis* females to reproduce naturally in a laboratory environment, precisely with the above aim of establishing this species as a future model in mind. This is important, since it would allow the most direct and explicit test of reproductive success. I discuss future directions for maintaining solitary pollinators in a laboratory setting, and provide proof of concept to the viability of behavioural assays with respect to foraging in a caged environment.

In Chapter 4, I try and root *O. bicornis* as a model species further, by testing microsatellite markers mined from the annotated genome (Beadle *et al.*, 2019). Where previous work has made use of partial genomic libraries (Neumann and Seidelmann, 2006), the use of a larger amount of markers is more informative, and provides a foundation for future genetic work in *O. bicornis*. I outline future applications for these markers.

In Chapter 5, I further aimed to establish *O. bicornis* as a model species, by providing a microstructural study of the ovary. I compare the *O. bicornis* ovary to that of the eusocial *A. mellifera* worker. This is central to further compare the use of conserved pathways in the reproductively constrained honey bee worker (e.g. germarial Notch signalling, section 1.2.4, and Chapter 6). I studied ovary structure in conjunction to the context of social environment, specifically mating status. This because reproduction and mating are intertwined in insects overall (Gillott, 2003; Colonello and Hartfelder, 2005; Avila *et al.*, 2011), as well as in queens of social species in particular (Patricio and Cruz-Landim, 2002; Tanaka and Hartfelder, 2004; de Souza *et al.*, 2007; Shukla *et al.*, 2013; Peso *et al.*, 2013). Furthermore, mating affects queen fertility signalling (Slessor *et al.*, 1990; Richard *et al.*, 2007; Oppelt and Heinze, 2009; Nino *et al.*, 2013; Jansen *et al.*, 2016), and queen pheromones may have been derived from sex pheromones (Oi *et al.*, 2015). Taken together with the fact that *A. mellifera* workers are reproductively constrained by a lack of spermatheca (constraint 3 - Table 1.2; Winston, 1991; Khila and Abouheif, 2010) — it raises the possibility that mechanisms associated with virginity underly QMP-mediated adult reproductive constraint in workers.

In Chapter 6, I demonstrate a robust optimisation of the *in situ* hybridisation technique in the ovary of *O. bicornis*[17] . I use this technique to investigate germarial Notch signalling in the *O. bicornis* ovary, and discuss its relevance with respect to QMP-mediated reproductive restraint in *A. mellifera* workers (Duncan *et al.*, 2016). I attempt to establish a functional link between germarial Notch signalling and the early control of oogenesis in *O. bicornis*, using a Notch inhibitor. Subsequently, I try to repress early oogenesis in *O.bicornis* through the use of both starvation (Drummond-Barbosa and Spradling, 2001; Bonfini *et al.*, 2015) and QMP (Lovegrove *et al.*, 2019). Finally, I test the effectiveness of QMP in repressing oogenesis in *A. mellifera* workers post-ovary activation to assess the reversibility of this phenotypically plastic repression.

Finally, in Chapter 7, I contextualise the overall results within the wider literature, and I suggest future avenues of research. I also propose a conceptual model for the evolutionary co-option of germarial Notch signalling into adult reproductive constraint of the honey bee, *A. mellifera*.

[17] Full protocols can be found in Chapter 2 (General methods)

Chapter 2 General Methods

2.1 Husbandry

2.1.1 *Apis mellifera*

A. mellifera workers were retrieved from a commercial stock which was kept according to standard practices (Dearden *et al.*, 2009b) in British National hives. Colonies were assessed weekly for egg-laying, queen cups, food stores and parasites. Supplementary feeding was supplied regularly. Queenless workers were obtained by placing frames of brood with several workers into a standard nucleus box, which typically activate their ovaries after two to four weeks in the absence of QMP (Duncan *et al.*, 2016). A queenless hive was considered reproductively active once 30% of dissected bees showed '*stage 3*' ovaries (Duncan *et al.*, 2016).

2.1.2 *Osmia bicornis*

O. bicornis is commercially available as a supplementary pollinator, and as a pollinator in greenhouses and orchards. The *O. bicornis* in this study were mainly obtained as cocoons from a commercial supplier (Dr Schubert plant breeding; Landsberg, Germany). While being a well-studied species, this species has typically been studied and kept in field or semi-field conditions. To date only a single study has managed to induce the complete set of foraging and reproductive behaviours, and facilitated a complete reproductive cycle in a full laboratory setting (Sandrock *et al.*, 2014). Chapter 3 deals with attempts to replicate the laboratory rearing of *O. bicornis*, and consequently aspects of husbandry for this species are treated in depth in Chapter 3.

2.2 Microdissection and tissue processing

Individuals were narcotised on ice, or at 4°C for 30 minutes since carbon dioxide narcosis is known to affect transcription (Koywiwattrakul *et al.*, 2005; whole abdomen RNA extractions). Ovaries were dissected using fine forceps in cold phosphate buffered saline (PBS). For *O. bicornis* females, the intima and ovariole sheath were removed using fine forceps to improve staining and image quality for microscopy samples. For *A. mellifera* females, large ovarioles from queen-less workers (QLW) were stripped similarly, while small ovarioles from queen-right workers (QRW) were individualised using fine forceps. All dissections were carried out within 20 minutes to limit degradation of nucleic

acids. For RNA extractions, tissue was placed on dry ice immediately after dissection and subsequently stored at -80°C.

Samples for immunohistochemistry (IHC) or *in situ* hybridisation (ISH) were placed in 400 µl PBS on ice. 100 µl of 40% formaldehyde and 500 µl heptane were added, and tissue was fixed on a nutating mixer at room temperature (RT). For IHC of *A. mellifera* ovaries, this was done for seven minutes for QRW, and ten to fifteen minutes for QLW. For *O. bicornis* ovaries, fixation was optimised as part of this study at twenty minutes for IHC (following honey bee queens: Dearden *et al.*, 2009a), and at one hour for ISH. Samples for ISH were rinsed three times with ice cold methanol (MeOH) and stored at -20°C.

2.3 RNA extraction

Tissue was weighed on a pre-cooled (dry ice) weighing boat and divided into pieces using a scalpel. The tissue was subsequently homogenised in Trizol (Invitrogen; 1ml reagent per 50 mg tissue) for 20-40 seconds at RT, and left to incubate for five minutes. Samples were placed on ice, and 200 µl of chloroform (Sigma-Aldrich) per 1 ml Trizol was added. Samples were vortexed for fifteen seconds and left to stand at RT for five minutes, after which they were centrifuged at a relative centrifugal force (RCF) of 12,000 g, at 4°C for ten minutes. 175µl of the aqueous upper phase (containing both RNA and DNA) was transferred, taking care not to touch the interphase and lower phenol:chloroform phase containing leftover tissue contaminants (proteins and lipids respectively).

RNA was subsequently purified using RNeasy (Qiagen) according to the manufacturer's instructions. 175 µl of 70% ethanol (EtOH) was added to dehydrate nucleic acids. The whole volume was pipetted onto the RNeasy mini column and centrifuged for fifteen seconds at 10,000 rotations per minute (rpm) at RT, precipitating both DNA and RNA onto the column. Salts were washed off using 350µl of the proprietary wash buffer and centrifuging for fifteen seconds at 10,000 rpm at RT. 80 µl of DNAse I was made up in buffer and added onto the membrane, and left to incubate for fifteen minutes at RT, after which it was washed off using 350 µl of wash buffer spun at 10,000 rpm for fifteen seconds at RT. The column bearing RNA was transferred to a collection tube, where 500 µl of a mild wash buffer was added and centrifuged for two minutes at 10,000 rpm to remove trace salts. This was performed twice, after which the column was transferred to a fresh microcentrifuge tube. The RNA on the column was eluted in 30 µl elution buffer and taken off the column by centrifugation at 10,000 rpm for one minute. 1.5 µl of sample was subsequently measured on a

spectrophotometer (NanoDrop 2000) to determine sample concentration and possible contamination. RNA was stored at -80°C.

2.4 cDNA synthesis

cDNA synthesis was performed using the Revert Aid first strand cDNA synthesis kit (Thermo Scientific) according to the manufacturer's instructions. 5 µg of RNA sample was placed in a thermal cycling tube on ice, and total volume was brought to 12 µl using nuclease free water (UltraPure, Invitrogen). 1x reaction buffer, 1 U of Ribolock RNase inhibitor, 1 mM dNTP mix, 5 µM random hexamer primer mix and 10 U of RevertAid M-MulV reverse transcriptase (RT) were added and the reaction mix was inverted gently and spun down briefly. Samples were incubated in a thermal cycler for five minutes at 25°C (annealing) and 60 minutes at 42°C (reverse transcription). The reaction was subsequently terminated at 70°C for five minutes. Resultant complementary DNA (cDNA) was stored at -20°C.

2.5 Primer design *in situ* hybridisation probes

Sequences were obtained from an unassembled *O. bicornis* transcriptome dataset, assembled by Dr E.J. Duncan using Trinity (Grabherr *et al.*, 2011; Haas *et al.*, 2013). Genes of interest were identified in the *A. mellifera* and *Megachile rotundata* (Megachilidae) genomes from previously published data (e.g. Duncan *et al.*, 2016) or by making use of the *Drosophila* sequence (Thurmond *et al.*, 2018). *O. bicornis* orthologs were identified within the *de novo* transcriptome using basic local alignment search tool (BLAST; Altschul *et al.*, 1990; Madden, 2013), and verified using reciprocal BLAST protein sequences to *A. mellifera* and *M. rotundata* (Megachilidae). This because *M. rotundata* is a more closely related species to *O. bicornis* with an available genome (accession number: PRJNA66515), and *A. mellifera* is subject to comparison in this thesis, in addition to having its genome well annotated (Weinstock *et al.*, 2006). Alignments were examined for assembly errors, and primer positions were selected outside of conserved and motif regions. Primers were designed with Primer3plus (Rozen and Skaletsky, 2000; Untergasser *et al.*, 2012), and devised to attain a product size of 800-1200 basepairs. Specificity was checked through Primer-BLAST (Ye *et al.*, 2012) to *A. mellifera* and *M. rotundata*.

2.6 Polymerase chain reaction (PCR)

PCR products intended for verifying transcription of a gene within tissue, and PCR product meant for ligation into a vector, and colony screening after transformation into *Escherichia coli* for subsequent ISH probe synthesis, were all performed using standard PCR. 20 µl PCR reactions were used, containing: 1x GoTaq flexi buffer (Promega), 250 µM dNTP mix (Promega), 3 mM MgCl2, 1 µM of forward and reverse primers, and 10-100 ng of template DNA. Thermocycling conditions varied (Table 2.1).

Table 2.1: Standard PCR conditions. T = temperature.

Stage			T	Time
Initial denaturation			94°C	3 minutes
x40		Denaturation	94°C	30 seconds
		Annealing	54-60°C	30 seconds
		Extension	72°C	1 minute
Final extension			72°C	10 minutes

In case of poor or unspecific amplification, troubleshooting consisted of adjusting template input, $MgCl_2$ concentration or the use of touchdown PCR (Korbie and Mattick, 2008). Product presence was verified on a 1% agarose gel alongside a 1 Kb Plus DNA Ladder (Thermofisher scientific), run at 150V in a sodium boric acid buffer (10 mM NaOH, pH balanced to 8.5 using BH_3O_3) for 10-15 minutes (Brody and Kern, 2004).

Gel extraction and purification were carried out prior to all ligations, using the QIAquick Gel Extraction Kit (QIAGEN). Band of the correct size was cut out under UV light, using a scalpel. The gel fragment was weighed, and three equivalent volumes of buffer QG were added. This was then incubated at 50°C for ten minutes. Sample was shaken, and one volume of isopropanol was added and the sample was mixed to dehydrate the DNA. The sample was transferred to a spin column and precipitated by centrifugation at 13,000 rpm for one minute. 500µl of QG was added again, and centrifugation repeated. Product was subsequently washed by adding 750µl of buffer PE and centrifugation at 13,000 rpm for one minute. The spin column was transferred to a new column in a fresh microcentrifuge tube to resuspend the DNA by adding 30µl of elution buffer and leaving to stand for one minute. Product was then taken from the spin column by centrifugation at 13,000 rpm for one minute. Product was subsequently verified by running 5µl on a new 1% agarose gel.

2.7 Cloning

Sticky end ligation or cloning was performed using the pEASY-T3 cloning kit (TransGen Biotech) following manufacturer's instructions. 2 µl of gel-purified PCR product was placed in a thermocycling tube along with 0.5 µl of pEASY-T3 cloning vector. This was incubated at RT for 30 minutes and subsequently placed on ice. Transformation was performed using sterile technique where appropriate. 50 µl of Subcloning Efficiency™ DH5α Competent Cells (Invitrogen; *E. coli*) was placed on ice in a culture tube and 0.85 µl of β-mercaptoethanol was added. Cells were incubated on ice for ten minutes, gently disturbing the mixture of cells every 2 minutes. 2.5 µl ligation mix was added, and mixture was left to incubate on ice for 30 minutes. The vector containing PCR product was then transformed into the competent cells by heatshocking at 42°C for 45 seconds. Mixture was then incubated on ice for another two minutes. 950 µl of pre-heated (42°C) super optimal broth with catabolite repression (SOC; 1 mM MgCl2, 1 mM MgSO4, 200 mM glucose in super optimal broth [SOB]) was then added to the cell mixture, and incubated at 37°C shaking at 220 rpm for one hour. 100 µl of the mixture was then plated onto a lysogeny broth agar plate (LB-agar) containing 50 µg/ml ampicillin. The LB-agar plate was then incubated at 37°C overnight. Individual colonies were screened by patch plating and PCR verification (section 2.6; Tm = 55°C; M13 primers).

Colonies containing a product of the right size were cultured in 3 ml LB containing 50 µg/ml ampicillin overnight at 37°C shaking at 220 rpm. The bacterial cultures were pelleted by centrifugation at 13,000 rpm for 30 seconds. Plasmid purification was then performed using a Plasmid Miniprep Kit (Monarch, New England Biolabs). Cultures were resuspended by vortexing pellets in 200 µl of resuspension buffer. Cells were lysed by adding 200 µl lysis buffer and inverting gently and leaving to incubate at RT for one minute. Plasmids were then renatured by adding 400 µl of neutralisation buffer (which also contains RNase A) and inverting gently and incubating at RT for two minutes. Contaminants were subsequently pelleted through centrifugation at 13,000 rpm for five minutes. The supernatant was transferred to a spin column and plasmid DNA was pelleted in the spin column through centrifugation at 13,000 rpm for one minute. Pellets were washed using the two separate wash buffers and centrifugation at 13,000 rpm for one minute once more. The spin column was transferred to a clean microcentrifuge tube and plasmid DNA was re-eluted by incubation in 30 µl elution buffer for one minute at RT. The sample was then centrifuged at 13,000 rpm for one minute. DNA concentration and possible contamination were then checked on a spectrophotometer. All samples

were sent for sequencing (M13 primers) by Eurofins Genomics, to verify the sequence and it's orientation in the plasmid.

2.8 RNA probe synthesis

Acquired sequences (section 2.7) were edited to contain only the ligated product using BioEdit (v 7.2; Hall, 1999). Edited sequences were put through a endonuclease restriction site mapper (http://restrictionmapper.org/) to select appropriate restriction enzymes that do not show any activity within the insert. 1 µg of plasmid was subsequently digested in 1x endonuclease specific buffer, using 0.2 U endonuclease in a total reaction volume of 50 µl. The digestion was incubated at 37°C for one hour. These reactions were done for both the antisense and sense directions[18]. Digestion was verified by running 5µl on a 1% agarose gel. The digested plasmid was then phenol:chloroform extracted by first adding 155 µl ddH2O and 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1). Samples were vortexed and centrifuged at 13,300 rpm for five minutes. 175 µl of the aqueous phase was then transferred into a sterile microcentrifuge tube without any carry-over from the lower- and interphase. 200 µl of chloroform was added, the sample was vortexed and the previous step repeated, this time transferring 150 µl of the aqueous phase.

The cut plasmid was then precipitated by dehydration by adding 15 µl of 3M sodium acetate (NaAc, pH 5.2) and vortexing briefly. Subsequently, 375µl of icecold 100% EtOH was added and left at -20°C overnight. Samples were then pelleted by centrifuging at 13,300 rpm for 20 minutes at RT and supernatant was decanted. The pellet was subsequently washed with 200 µl of 70% EtOH (RNAse free) and centrifugation at 13,300 rpm for five minutes. Supernatant was decanted and DNA was left to airdry. The pellet was then resuspended in 30 µl nuclease free water, left to stand for 5 minutes. Concentrations were measured and contamination checked using 1.5µl of sample by spectrophotometry.

Digoxigenin-labeled (DIG) RNA probes were transcribed *in vitro* in a 50 µl reaction containing 1x transcription buffer, 2 µl DIG RNA labelling mix (Roche), 80 U Ribolock RNAse inhibitor (ThermoFisher Scientific), and 40 U of

[18] Antisense probes are '*treatment*' probes, as the antisense (reverse complement) should bind specifically to the sense direction mRNA present in the tissue. Sense direction probes are a commonly used negative control, which should show little to no staining, and will help check for probe trapping inside the tissue. Other negative controls are typically a '*no probe controls*' or a '*scrambled probe controls'*. In the latter, the antisense sequence is scrambled (completely synthetic sequence) which can be used instead of sense controls when gene regulation by antisense RNA is suspected (Green *et al.*, 1986), and antisense strands are present in tissue.

the relevant T7 or SP6 RNA polymerase (ThermoFisher Scientific). The transcription reaction was incubated at 37°C for four hours, after which 1 U of DNAse I (ThermoFisher Scientific) was added and left to incubate for fifteen minutes at 37°C to degrade the template DNA. RNA probes were then precipitated by adding 5 µl of 3M NaAc (pH 5.2) and vortexing briefly, and subsequently adding 125 µl of icecold 100% EtOH and incubating at -20°C overnight. RNA was then pelleted by centrifuging at 10,000 rpm for 30 minutes at 4°C. The supernatant was removed and the pellet washed twice with 200 µl of 70% EtOH (RNAse free) by centrifugation at 10,000 rpm for five minutes at 4°C. After decanting the supernatant, the pellet was resuspended in 10 µl of nuclease free water. 1 µl of the sample was diluted 1:10 to be analysed using spectrophotometry and running on a 1% agarose gel to verify the product. 50 µl of hybridisation buffer was added to the remaining undiluted product which was stored at -20°C.

2.9 Whole-Mount *In Situ* Hybridisation (ISH)

Tissue samples (section 2.2) were rehydrated in MeOH and 0.1% Tween 20 (Sigma-Aldrich) in PBS (PTw). This was done gradually over a MeOH and PTw series (3:1, 1:1, and 1:3), each step nutated for five minutes at RT. Samples were then washed three times in PTw for five minutes nutating at RT. Proteinase K was added to 0.02 mg/ml, and samples were left to incubate at RT for 20 minutes to puncture cell membranes. Samples were then re-fixed in a 4% formaldehyde:PTw solution by nutating for fifteen minutes at RT. Samples were rinsed six times in PTw and placed in 1 ml of hybridisation buffer (50% deioinised formamide, 4 × SSC buffer, 1 × Denhardt's solution, 250 µg / ml tRNA, 250 µg / ml boiled ssDNA, 50 µg / ml heparin, 0.1% Tween 20, and 5% dextran sulfate in DEPC treated water). Samples were then pre-hybridised for at least four hours at 52°C, to prevent non-specific binding of the probe. Optimal probe concentration was determined empirically for each gene, and optimised for the signal to noise ratio. Generally, 0.5 to 4 µl of (undigested) probe (section 2.8) was added to the tissue samples. Probe annealing was incubated overnight at 52°C. Samples were subsequently washed seven times at 52°C using wash buffer (50% formamide, 2 × SSC buffer, and 0.1% Tween 20) for set periods of time (2x 5 minutes, 10 minutes, 15 minutes, 30 minutes and 2x one hour, followed by an overnight wash) following Dearden *et al.* (2009c) to reduce background staining (removing unbound probe).

Samples were rinsed three times in PTw, and subsequently nutated in PTw with 0.1% bovine serum albumin (PBTw; made fresh) at RT for at least two hours to block (i.e. prevent non-specific binding of the antibody). This PBTw

was then replaced with a 1:2000 anti-DIG AP antibody (Roche) solution in PBTw, and incubated for at least four hours at RT. Samples were rinsed three times, and subsequently washed for ten, fifteen and thirty minutes, and one hour in PTw by nutating at RT. Samples are then washed twice in alkaline phosphatase buffer (AP buffer; 100mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, and 0.1% Tween 20) nutating for five minutes at RT, after which tissue is transferred to a staining dish. 20 µl of NBT/BCIP solution (Sigma-Aldrich; solution of 18.8 mg/ml nitro-blue tetrazolium chloride and 9.4 mg/ml 5-bromo-4-chloro-3'-indolylphosphate toluidine salt in 67% DMSO) was added to 500 µl of AP buffer. This mixture was added to the samples in the staining dish, initiating staining. The staining reaction was stopped when the stain had developed to satisfaction by rinsing in PTw three times. Tissue was destained in 100% MeOH until the tissue looked clean and staining had transformed from a purple to a blue colour. Tissue was then transferred to a microcentrifuge tube and rehydrated by nutating for five minutes in 1:1 MeOH:PTw. Tissue was rinsed four times in PTw, stained in the dark with 1 µl DAPI (5 mg/ml; 4′,6-diamidino-2-phenylindole; Invitrogen ThermoFisher Scientific) for ten minutes, and washed twice in PTw for five minutes. Samples were then incubated in 80% glycerol overnight and mounted on microscopic slides.

2.10 Immunohistochemistry (IHC)

Freshly, fixed tissue (section 2.2) was rinsed three times in PBS with 0.1% Triton X-100 (PTx; Sigma-Aldrich) and left nutating for two hours at RT to permeabilise the tissue facilitating antibody penetration. Samples were blocked PBTx for one hour at RT while nutating. PBTx was replaced, and between 1:20 – 1:200 primary antibody was added, and left to incubate overnight at 4°C. Tissue was washed four times in PTx while nutating for thirty minutes at RT. Tissue was blocked once more in PBTx for one hour while nutating. Subsequently PBTx was replaced with a 1:20 - 1:1000 dilution of the secondary antibody and incubated in the dark at 4°C overnight. Samples were washed four times in PTx while nutating at RT for 30 minutes to reduce background staining. Both DAPI and phalloidin were used as counterstains. 0.33 µM Phalloidin Dylight 488 (Thermo Fisher Scientific) was incubated for three hours in the dark, followed by three five minute nutating washes in PTx. Then 1 µl DAPI (5 mg/ml, Invitrogen ThermoFisher Scientific) was added and incubated in the dark for ten minutes. Samples were washed three times for five minutes in PTx whilst nutating once more. Samples were then incubated in 80% glycerol overnight, and mounted and imaged the next day.

Chapter 3 Establishing *Osmia bicornis* as a laboratory model

3.1 Summary

Valid empirical tests of the reproductive ground plan hypothesis (RGPH) require a solitary species as a reference point. Hence, the absence of a solitary model species that is tractable in a laboratory environment — particularly within the European temperate climate — is limiting to investigations into the RGPH. In this chapter I attempt to replicate a 2014 study, in order to try and reliably establish *O. bicornis* in a controlled laboratory environment. I first outline the life-history of *O. bicornis,* explore previous attempts at accomplishing this, and identify the specific challenges surrounding the use of a solitary pollinator in the laboratory. Informed by the general ecology of *O. bicornis* and the wider plant-pollinator literature, I trialled numerous set-ups to induce egg laying within flight cages. Using behavioural observations of small preliminary successes, I refined parts of the set-up. My results indicate the difficulty of utilising a pollinator with a complex life-cycle in a laboratory. I provide proof of concept for some behavioural assays that may be possible in future for this species in the lab. Finally, I identify major pitfalls and lay out future recommendations for further establishing the species within the laboratory.

3.2 Introduction

In order to facilitate a cross-species comparison of the reproductive biology of *A. mellifera* workers and *O. bicornis* females, it is imperative to establish the *O. bicornis* in a laboratory setting. This would allow for experimental manipulation; RNAi feeding for instance, could allow for functional tests through gene-knockdown; (e.g.: Maleszka *et al.*, 2007; Marco Antonio *et al.*, 2008; Hunter *et al.*, 2010; Jarosch and Moritz, 2011, 2012; Garbian *et al.*, 2012). Not only has *A. mellifera* been domesticated for some 9000 years (Crane, 1983; Bloch *et al.*, 2010), it has also emerged as a model organism (e.g.: Dearden *et al.*, 2009b; Williams *et al.*, 2013). *Osmia* have likewise enjoyed extensive study as they have attracted substantial interest for their potential as a commercial pollinator for use in greenhouses and with fruit crop (e.g.: Holm, 1974; Roth, 1990; van der Steen and Ruiter, 1991; Krunić *et al.*, 1995; Bosch and Kemp, 2002; Teper and Bilinski, 2009; Gruber *et al.*, 2011; Fliszkiewicz *et al.*, 2011b; Hansted *et al.*, 2014; Ahrenfeldt *et al.*, 2019; Ryder *et al.*, 2019). However, *O. bicornis* has typically been studied in field (e.g.: Ryder *et al.*, 2019; Ahrenfeldt *et al.*, 2019) or semi-field conditions (e.g.: Dietzsch *et al.*, 2015; Dietzsch *et al.*, 2019; Strobl *et al.*, 2019) precisely because of its

promise in commercial pollination. Other studies have examined the effects of pesticides on *Osmia* (Sandrock *et al.*, 2014; Beadle *et al.*, 2019; Azpiazu *et al.*, 2019; Sgolastra *et al.*, 2019), and the species' general ecology (Raw, 1972; Seidelmann, 1995; Ivanov, 2006; Fliszkiewicz *et al.*, 2015; Giejdasz *et al.*, 2016; Coudrain *et al.*, 2016; Persson *et al.*, 2018; Filipiak, 2019).

Some experiments have been performed in laboratory environments on *O. bicornis*, typically pertaining to a single aspect of its ecology (generally, mating: Fliszkiewicz *et al.*, 2013; Seidelmann, 2015; Conrad and Ayasse, 2019; or diapause and emergence: van der Steen and Ruiter, 1991; Krunić and Stanisavljević, 2006; Wasielewski *et al.*, 2011a; Fliszkiewicz *et al.*, 2012a; Dmochowska *et al.*, 2013; Giejdasz and Fliszkiewicz, 2016; Strachecka *et al.*, 2017; Beer *et al.*, 2019).These types of studies involve either bringing adults into the lab (from nest trapped cocoons in established populations or commercially bought cocoons), or manipulations and measurements on overwintering nest trapped bees (from established populations). Raw (1972), van der Steen (1997) and Sandrock *et al.* (2014) are the only studies to date, that attained a full life cycle in a laboratory set-up. Securing offspring within the confines of a controlled laboratory setting, would provide future experiments with a direct and powerful measure of reproductive success, by being able to assess egg to adult survival of said offspring (as in Sandrock *et al.*, 2014), free from environmental variation.

3.2.1 Life history of *O. bicornis*

Model organisms typically have a relatively short life cycles (e.g. *Drosophila melanogaster*), and are therefore often easy to maintain. Indeed, in insects many model species are also pest species, e.g.: *Tribolium castaneum*, *Acyrthosiphon pisum*, and *Schistocerca gregaria*). *O. bicornis* on the other hand — while being a common and generalist pollinator (Falk, 2015) —has an annual life cycle and a more particular life-history and niche (Figure 3.1).

In spring, adult *O. bicornis* emerge from their pupae. Males can hatch one to two weeks prior to females. During this time they will forage (nectar and pollen), search for females around nesting and foraging sites (Raw, 1976; Seidelmann, 1999b), and overnight in vacant nest sites or other small cavities (O'Toole, 2000). Females emerge from their pupae,generally around the beginning of May, though dates will shift with both region and year (Netherlands and Germany, mid April - start May: Vleugel, 1952; UK, start - mid May: Raw, 1972). Adult females typically survive four to six weeks, while males will only survive three to four weeks due to intense competition for mates (O'Toole, 2000). During the week following hatching, females fly around to look for

cavities that serve as both shelter and nest sites, forage, and a mate (van der Steen, 1997; O'Toole, 2000). After this period females start building and provisioning inside their respective nests (May-June; Raw, 1972). Nests are made in holes and crevices in walls, in reed stems and bee hotels[19] (Raw, 1972). The provisioning female first creates a smooth hemispherical layer of mud at the end of her elongate nest, to line it (Figure 3.1; Ivanov, 2006). She will then build the outline of the first septum (Fabre's threshold, Figure 3.1; Ivanov, 2006; Raw, 1972). She will gather pollen, predominantly *Ranunculus* and *Quercus*, although she is polylectic (Raw, 1974; Teper, 2007). Nectar is acquired alongside pollen and mixed in the nest to create pollen loaves. An egg is then oviposited on top of the pollen loaf, after which the septum is completed. Approximately one egg is provisioned and oviposited per day in this way (Raw, 1972). The amount of provisioning will determine the size of the offspring (Seidelmann, 2006; Seidelmann *et al.*, 2010; Seidelmann, 2014b), and the provisioning efficiency of a female will in turn increase with its body size (Seidelmann *et al.*, 2010).

The amount of provisioning also decreases when building cells closer towards the cells at the entrance of the nest (with a steep decline from female to male). By placing male offspring — who require less provisioning — closest to the nest entrance, females reduce the time spent away from the unguarded nest (Seidelmann, 2006). Females rest in their nest facing the entrance when not foraging; to guard against parasitoids and kleptoparasites (Krombein, 1967; Brechtel, 1986; Westrich, 1989), as well as nest take-overs by conspecifics (Raw, 1972; Strohm *et al.*, 2002; with the possibility of both the nest and its provisioning being commandeered). Hence, males are thought to be placed last in the nest to counteract parasitism. Provisioning efficiency also declines with maternal age and thus nest progression, leaving males to be deposited nearer nest entrances (Raw, 1972; Ivanov, 2006; Seidelmann, 2006). While the weight of provisions declines closer to the nest entrance, the weight of the mud partitions increases (Ivanov, 2006), further corroborating the role of parasitism avoidance to male placement in the nest. An additional contributing factor to the order in the nest, may be that males emerge from pupae earlier than do females. Ultimately a vestibulum and terminal plug secure the finished nest completely (Figure 3.1; Seidelmann, 1999a). During summer, the eggs hatch, and larvae start feeding on the provisions. These then spin their cocoons which

[19] A bee hotel is a manmade insect hotel, aimed specifically at pollinators such as solitary bees and wasps to nest and shelter in. They usually comprise collected reed stems in a half-open container (Fabre’s hive) or wood with drilled holes. The specificity of the insect hotel refers to the dimensions of the cavities.

will contain adults by the end of September (Raw, 1972). The pupae enter diapause and emerge the following spring.

The purpose of this chapter was acclimate *O. bicornis* to a laboratory environment, to allow for experimental studies and to acquire a direct measure of reproductive success (i.e. number of offspring and their survival). This, with the aim of determining what environmental factors govern the control of reproduction in this species, and whether and how the mechanisms controlling reproduction relate to QMP mediated adult reproductive constraint in the eusocial honey bee.

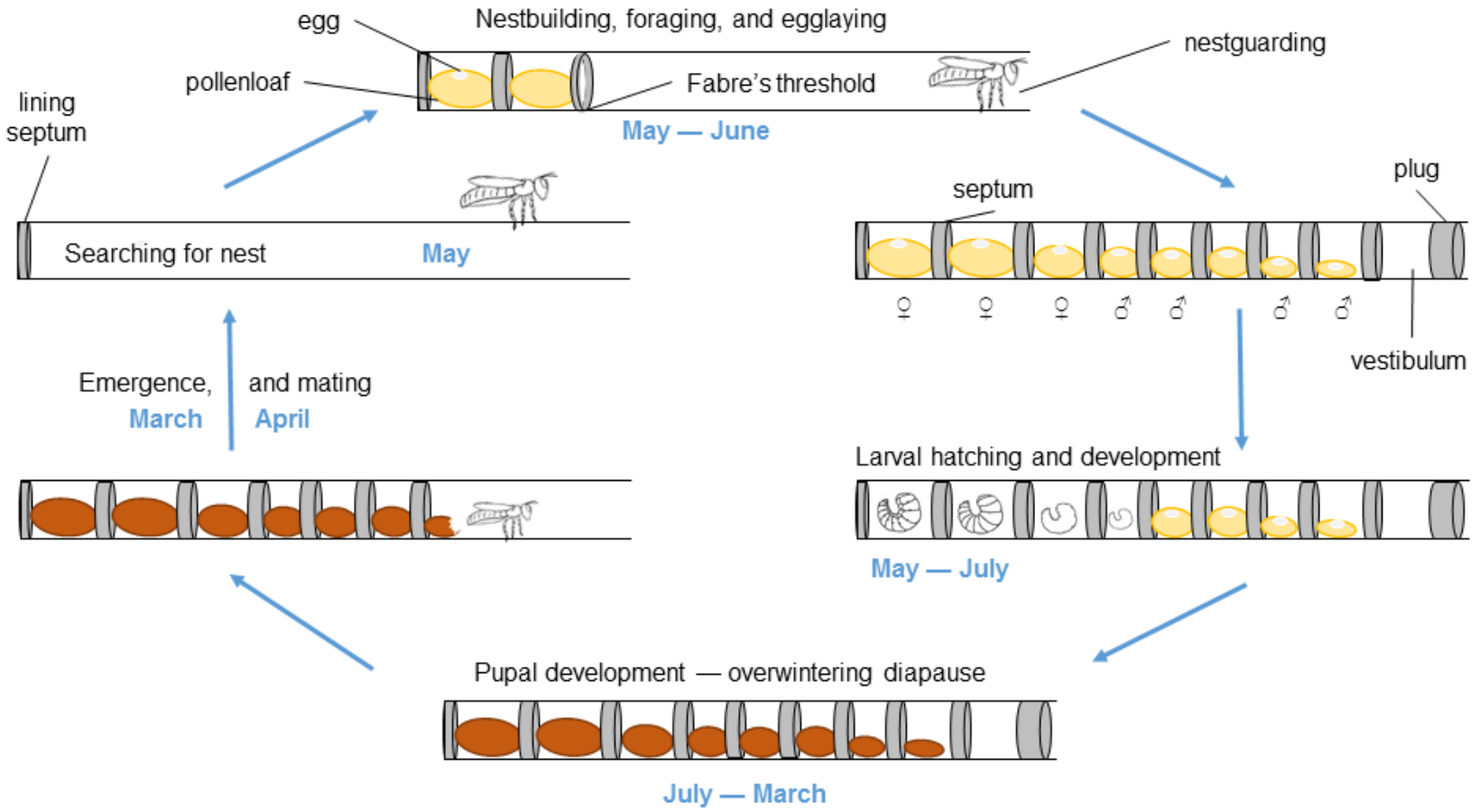

Figure 3.1: Life cycle of *O. bicornis*. Images are illustrative only. For a more detailed diagram see Seidelmann and Rolke (2019; specifically Fig. 7 therein), and the work of Radmacher and Strohm (2010, 2011) for specifics on larval development.

3.2.2 Previous work

To attempt a laboratory set-up, I contacted various people in the community working on and with *O. bicornis*. These personal communications (PC) are provided as supplementary material (Appendix A) and may be referred to throughout this chapter.

As mentioned earlier, only three studies have managed to achieve egg laying in a laboratory set-up to date (Raw, 1972; van der Steen, 1997; Sandrock *et al.*, 2014). Raw (1972) managed to get nesting in an insectary '*simulating natural conditions*' (paraphrased). Further descriptions of the insectary are lacking, but given the dimensions it is presumed to have been inside a greenhouse. Within the insectary he maintained an additional three cages with one individual in each. The egg laying success of these individually caged bees was not reported on, but females in the insectary managed to lay eighteen eggs on average (Raw, 1972). A different approach was used by van der Steen (1997), whom provided *O. bicornis* with gelatin capsules filled with a pollen paste. Additionally, van der Steen (1997) provided very fine and dry pollen, which females used to dust the pollen paste, after which they laid their eggs upon it (PC van der Steen). Out of 263 females, 131 females were actively flying around, and 113 capsules contained eggs (reported as 0.9 eggs per active female, over a period of three weeks: van der Steen, 1997). In contrast, Sandrock *et al.* (2014) managed to get females completing whole nest-tubes in their experiment. For treatment groups of 125 females each, they managed to get 151 completed nests in neonicotinoid treated females, and 194 completed nests in their control group. Considering a median of four eggs per nest tube (as in Fig. 3 in Sandrock *et al.*, 2014), this equates to roughly 6.2 eggs laid per female in their control group, over a period of four weeks.

While Raw (1972) attained the highest reproductive success (18 egg/female) of these three studies, the set-up of Sandrock *et al.* (2014) was trialled here. This given the level of detail provided in the study, the lack of an insectary at my disposal (as in Raw, 1972), and the fact that it provided better and more consistent egg-laying than the van der Steen (1997) study. Indeed, the Sandrock *et al.* (2014) study demonstrated the feasibility of studying reproductive success with their method. Hence I mainly attempted to replicate their results, though van der Steen's (1997) method was also briefly trialled.

3.3 Methods

3.3.1 Factors underlying species ecology

Tables 3.1 and 3.2 summarise the various aspects of the ecology of *O. bicornis* relevant to establishing the species in the laboratory. Even under the naive assumption that there are only nine dichotomous and relevant aspects to be investigated, it would take considerable time testing each individually whilst keeping all other components constant. Given that few of these ecological aspects are actually dichotomous (e.g. the complexity of natural light alone: dominant wavelength, polarisation[20] and intensity all change throughout the day), and given that *O. bicornis* further possesses an annual life cycle with a limited flight season (Raw, 1972); tackling each aspect of its ecology separately was deemed unfeasible. Furthermore, as long as females did not display certain (nesting) behaviours that could be consistently recorded and/or measured, there is a reduced capacity for discerning preferences.

Therefore several trials (mainly 2016-2017) were performed in sequence, and more than one aspect was changed between these trials based on limited behavioural observations, as well as literature reviewed. Changes to the setup were sometimes even made during the trials due to the constraint of time. The trials and their set-ups were focused on those aspects of Table 3.1 and 3.2 that were within my control. The setup of the individual trials is summarised in Table 3.3, and an example of the last setup used is depicted in Figure 3.2. Trials mainly consisted of providing variations on the following themes: pollen and how it is presented, sugar solutions and how they were presented, the provision of different nesting materials (mud and nesting tubes), cage dimensions, light sources and light dark cycles. For all these trials and further laboratory experiments in later chapters, *O. bicornis* were obtained as cocoons from a commercial supplier (Dr Schubert plant breeding; Landsberg, Germany).

[20] E.g. the Rayleigh sky model with changing degrees and angles of polarisation.

Table 3.1: Summary of relevant ecological aspects of *O. bicornis*.

Ecological aspects			Notes
Foraging	Pollen	Presentation	Colour, odour and other floral traits (Proctor and Yeo, 1973)
		Preference	Polylectic but prefers *Quercus*/*Ranunculus* (Tasei, 1973; Raw, 1974; Teper, 2007); Quality — e.g. trace elements (Radmacher and Strohm, 2010; Filipiak, 2019)
	Nectar	Presentation	Colour, odour, nectar guides and other floral traits (Proctor and Yeo, 1973)
		Composition & concentrations	Sucrose, glucose, fructose, amino acids, and vitamins (Haydak and Palmer, 1942; Degroot, 1953; Proctor and Yeo, 1973; Zahra and Tallal, 2008; Geister *et al.*, 2008; Costa and Venturieri, 2009)
Nesting	Substrate	Material	Reed, cardboard, wood, straws, and styrofoam (Raw, 1972; Strohm *et al.*, 2002; Wilkaniec and Giejdasz, 2003)
		Diameter	$\bar{x}$ = 8 mm (Wilkaniec, 1998; Ivanov, 2006; Seidelmann *et al.*, 2016)
		Length	$\bar{x}$ = 23 cm (Wilkaniec, 1998; Ivanov, 2006; Seidelmann *et al.*, 2016)
		Orientation	Sheltered sunny spots, SE or SW facing, 75cm high (O'Toole, 2000)
		Presentation	Attracted to brick or mud coloured exterior (O'Toole, 2000)
	Masonry	Composition	Silt, clay and silica (Sandrock *et al.*, 2014)
		Wetness	Gradient (Sandrock *et al.*, 2014)

Table 3.2: Summary of relevant ecological aspects of *O. bicornis* (*continued*).

Ecological aspects			Notes
Environment	Space	Cage dimension	40*40*40 cm — 4.3*2.4*1.8 m (van der Steen, 1997; Sandrock *et al.*, 2014)
		Density	Individuals in flight cage affects survival (Fliszkiewicz *et al.*, 2013)
	Humidity	RH	80-90% RH for larval development (i.e. in nest; van der Steen, 1997)
		Graduated	Sandrock *et al.* (2014); PC van der Steen
	Light	Graduated	Gradual fluctuation (Sandrock *et al.*, 2014) sets and maintains circadian rhythm post eclosion (Beer *et al.*, 2019); daytime length sets nest construction hours (Seidelmann, 2006)
		Quality	Daylight is essential (PC van der Steen); polarisation underlies navigation (Von Frisch, 1954; Mazokhin-Porshniakov, 1969; Wehner, 1984); composition and intensity affect phototaxis (Chen *et al.*, 2012)
	Temperature	Temperature	≥18°C for nest construction (Seidelmann, 2006); >12°C for emergence (Raw, 1972)
		Cyclicity	Pre-emergence circadian rhythm is synchronised through temperature fluctuation (Beer *et al.*, 2019), facilitating morning emergence
	Social	Mating	1:2 — 1:3 under caged conditions (male : female; Fliszkiewicz *et al.*, 2013)

Table 3.3: Overview of the set-up of each trial.

Trial	T1	T2	T3	T4	T5	T6	T7	T8
Days	28	29	22	17	15	55	33	24
Pollen[a]	Petri dish	50 ml Falcon	MCT; C	MCT; C	MCT; C	MCT; C	MCT; C	MCT
Protein[b]	Suppl; plant	Suppl; plant	Suppl	Suppl	Suppl	Suppl; PL	NA	NA
Sugar[c]	50 ml Falcon 1:1 & 2:1 S; 1:1 & 2:1 SGF	50 ml Falcon 1:1 & 2:1 S; 1:1 & 2:1 SGF	MCT; N; 1:1 & 2:1 S; 1:1 & 2:1 SGF	MCT; N; 1:1 & 2:1 S; 1:1 & 2:1 SGF	MCT; N; 1:1 & 2:1 S^{B}; 1:1 & 2:1 SGF	MCT; AA; B_2-B_5-B_9; 1:1 SGF	PCRT 1:1 S^{FS}	PCR 1:1 S
Nest[d]	Reeds; NB	Reeds; NB	Card	Card	Card	Card	Card	Car
Mud[e]	70/30; 60/40; 50/50; 40/60	70/30; 60/40; 50/50; 40/60	70/30; loam	70/30	70/30; natural mud	70/30	70/30	70/3
Cage (cm)	60·60·90	60·60·90	60·60·90	60·60·90	60·60·90	65·90·140	65·90·140	65·90·
Cocoons used	8	13	40	31	32	55	82	24
Light[f]	S/H	S/H	S/H	S/H	S/H	TL-D	TL-D	TL-
L:D hrs	13:11	13:11	13:11	14:10	14:10	18:6	18:6	18:
Temp.[g] (°C)	16-17; IL	>18; HM	>18	>18	>18; CT	21-23; CT	21-23	21-2

(a) Ground *A. mellifera* pollen pellets (Wholesome Bee Pollen, Livemore; were ground using Delonghi coffee grinder). Cut 50ml Falcon tubes or micro centrifuge tubes (MCT) without lid were decorated with makeshift flower petals (Premium photo paper, satin; UV reflecting) and coloured; these artificial flowers had pollen either: loose in the container (T1-4), on ruffled cotton bulbs (T5-6) or on pipecleaner (T7-8 Artificial catkins (AC) were made of pieces of knotted string and immersed in ground pollen (to exploit *Quercus* preference; Raw, 1974; Teper, 2007). Essential oils (Miaroma Geranium; and Tisserand Lave as attractants for the artificial flowers (Proctor and Yeo, 1973; T6-8). (b) Honey bee supplementary feed (= Suppl; Candipolline Gold; sterilised and contains sugar/protein/vitamins) was often added to trials Petri dish. Fresh and available flowers, potted (*Vicia faba*) or cut (*Ranunculus acris*), were also tried (T1-2; PC Raw). PL = pollen loaves consisting of either Candipolline Gold or a 50:50 mix of sucrose and were placed in open gelatin capsules (00; following van der Steen, 1997; T6). (c) Sugar solutions were presented using 50ml Falcon tubes with holes, decorated with UV reflecting paper (Premium photo pa satin), and possessed cardboard landing zones (as in Sandrock *et al.*, 2014; T1-2). Micro centrifuge tubes (MCT) without lids were also used (T3-6), because they were easier to maintain (e.g. autoclavable decorated with makeshift flower petals. Finally (T7-8), PCR tubes were used (avoids bees drowning and facilitates treatments), without decorations since bees showed no preferences. 2:1 or 1:1 (sugar : dis water) mixtures were mainly tried, using commercial sugar (S = sucrose; Fairtrade white granulated sugar by Sainsbury's), sugar mix (SGF; 33% of sucrose, D-glucose and D-(-)fructose each; Proctor and Y 1973), or Agave nectar (N; the Groovy food company). S^{FS} = filter-sterilised solution (0.22 µm; Millipore) and S^{B} = boiled solution (30 min to invert sugars and kill bacteria). Vitamins (B_2-B_5-B_9) were also trie mg/l folic acid, 0.01 mg/l D-pantothenic acid and 1.1 mg/l riboflavin; Pearson and Burgin, 1941; Haydak and Palmer, 1942; Pearson, 1942; Zahra and Tallal, 2008; Geister *et al.*, 2008; Costa and Venturieri, as well as amino acids (0.500g/l hydrolysed Casein and 0.100g/l tryptophan; Mueller and Johnson, 1941; Groot, 1953). (d) Nest substrates used were reeds, observation nest box (NB ;Nurturing nature Ltd. Fabre's hives containing cardboard tubes with paper linings (Card; Oxford bee company Ltd.). (e) % clay/silica mixtures (Sandrock *et al.*, 2014). Using fine calcium bentonite powder (Fuller's earth, Intralabs natural white silica sand (Cristoballite™). Additionally, natural mud from a river bank in Leeds, and loam (Wilco, Graded top soil) were trialled. (f) Light sources used: sodium/halide (S/H; 230-240V ~AC 50 1.25 amps; Maxibright; T1-5) and fluorescent (TL-D 58W/865, Phillips, 6500K white light, 150cm; T6-8; in a graduated cycle). (g) Temperature (along with humidity) could not really be controlled in the lab. I efforts were made to increase heat (e.g. IL = infrared lamp, Exoterra, 50W; and HM = heat map) and smaller cages trialled in constant temperature rooms (CT; 20 and 25°C). But temperature was higher an more stable T6-8 when moving to different lab that was made accessible.

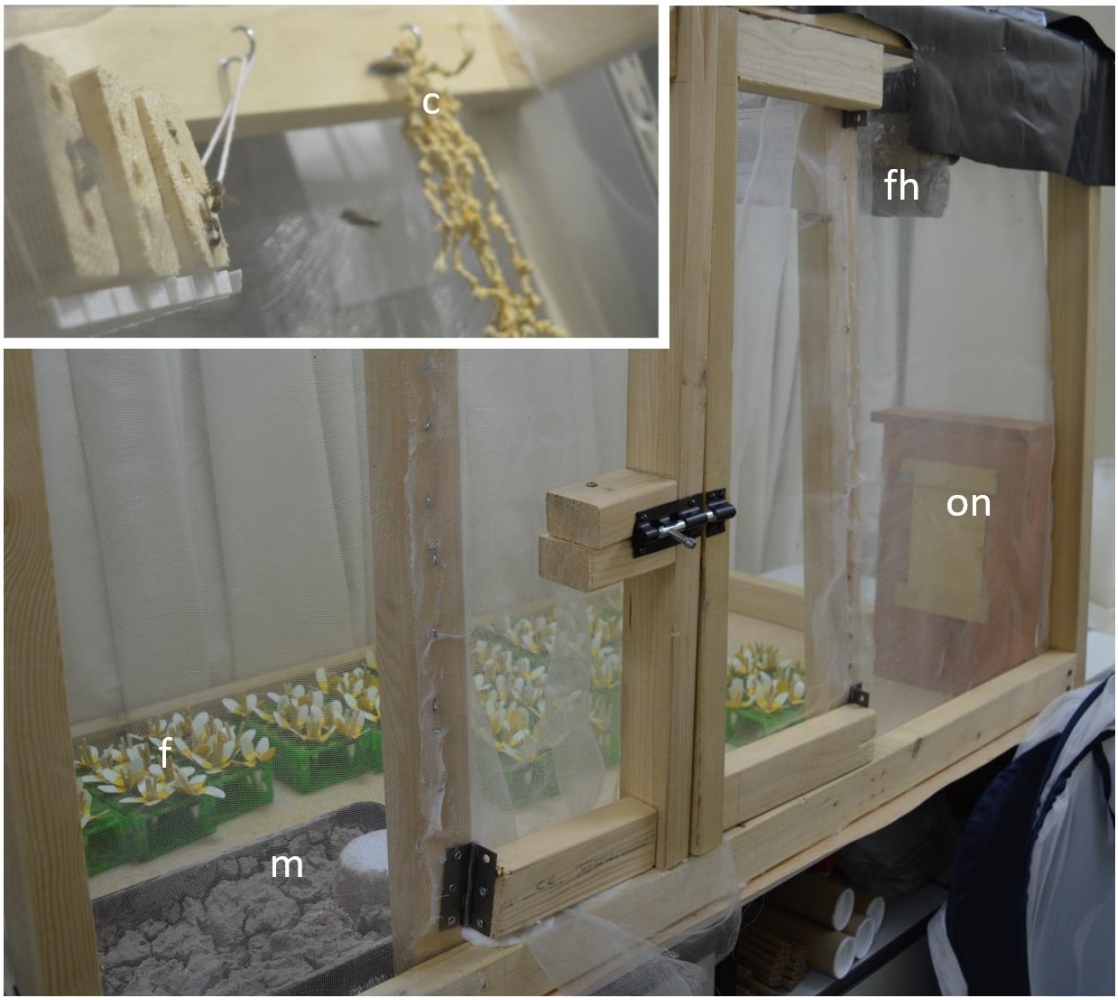

Figure 3.2: Example of setup in trials 7 and 8. c = artificial catkin, f = artificial flowers, fh = Fabre's hive, on = observation nest box, m = 70% clay 30% sillica mud.

3.3.2 Measurements

Nesting activity was quantified by counting the number of terminal plugs in nests, the number of Fabre's thresholds (Figure 3.1), the number of provisions (pollen breads or loafs; both with and without oviposited eggs), the number underdeveloped cocoons, and the number of developed adults per trial.

During all trials, cocoons were initially sexed based on both weight, size and shape of the cocoon. The heaviest cocoons tend to contain females, additionally possessing more rounded shaped cocoons. During two trials (T7-8), cocoons were hatched individually and cocoon weight could be linked to adult sex directly (adults can be sexed easily, as males have a white tuft on the frons; whereas females possess two horns)[21]. During these same two trials, cocoons

[21] The name, *O. bicornis*, refers to the double horns of the females. From the Latin stems: *bis* = twice, and *cornu* = horned.

were checked upon twice a day (09:00 and 19:00), to estimate the time it took from being placed at RT until eclosion from the cocoon (activation time). If cocoons had not hatched after a two week period, they were considered deceased, and were cut open to check for parasitism. Uneclosed but intact individuals were counted as pupal mortality, parasitized cocoons were disregarded from analysis. Both the time until eclosion dataset, as well as mortality dataset (or reversely: the pupal/diapause survival data) were extended with the data from van der Steen and Ruiter (1991) for analysis.

Behavioural observations were carried out for preferences in the presentation, concentration and composition of sugar solutions (T1-3). Sugar solution visitation data lacked rigid experimental design, as it was an exploratory study. Consequently, the data was explored graphically, using factor analysis for mixed data (Chavent *et al.*, 2014). Qualitative/categorical factors were the colour of the petals, the background colour (microtube rack), sugar composition (sucrose or a 1:1:1 mix of fructose:glucose:sucrose), and the presentation method used (50 ml Falcon tube or microcentrifuge tubes). Quantitative data used were the total number of bee visits, the number of petals used, and the sugar concentration.

Behavioural observations on the preferences in the presentation of pollen on artificial flowers with differing colour combinations, and the presentation of pollen on articiial catkins were carried out during trial 7. For the artificial flowers, four petals were used for the sake of consistency, and common colour combinations were tried (Proctor and Yeo, 1973). Coloured paper was used to avoid toxicity associated with unevaporated solvents (Dafni et al., 2005), and UV reflection was simulated using photo paper (Premium photo paper, satin) to which colours were be added using fluorescent markers. In retrospect, since UV is part of bee colour vision, adding colour to the photo paper complicated analysis. Additionally, UV reflection was likely minimal due to the lighting used (even though fluorescent lighting creates UV: Maxwell and Elwood, 1983; it is generally blocked of using filters). Therefore, the use of UV reflecting photo paper was treated as a separate factor in analysis.

Behavioural observations on the preference of mud were carried out during all trials, whenever this aspect of nesting behaviour could be observed. Clay:silica mixtures were presented in a large black tray, supplied with a wick that was kept moist by a container of water (bottom left of Figure 3.2). The water was covered in mesh to prevent bees drowning.

3.3.3 Statistical analysis

All statistical analysis was carried out using R 3.5.1 (R Core Team, 2016). Non-normal weight and sexing data was analysed using Wilcoxon's rank sum test. Linear (emergence data) and generalised linear models (diapause/pupal survival and pollen floral resource data; using quasibinomial and quasipoisson distributions respectively) were constructed. Assumptions were investigated following Zuur *et al.* (2010). Analysis of variance was performed on linear models, and generalised linear models were tested through model comparison Bates *et al.* (2015). Factors of generalised linear models were tested likelihood ratio tests (Whittingham *et al.*, 2006; Mundry and Nunn, 2008; Forstmeier and Schielzeth, 2011; Bates *et al.*, 2015), leaving out interaction effects only where appropriate (Engqvist, 2005). Sugar solution preference data was explored using multivariate analysis of mixed data (PCAmixdata; Chavent *et al.*, 2014). Abbreviations in the text and figures are as follows: $\bar{x}$ refers to the mean, s refers to the standard error of the sample, and σ refers to standard error of the population.

3.4 Results and discussion

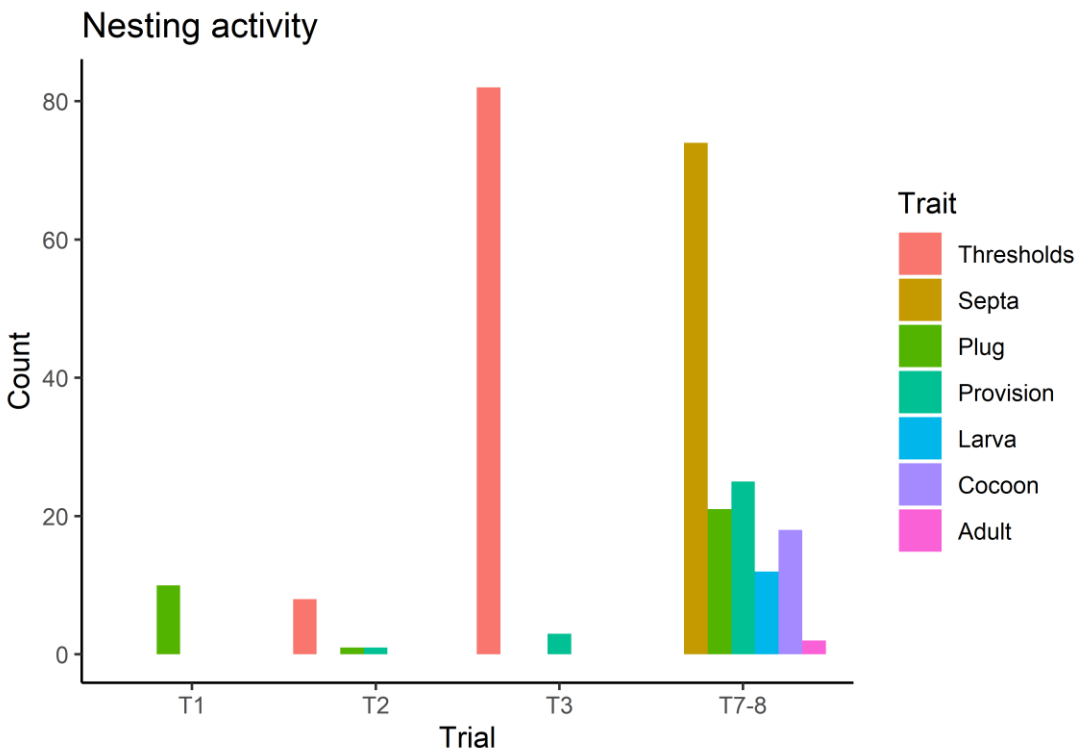

Figure 3.3: Nesting activity of *O. bicornis* females during trials. Counts indicating nesting activity of the overall trial are presented in sequence. Masonry: thresholds = Fabre's thresholds (outline future septa), septa = cell walls, plug = terminal septum. Provision = the presence of a pollen loaf (usually containing at least an egg outline). Development: larva = developing or petrified larvae, cocoon = undeveloped cocoon, adult = cocoon with a pharate. Note: trials T4-5 were arrested prematurely due to exceptionally low activity; T7-8 were pooled since nest tubes were not swapped out.

Figure 3.3 shows the increase in nesting activity throughout the trials. Masonry was easily elicited within the laboratory. Oddly, in T1, many reeds were plugged off with mud, without any other sign of cells being built. In T2 mud was provided with variations in sand and clay composition, resulting in Fabre's thresholds appearing. In T3 the use of artificial catkins (Figure 3.4) elicited the first provisioning and egg laying in a female. Nesting activity seen in T7-8, corresponds to several changes, mainly: a switch in laboratory with a higher and more consistent ambient temperature, diffuse and partially graduated lighting, use of a larger cage, and an increase of floral and nesting resources. In what follows, preferences on the basis of behavioural observations are presented and discussed. It should be noted that in any one trial; no more than five females were active at any one time. Additionally, individual marking was abandoned to avoid chilling (Giejdasz *et al.*, 2016) and handling stress. Consequently, statistical tests shown are naive, without a repeated measurements structure. Sample sizes are thus inflated, and tests should be treated with circumspection.

Figure 3.4: Example of artificial catkins and flowers used.

3.4.1 Sex, diapause and emergence

O. bicornis can generally be sexed from the size or weight of their cocoons, because females are provisioned more than are males (Raw, 1972; Seidelmann, 1995; O'Toole, 2000; Ivanov, 2006). This facilitates control over sex ratio when setting out cocoons for emergence in for instance greenhouses or a laboratory environments (e.g.: van der Steen and Ruiter, 1991,

approximated a 1:1 sex ratio by selecting 8 and 10 mm cocoons). Similarly, I found that females were on average 37.52 mg heavier than males (Wilcoxon's rank test: W = 4307, p < 0.001; Figure 3.5). Notwithstanding, female *O. bicornis* in particular, showed a greater variance in weight ($\bar{x}_f$ = 118.9 mg, s_f = 22.25; and $\bar{x}_m$ = 81.35 mg, s_m = 14.28). Seidelmann *et al.* (2010) similarly reported almost twice as much variance in birthweight for females ($\bar{x}_f$ = 108.4 mg, σ_f = 23.96) as opposed to males ($\bar{x}_m$ = 64.07 mg, σ_m = 13.88). This may be down to the strong correlation between the weight of a provisioning mother to her daughter (Seidelmann *et al.*, 2010). Especially since provisioning mothers at either extreme of the weight distribution show distinctly more variance in provisioning for their daughters (see heavy tailed distribution for females in Fig.3 in Seidelmann *et al.*, 2010, specifically the wider standard deviations around tails).

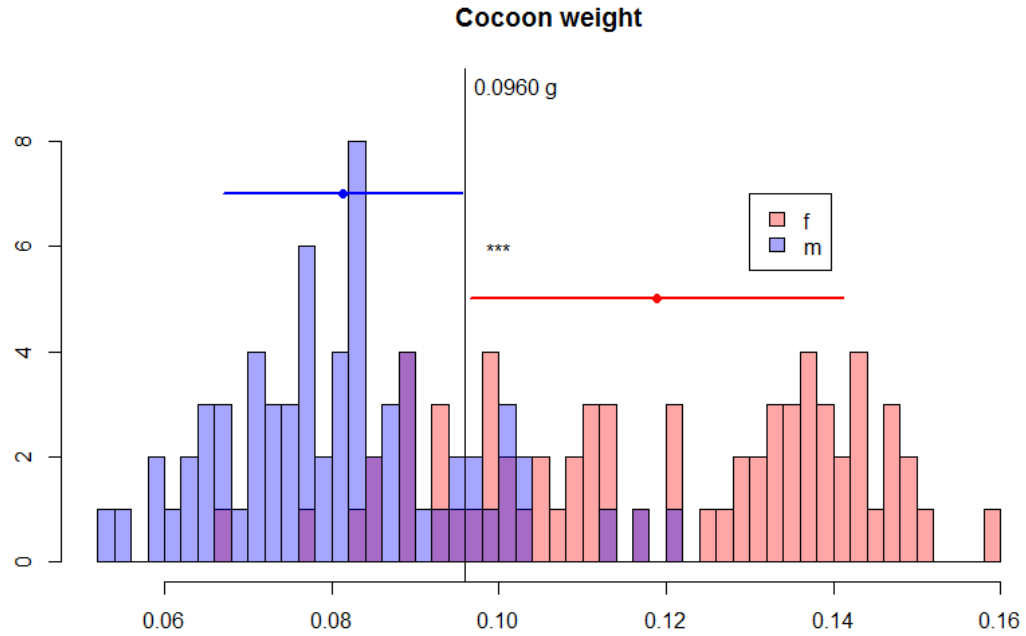

Figure 3.5: Sex-specific cocoon weight. Weight distribution (g) of male (m) and female (f) cocoons. Horizontal lines are standard deviations, and points are means which differed significantly (Wilcoxon's: W = 4307, p < 0.001). Female weight was non-normal (Shapiro-Wilk's: W = 0.952, p = 0.010) and females displayed a significantly more variance (Levene's: $F_{1,135}$ = 25.07, p < 0.001). The black vertical line shows an approximate cut-off point for sexing cocoons in practice.

Further sources of variance in weight are attributable to seasonal variation (Ivanov, 2006; Seidelmann *et al.*, 2010), errors in the fertilisation of eggs (Raw and O'Toole, 1979), and the cleptoparasite *Cacoxenus indagator* which usually results in the starvation of the larva but can in cases simply lead

to underweight adults developing (Raw, 1972). Consequently, care should be taken when sexing by weight. Individual hatching might be considered, since individuals can be sexed after hatching, and female virginity can be assured if needed (e.g. Chapter 5). Do note that females are most attractive to males when newly eclosed, and lose their attractiveness within three days (Seidelmann, 2014a).

Furthermore, diapause and emergence have been studied extensively in this species (van der Steen and Ruiter, 1991; Wasielewski *et al.*, 2011a; Wasielewski *et al.*, 2011b; Dmochowska *et al.*, 2012, 2013; Fliszkiewicz *et al.*, 2012a; Giejdasz and Fliszkiewicz, 2016; Schenk *et al.*, 2018a; Schenk *et al.*, 2018b; Beer *et al.*, 2019) and other *Osmia* (Bosch and Kemp, 2004; Krunić and Stanisavljević, 2006). This mainly with a view to pollination in greenhouses and orchards. A mismatch between bee emergence and inflorescences could for instance lead to bees emigrating from the orchard they were set out in (foraging range 500-600m: Rathjen, 1994; Gathmann, 1998; Gathmann and Tscharntke, 2002; Everaars *et al.*, 2011), or bees could simply starve under greenhouse or semi-field conditions (e.g. Schenk *et al.*, 2018a).

The heavier females take longer to eclose from their cocoons than do the lighter males. In line with Holm (1974) I found that this effect is not likely to be driven by weight itself and is purely sex-specific (when tracking individual females: $F_{1,30} = 2.819$, $p = 0.103$; and males: $F_{1,28} = 0.651$, $p = 0.427$; Figure 3.6A). The main factors driving emergence time seem to be sex and days spent in diapause (Holm, 1974; van der Steen and Ruiter, 1991). Extending the dataset from van der Steen and Ruiter (1991) with my own, I found no interaction effect between time spent in diapause and sex ($F_{1,30} = 1.351$, $p = 0.254$), and can only corroborate the independent effects of sex ($F_{1,30} = 7.011$, $p = 0.013$) and diapause length ($F_{1,30} = 78.79$, $p < 0.001$; Figure 3.6B). When extending van der Steen and Ruiter's (1991) pupal survival data with my own, I could likewise not find an interaction effect of sex and diapause length ($\chi^2_{1,30} = 1.6644$, $p = 0.825$), nor an effect of the study ($\chi^2_{1,31} = 1.524$, $p = 0.829$), nor of sex ($\chi^2_{1,31} = 48.14$, $p = 0.224$), nor of diapause length ($\chi^2_{1,31} = 61.51$, $p = 0.169$) on pupal survival (i.e. proportion eclosed; Figure 3.6B). The pupal survival data, like the emergence time data, is largely in agreement with my own, apart from a plummet in diapause survival at 192 and 210 days in the van der Steen and Ruiter (1991) dataset (these were their final datapoints). When combining this last dataset, I made the assumption that their sexing of cocoons by length (8 mm = male and 10 mm = female) was correct since sex specific eclosion was not reported. Yet, the overall emergence was likewise low (30 and 48% respectively), and no increased levels of parasitism were recorded for these

cocoons (1 and 4% respectively). Without these outliers — which cannot be explained away and therefore cannot be removed — there may have been an effect of time. Additionally, since the diapause survival data here extends beyond 210 days, male survival can also be seen to trail off before that of females (Figure 3.6B).

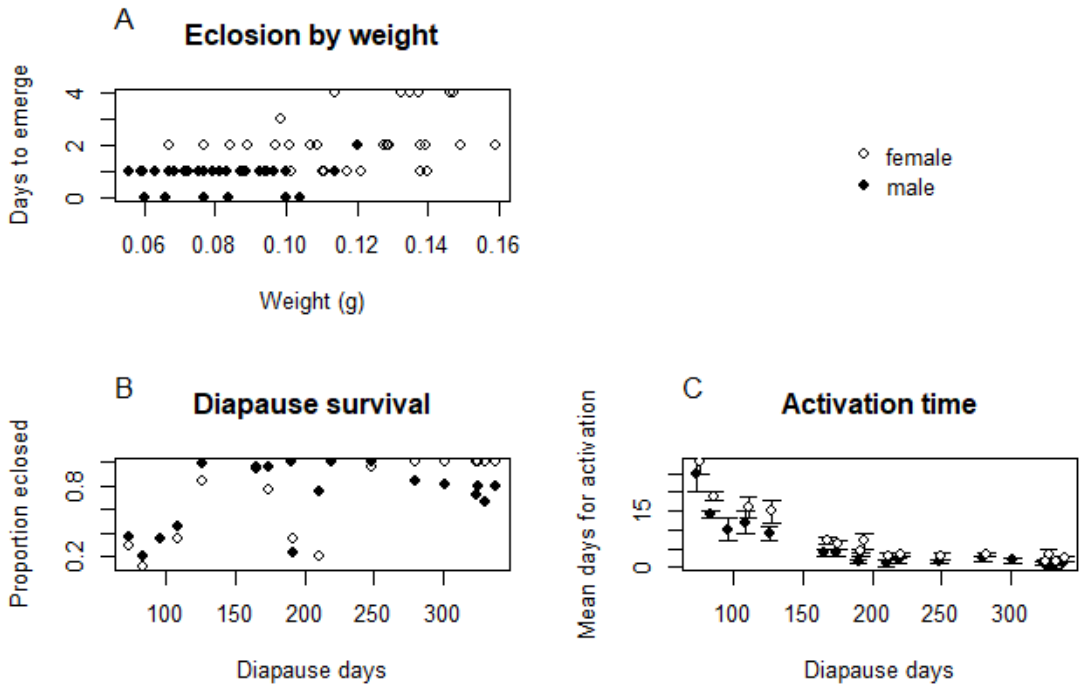

Figure 3.6: Sex specific diapause and emergence. A) individual weight did not explain days to emergence in females ($F_{1,30} = 2.819$, $p = 0.103$), nor males ($F_{1,28} = 0.651$, $p = 0.427$). B) combined data from this study and van der Steen and Ruiter (1991) showing time to eclosion in relation to the length of diapause (error bars = s; female points were shifted right by two days to avoid overlap). C) combined data from this study and van der Steen and Ruiter (1991) showing survival with diapause length. The proportion eclosed data van der Steen and Ruiter (1991) was assumed to have even sex ratios. Survival plummeted around 200 diapause days in van der Steen and Ruiter (1991), contrary to the present study. Diapause days = days from November 9 (as in van der Steen and Ruiter, 1991).

Overall, the individuals used here, could diapause late into summer when kept at 5°C. It should be noted that, as reported in van der Steen and Ruiter (1991), females diapausing for such a long period also suffered increased mortality and decreased vitality. It is for this reason that an increasing amount of cocoons was often set-out for trials that occurred later in the year (Table 3.3). Finally, the duration of the summer dormancy (pre-winter diapause) affects diapause in *O. cornuta* and *O. lignaria* (Sgolastra *et al.*, 2010). Hence, if full life

cycles are attained in a laboratory environment, more control could be exerted over diapause. For instance, placing developing brood at fluctuating temperatures accelerates development, and using lower temperatures should reduce adult mortality (Radmacher and Strohm, 2011).

3.4.2 Foraging

With regard to floral resources, Raw (PC) stressed using freshly cut flowers, while Sandrock (PC) initially planned for the use of potted poppies which the bees ended up destroying the plants by biting/cutting them. Since *O. bicornis* is polylectic (Tasei, 1973; Raw, 1974; Teper, 2007), *Vicia faba* (T1) and cut *Ranunculus acris* (T2; *O. bicornis* prefers *Ranunculus* Table 3.1) were trialled, but the bees showed little interest. This may have been due to other conditions in those initial trials. But their use was also considered impractical, since *O. bicornis* also tend to aquire pollen and nectar from separate species (Tasei, 1973). Hence, all further trials maintained artificial floral resources exclusively.

Sugar solutions easily attracted the interest of females. Record was made of visits to sugar resources during three trials (T1-3; with different choices available). Visits were infrequent, and bees mostly frequented the same sugar resources. Given the sparsity of data and lack of experimental design, no tests were performed and an exploratory technique was used (factor analysis for mixed data; FAMD; Figure 3.7A,B). The floral visits seemed to mostly correspond to sugar composition, with most total visits in favour of sucrose (S) only solutions as opposed to fructose/glucose/sucrose solutions (FGS; note that both types of solutions were always offered in equal measure in all early trials; Figure 3.7C).

The number of visits with regard to the number of flower petals also stood out (Figure 3.7D; although the number of petals on display was not consistent among trials). Tasei (1973) recorded nectar visits to: *Pulmonaria sp.* (5 fused petals), *Glechoma hederacea* (6 fused and modified purple petals), *Ajuga reptans* (6 fused and modified blue-purple petals), *Onobrychis sativa* (4 fused and modified pink petals), *Medicago sativa* (4 fused and modified purple petals), and *Hyacinthoides non-scripta* (formerly *Endymion nutans*; 5 fused and modified blue-purple petals). Given those recordings, and the data here, 4-6 blue-purple flower petals seem evident to make sugar solutions more attractive. Another method that has been suggested to help feed bees treatments involves simply placing ampules inside flowers (Ladurner *et al.*, 2003).

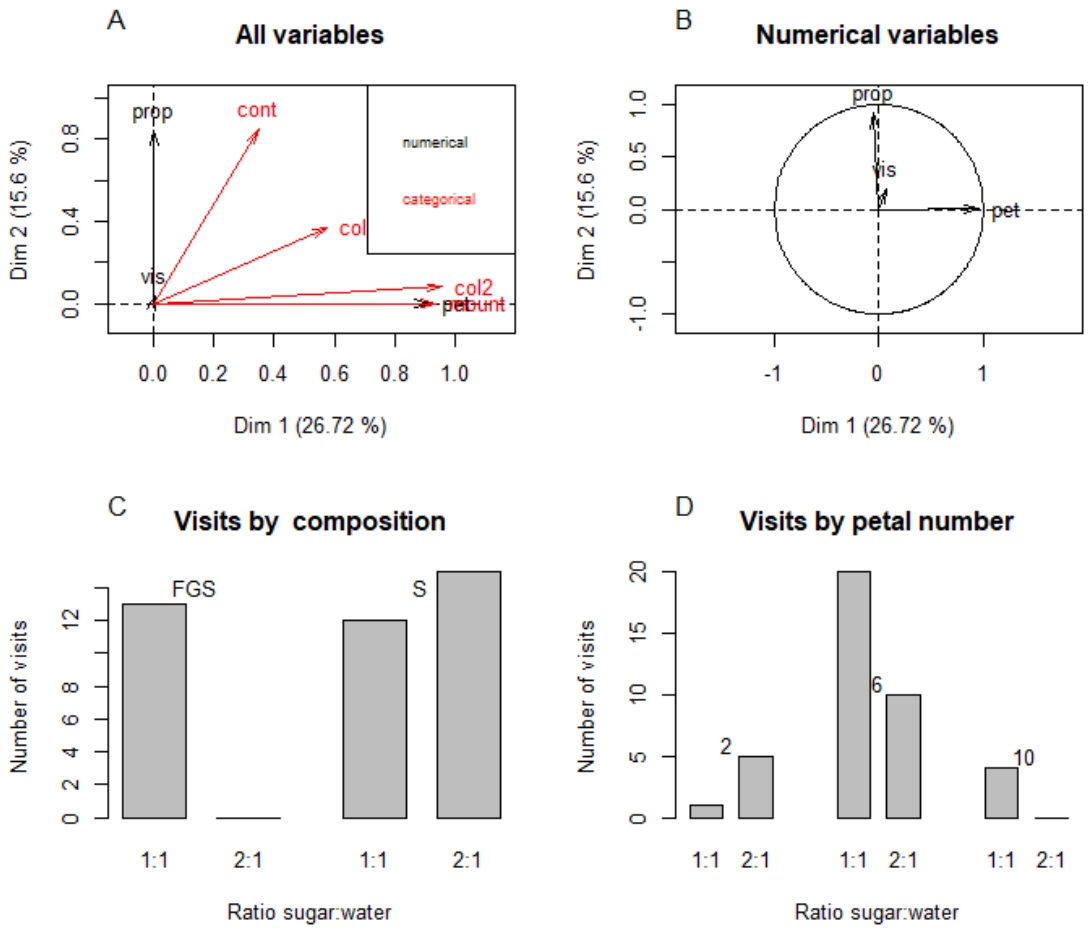

Figure 3.7: Factor analysis for sugar solutions. A) The number of visitations (vis) shows little variance (limited dataset); but seems to correlate mostly to the sugar concentration (prop; numerical) and sugar composition/content (cont; categorical; sucrose only versus fructose:glucose:sucrose). Colour (col and col2) seemed to matter little with regard to bee preference, nor did the mount (50ml Falcon tube versus microcentrifuge tube), nor the number of petals (pet) used. Do note that this is based on a limited dataset. B) Regarding the numerical variables separately, further illustrates the importance of sugar concentration (prop) over petal number (pet) with regard to preference (vis; number of visits). C) The sucrose only composition (S) of sugar solution appeared to be the most robust over concentrations. The composite sugar solution (FGS; fructose:glucose:sucrose) at high concentrations (2:1) seemed particularly ill-favoured. D) The petal numbers used, when regarded separately did seem to have had some effect on preference. Makeshift flowers with six petals were favoured, regardless of their respective sugar concentration (1:1 or 2:1).

Finally, thermocycling tubes were used without further stimuli. Since, regardless of their level of nesting activity, bees survived and always had sucrose solution in their crop upon dissection. Indicating that bees had no trouble locating sugar resources in the flight cage. Further considerations were again mostly practical. PCR tubes are ideal since the smaller volumes can save

on costly chemicals in treatment groups (sugar solutions go off after roughly two days), bees would often drown in open microcentrifuge tubes (especially when not filled regularly, even though *Osmia* are long tongued: Proctor and Yeo, 1973), and falcon tubes needed to be suspended (clamp stand) and suffered spillage. During T3, an embryo was found, yet it was found to be developing abnormally (observation Dr E. Duncan), hence boiled sucrose solution was briefly trialled, but not favoured by bees (boiling inverts sugars, but also forms hydroxymethylfurfural, which is toxic to bees). Agave nectar was also briefly trialled but not favoured. Finally, 50% (or '1:1') filter sterilised sucrose solution (as is the case in nectar: Schoonhoven *et al.*, 2005; 0.6148 mg/µl sugar: Dafni *et al.*, 2005; stored frozen) was used in trials T7-8 where full development to adults was achieved. Overall, presenting sugar resources was robust, and no amino acids or vitamins (T6) needed to be added to induce egg-laying (proline, glycine and phenylalanine in nectar are attractive: Nicolson, 2011). *O. bicornis* likely retrieves nutrients and trace elements through pollen (pollen consist of 15-60% protein and other essential elements: Schoonhoven *et al.*, 2005), as is the case in *Osmia californica* (Cane, 2016). The eliciting of pollen collection was further considered the more limiting step towards achieving nesting and oviposition in a laboratory environment (the majority of nesting activity comprises mud and pollen collection: Raw, 1972).

For practical purposes, *Apis* pollen pellets (Wholesome Bee Pollen, Livemore) were ground using a coffee grinder, and presented on artificial floral resources. This not only provides more control, but the amount of pollen needed for finishing a single pollen loaf is substantial, and it follows that the number of cut or potted flowers needed should be substantial (PC Sandrock). The fineness and dryness of the pollen seemed important as suggested by van der Steen (PC), and pollen pellets need to be ground using a coffee grinder for a at least five minutes (mortar and pestle were insufficient). This is not only necessary for females to be able to collect the pollen, since pollen is compacted somewhat to stick in-between scopa (O'Toole, 2000; and hairs are unbranched: Proctor and Yeo, 1973), but just before ovipositing the pollen loaf is also dusted with a layer of very fine pollen (van der Steen, 1997). The quality of pollen — the presence of trace elements in pollen differs between plant species for instance— might also be important with regard to larval diet (Filipiak, 2019), and larval development by extension (Filipiak, 2019).

With the use of unsterilized ground *Apis* pollen pellets I managed to acquire fully developed adults (T7-8). It should be noted that commercial *Apis*

pollen pellets may vary seasonally in their composition, and no palynological[22] analyses were carried out here. *Apis* pollen pellets may furthermore contain unwanted traces of pesticides (Chauzat *et al.*, 2006; Škerl *et al.*, 2009; Bernal *et al.*, 2010; Kasiotis *et al.*, 2014; Calatayud-Vernich *et al.*, 2018). With the above considerations in mind: if *Quercus* is bountiful and in bloom (as preferred by *O. bicornis*: Tasei, 1973; Raw, 1974; Teper, 2007), pollen might easily be collected[23] by placing plastic bags over branches with inflorescences and subsequently shaking. Or by fitting a vacuum cleaner with filters of differing mesh sizes (King and Ferguson, 1991; Johnson-Brousseau and McCormick, 2004). Manually collecting pollen in this way should eliminate the need for grinding pellets and should provide finer pollen. In addition, providing *O. bicornis* with its preferred pollen resource may elicit more nesting behaviour in more females. It should further eliminate unwanted variation in pollen composition within experiments or across replicates, as well as reduce the possibility of trace pesticides. While I think this is a worthwhile avenue to pursue, possible downsides are the amount of pollen that can be acquired at any one time, and the possibility of an unbalanced larval diet (Filipiak, 2019).

From behavioural observations I mainly found that the presentation of pollen matters substantially. I was unable to replicate previous successes made with simply presenting pollen on a Petridish (van der Steen, 1997; Sandrock *et al.*, 2014). Provisioning first started when presenting artificial catkins comprised of knotted string (Figure 3.4) to exploit *O.bicornis' Quercus* preference (Tasei, 1973; Raw, 1974; Teper, 2007). A female could be seen 'abdominal drumming' (actively placing pollen on her scopa; Cane, 2016) quite quickly. When presenting pollen on these catkins[24] — as well as on flowers with stamen being mimicked by ruffled out cotton bulbs or pipecleaner[25] — it increases surface area. This prevents the clumping together of pollen, as it does in petridishes, left pollen to dry (ground pollen was stored in batches at -20°C), and made it easier to collect for the bees overall (personal observation). Dafni *et al.* (2005) states that while there is no strict need to emulate floral organs, doing so does increase the likeliness of an species to visit without training. Indeed many species require training before they visit an artificial flower (Dafni *et al.*, 2005; Ladurner *et al.*, 2005).

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[22] Referring here to the study of pollen specifically. I.e. pollen was not analysed microscopically, genomically (barcoding), nor chemically (e.g. trace elements) in the current study.

[23] *Quercus* are anemophilic species, with consequently high pollen production.

[24] Catkins were thoroughly rinsed with tap water, dried in a drying over overnight and subsequently autoclaved for reuse between trials.

[25] Pipecleaner being seemingly the most effective (personal observation).

Natural floral resources exist in a complex sensory landscape. Traditionally, vision is considered the long range attractant, whereas scent is the close ranged one (Proctor and Yeo, 1973). Essential oils (Miaroma Geranium; and Tisserand Lavender) were briefly trialled as close range attractants (T6-8). Drops of these were placed at random, at the bottom of the microcentrifuge tubes of the artificial flowers. While at times, bees may have seemed attracted to it when applying them, I cannot state it with objective certainty. Additionally, interested bees did not start foraging when investigating, nor did the addition of scent lead to an apparent increase in the overall activity of increase in the number of bees foraging. The olfactory landscape is very complex, with over 700 known compounds (Knudsen *et al.*, 1993), and odours serve not only to attract obligate visitors but exist to repel facultative ones as well (Junker and Blüthgen, 2010). Using lavender oil to test artificial flower design, Ladurner *et al.* (2005) could not find an effect of scent for their three tested species (which included *Osmia lignaria*). In fact, unaccustomed scents can prevent pollinators from visiting, rather than encouraging them (i.e. synergy of visual and olfactory cues: Proctor and Yeo, 1973; Raguso and Willis, 2002). Given this underlying complexity, standalone visual cues are more reliable and easier to test.

Table 3.4: The complexity of (bee) pollinator-plant visual cues. This table is likely not exhaustive, and further complexity is added through synergy among visual cues as well as with scent.

Visual cue	Reference
Figural intensity[a]	Proctor and Yeo (1973); Barth (1985)
Figural quality[b]	Barth (1985)
Dimensionality[c]	Proctor and Yeo (1973); Schoonhoven *et al.* (2005); Dafni *et al.* (2005)
Angle/incline	Barth (1985)
Colour (spectrum)[d]	Proctor and Yeo (1973); Barth (1985); Schoonhoven *et al.* (2005); Dafni *et al.* (2005)
Colour purity	Lunau (2000)
Colour combination[e]	Barth (1985)
Nectar guides[f]	Schoonhoven *et al.* (2005)
Symmetry	Schoonhoven *et al.* (2005)

a) contour length, edginess; compound vs primitive. b) round, square, or composite. c) 3 dimensionality and shade. d) Main colour groups = UV, yellow and blue. And colour tend to be easier to learn than shapes. e) Yellow-blue, orange-blue, yellow-purple, yellow-violet, and white with various colours are common (K. von Frisch). f) Both scented and tactile.

While more tangible, visual cues enjoy their own complexities (Table 3.4). I mainly focused on colour during my trials, since the combinations in Table 3.4 are myriad, and because colour tends to be easier to learn than shape (Schoonhoven *et al.*, 2005). Colours perceived by bees are different than those perceived by humans (Proctor and Yeo, 1973). The three main colours perceived by bees are yellow, blue and UV (Figure 3.8C; Proctor and Yeo, 1973). This is also the case for *O. bicornis*, who's green receptor is closer to yellow than it is in other insects (peak sensitivity: UV receptor = 348nm, blue receptor = 436nm, and green receptor= 572nm; Menzel *et al.*, 1988). In comparison to human vision, this is mainly a shift towards the shorter wavelengths of the spectrum, and it was assumed bees could not perceive red (Proctor and Yeo, 1973), but this has been contested (Chittka and Waser, 1997). For bees, flowers that reflect all light except for UV appear coloured (while appearing white to humans), whereas all wavelengths including UV would appear as 'white' to a bee (Proctor and Yeo, 1973). For these colours: yellow - UV is known as 'bee purple', UV-blue is known as violet, and yellow-blue is known as blue-green (Proctor and Yeo, 1973). While attempts have been made to name bee colour space (Chittka et al., 1994), these differences in colour perception tend to complicate experimental design somewhat. All colours mentioned throughout the text refer to the human perception of them, and only the main bee colour groups are coloured in Figure 3.8 for the sake of emphasis.

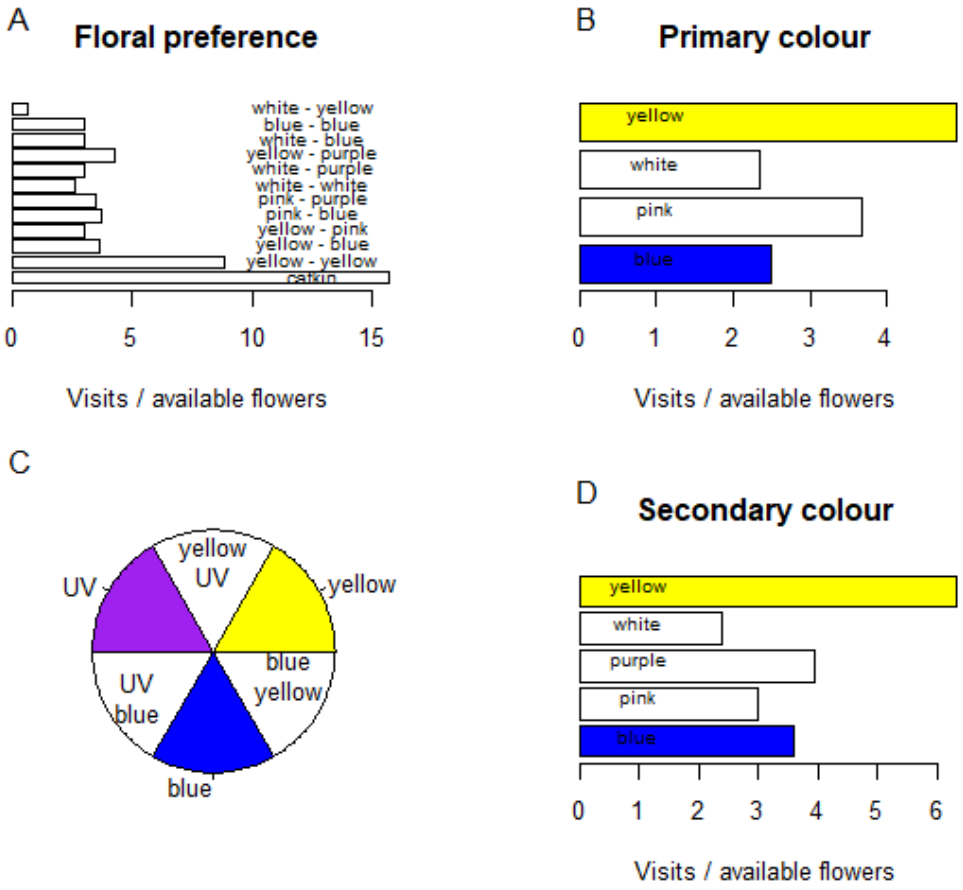

Figure 3.8: Floral colour preference of *O. bicornis* females. Behavioural observations of *O. bicornis* females alighting artificial flowers (T7). Data were normalised by the number of artificial flowers available of that type. All colour combinations are presented in (A). This same data is further presented by primary floral colour (B), and secondary floral colour (D), for ease of view. Primary colours (B) refer to the colour used closest to the makeshift 'stamen' (pipe cleaner). Secondary colours refer to the colours used on the distal side of the petals (outermost colours). Both primary and secondary colour indicated a preference for yellow, suggesting a general prediliction for yellow. C) Provides an overview of the bee-colour spectrum following Proctor and Yeo (1973), with the main colours (UV, blue and yellow; which correspond to their relative photoreceptors: Menzel *et al.*, 1988) being coloured in for emphasis.

Bees are exceptionally sensitive to UV (Proctor and Yeo, 1973). Additionally, UV is the most attractive colour to bees in general (Barth, 1985). In spite of this, UV-reflecting photo paper used on the outer parts of the petals had no discernible effect on the number of visitations ($\chi^2_{1,5}$= 90.98, p = 0.095). A likely explanation for these results is that while fluorescent lamps give off UV radiation (Maxwell and Elwood, 1983), it is usually blocked off by filters on the

lamp (as was the case here; ~20 µW x 5 nm^{-1} x lm^{-1} at ~360nm)[26]. In addition to the apparent indifference to UV, no significant effect of primary colour could be find either (i.e. colour used closest to the stamen; $\chi^2_{3,7}$= 247.49, p = 0.056; Figure 3.8B). Only the colour used furthest away from the emulated stamen seemed to matter (i.e. secondary colour; $\chi^2_{4,8}$= 495.5, p = 0.004; Figure 3.8D). The individual colour combinations could not be tested, due to a lack of statistical power (even with already inflated sample sizes due to disregarding repeated measurements). Nor were colour combinations deemed important here, since the aim was not to investigate pollinator behaviour and colour synergy in depth, but simply optimise a laboratory design. Statistical considerations aside, both Figure 3.8 C and D show a distinct preference for yellow in flowers, for those bees that foraged in my setup. This is in line with canonical visual cues for pollen (Lunau, 2000). Pollen is predominantly yellow, and the colour patterns of flowers reflect this (e.g. yellow flower centres, shorter wavelengths on petal perifery for contrast, and increasing colour purity moving centripetally: Lunau, 2000). Pollen also possesses protective UV-absorbing (poly)phenolics (Torabinejad *et al.*, 1998; Rozema *et al.*, 2001), which make pollen contrast with UV reflection on petal periphery, creating an ultraviolet bull's-eye (Lunau, 2000). UV- free spaces are also known to initiate head proboscis reactions (Barth, 1985).

Most striking is the agreement between the top left graph in Figure 3.8 here with Fig. 3 in Tasei (1973), with ‘catkins’ and ‘yellow – yellow’ representing *Quercus* and *Ranunculus* pollen collection respectively. In order to increase nesting in future setups, the *Quercus* and *Ranunculus* preference might be exploited further (e.g. 5 petals with more accurate dimensions could be used; and if UV light is present, UV reflecting patterns for Ranunculus may be found in Fig. 1 of Koski and Ashman, 2014). A further consideration may be the amount of floral resources presented to the cage bees, as both Cane (2016) and Sandrock (PC) noted pollen depletion as problematic. Regardless of pollen being refreshed daily, resources perceived by the bee (and perceived competition) may be important.

[26] LDPO_TL-D8G_865-Spectral power distribution on the datasheet shows low emission in the UV region (10-380 nm); https://www.assets.signify.com/is/content/PhilipsLighting/fp927983286536-pss-global; last updated 13/12/2019; access date 31/01/2020

3.4.3 Nesting

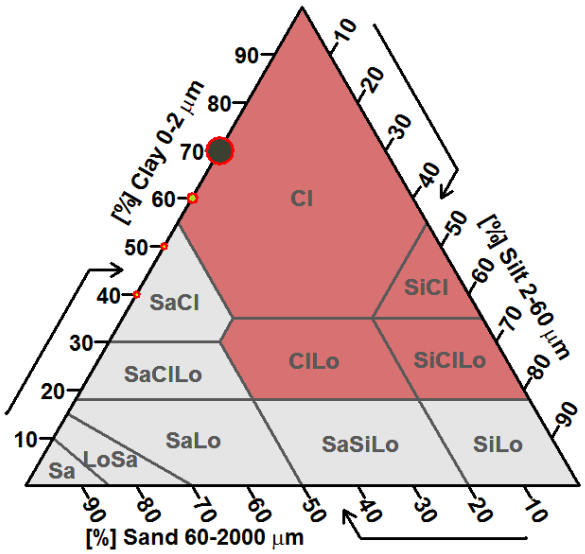

Figure 3.9: Masonry in *O. bicornis*. Soil texture triangle (Moeys, 2018) using the UK Soil Survey of England and Wales texture classification[27]. Number of recorded visitations during T1-2 are plotted as bubbles. Choices presented to bees were 40/60, 50/50, 60/40, and 70/30% clay and silica respectively. Smallest points represent no visits. Silt was not readily available. Red-shaded areas are recommended for future trials involving silt.

Nesting in *O. bicornis* consists of two major components. The acquisition of a suitable nest, and the aspect of masonry. O'Toole (2000) stressed the importance of good mud and further implied that preference is stable across individuals (anecdotal evidence). Figure 3.9 indicates a distinct preference of *O. bicornis* females for clay rich mud. Unfortunately, silt was not readily available and areas shaded in red (Figure 3.9) indicate prospective avenues for future trials which include silt. Another important factor with regard to mud collection is moistness. Sandrock (PC) recommended using a wick suspended in water to keep the mud moist, which works well, though care must be taken with water in closely confined cages since bees were prone to drown (personal observation; PC Sandrock). O'Toole (2000) stated that females may break apart soil and are able to moisten it with saliva. While I did not notice any females breaking dry soil, females often formed little excavation sites to find mud with a moistness and consistency to their liking.

Other sources of masonry were offered aside from the clay (Fuller's earth by Intra Laboratories) and silica (white silica sand by Cristobalite) mixtures. Cat litter was briefly attempted, and bees even showed interest, but unwanted chemicals may be used in such a product. Loam soil (Wilco, Graded top soil)

[27] Defra – Rural Development Service – Technical Advice Unit 2006 [9] (Technical Advice Note 52 – Soil texture).

supplemented with clay was also trialled with no preference shown. Finally, natural mud from the bank of a stream in Leeds was collected and placed inside the cage in a transparent container, but bees showed no interest. Additionally, bringing mud with high organic content into an environment of 23°C was not ideal and quickly turned foul. The 70/30% clay/silica mixture presented seemed sufficient for the bees to carry out their nest building, and was preferred to the 50/50 mixture used in Sandrock *et al.* (2014; mixture composition not mentioned in paper, but in PC). The lack of organic content also made it a practically sustainable source of mud. Fungal growth occurred frequently when pollen was spilt on the provided mud, but is easily scraped off, and mud was generally replaced every 1-2 weeks.

O. bicornis also shows certain preferences when it comes to nesting substrates. These are better studied fortunately. Females nest in various cavities, including beetle borings in wood, cut reeds, cardboard tubes, plastic straws, and styrofoam (even in glass tubes, although this does not come recommended: Raw, 1972; Strohm *et al.*, 2002; Wilkaniec and Giejdasz, 2003). Nest tube diameter and length are both known to affect reproductive output (Wilkaniec, 1998; Ivanov, 2006; Seidelmann *et al.*, 2016). Both parameters mainly matter with regard to sex ratio. Larger tube diameters will facilitate more daughters, since these require more provisioning and will be broader (Ivanov, 2006; Seidelmann *et al.*, 2016). Ivanov (2006) found that once tube diameter exceeded 7 mm, male bias disappeared and the sex ratio averaged out (Fig. 14 in Ivanov, 2006). Seidelmann *et al.* (2016) considered tube diameters of 8-10 mm to be ideal, with wider diameters increasing the costs associated with both masonry and parasitism. Ivanov (2006) sometimes found that nests with diameters wider than 12 mm, cells would sometimes lie in two rows instead of one. Tube length affects the total number of cells that can be made, and since females are preferentially placed at the back of the nest; longer tubes lead to more daughters being produced (Ivanov, 2006; Seidelmann *et al.*, 2016).

In trials T1-2, I used reeds provided by the supplier of the bees. During T2 I recorded inspections to these reeds, as well as occupancy at the end of the trial (i.e. whether a female was present; Figure 3.10A). While the same reeds were inspected several times, there seemed to be no clear pattern. Several tubes of 6.5 mm diameter were inspected frequently (Figure 3.10A), but were not found to be occupied at the end of the trial (Figure 3.10C). During a later trial (T6-7) where cardboard tubes (Fabre’s hives; Oxford bee company) were used and occupancy was similarly investigated (tubes were not marked and hence no nest inspections were recorded for this trial; Figure 3.10D). Similarly no clear pattern could be discerned.

Trap nests such as those used here, are generally designed with the intention of getting clear and unbiased ecological estimates (e.g. of sex ratios or parasitism: Seidelmann *et al.*, 2016; Staab *et al.*, 2018). But such studies do not necessarily reflect the preference of the bees themselves, which relate more to their own body size (e.g. with respect to nest defence: MacIvor, 2017). Wilkaniec and Giejdasz (2003) recorded a 100% acceptance rate for reed nesting substrates over two years, even though reeds did not always provide the highest reproductive success. With the aim of getting females to nest in a laboratory environment, it may be better to provide reed sources, however much they may vary in diameter and length. Additionally, it is recommended to provide multiple sources of nesting material, of varying diameters and lengths. In other words, saturating the bees with opportunities for nesting may be ideal, even if it leads to a loss in standardisation. The objective may be to decrease competition over nesting resources (competition over nests is known to exist: Strohm *et al.*, 2002). For instance, during experiments relating to Chapter 6, smaller cages with fewer tubes were used — yet the number of cardboard tubes present still greatly outnumbered the number of females — and up to three bees were regularly found in single tubes while many others were left vacant. van der Steen (PC) also stressed supplying sufficient nesting substrate. Another observation concerns paper linings in the cardboard tubes (Oxford bee company). These are used to be able to remove cells and cocoons from the nest tubes without destroying the cardboard tube itself. Bees often destroyed the paper by chewing holes in it. Whether this was behaviour related to stress, or a removal of ill-favoured material is unclear. Finally, a pheromonal spray[28] which was developed for use in agricultural settings to prevent *Osmia* from searching out new nests after emerging from cocoons, was also tried (applied to nest entrances as per instruction; T7-8), but elicited no discernable increase in nesting activity.

[28] Invitabee PLUS+ mason bee attractant; no peer reviewed or other documentation could be found; https://portal.nifa.usda.gov/web/crisprojectpages/0222890-development-of-a-nest-attractant-for-the-blue-orchard-bee.html; last updated: could not be accessed; access date: 31/01/2020

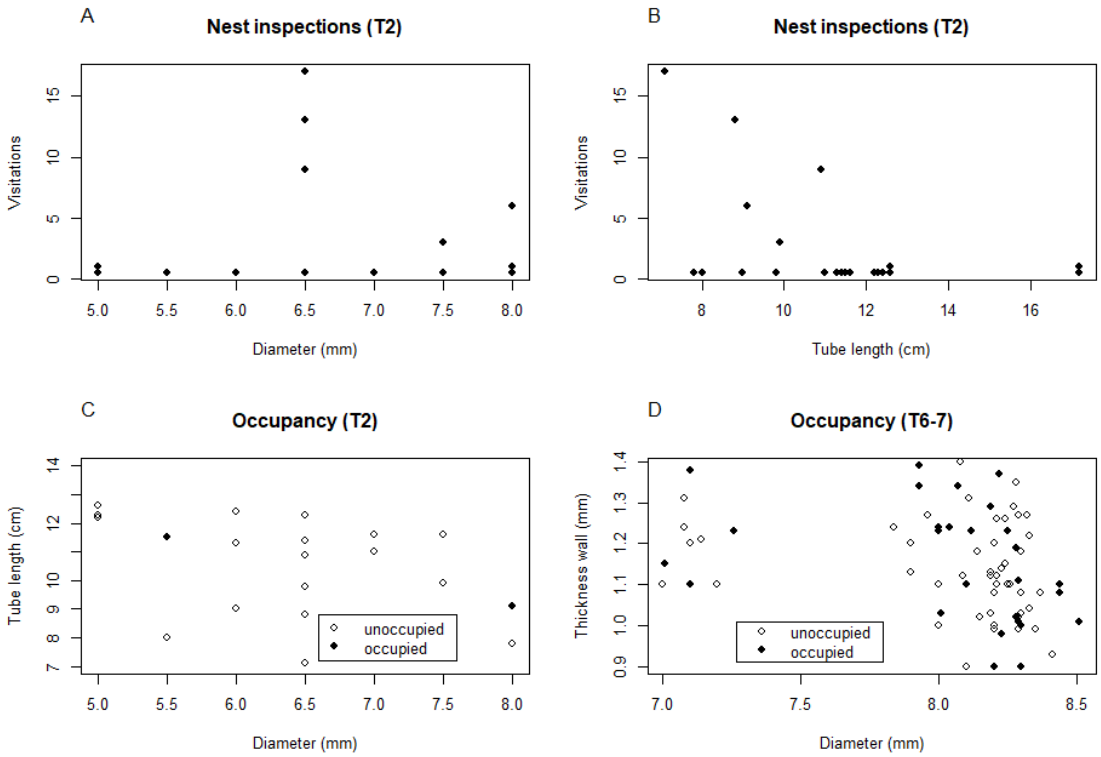

Figure 3.10: Nest preference in *O. bicornis*. A-B) Behavioural observations of nest inspections by females, during T2 did not show any clear patterns in nest preference for tube diameter (A), nor tube length (B). A select few nest tubes received the bult of the visitations. C-D) The nest tube occupancy at the end of T2 (C) and T6-7 (D), likewise did not indicate any patterns of preference for tube diameter.

3.4.4 Environment

Environmental cues are likely to be important in the establishing of *O. bicornis* in a laboratory environment, and abiotic factors seem to matter more than biotic ones. The optimal conditions for mating in the lab have already been studied (Fliszkiewicz and Wilkaniec, 2009; Fliszkiewicz *et al.*, 2011a; Fliszkiewicz *et al.*, 2013). From personal observation, I would add that males could be removed to prevent harassment of females by males (though this may depend on the size of the flight cage). Ideally after three days, since females become unattractive and unreceptive after this time, regardless of already having mated (Seidelmann, 2014a). The importance of mating to reproduction in females is further discussed in Chapter 4. The density of bees is also relevant (competition). The flight cage should not be made too crowded (PC van der Steen; roughly 20 females at a time).

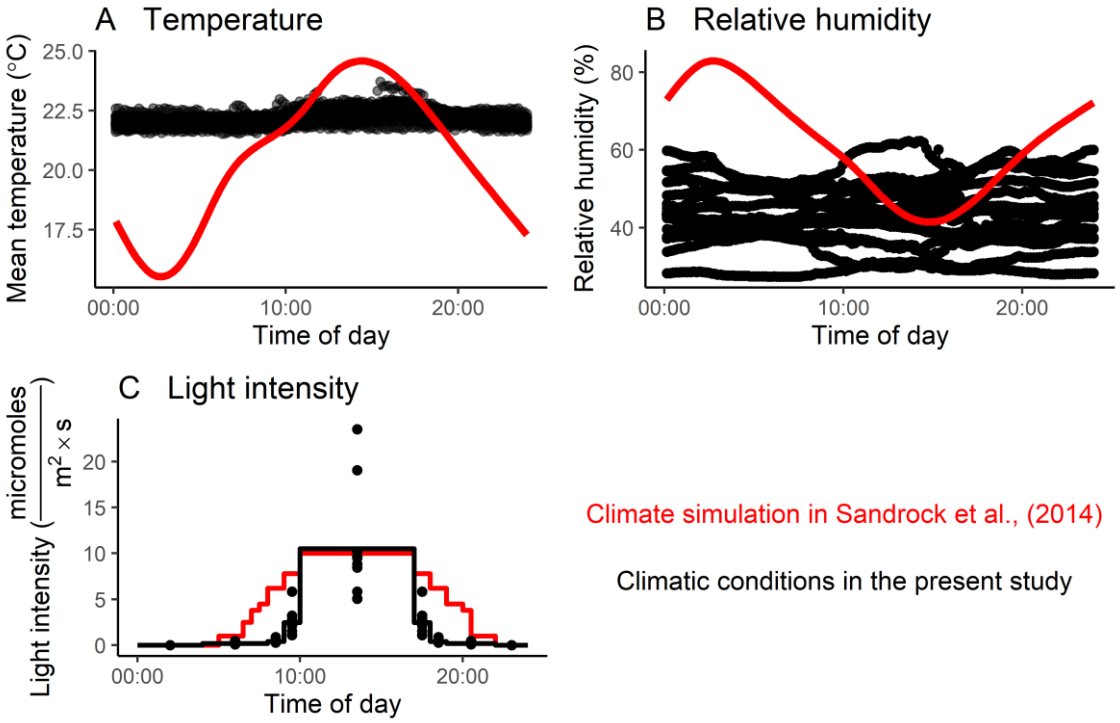

Figure 3.11: Abiotic environmental factors of trials T7-8. Graphs showing temperature A), relative humidity (B) and light intensity (C) fluctuations in T7-8 of the present study (black) and in the climate controlled rooms in Sandrock *et al.* (2014). Neither temperature, nor relative humidity could be controlled in the present study, though temperature was stable. Light intensity was measured using a light intensity meter (Skye) at various positions inside the cage (black points; high measurements are at the top inside the cage). These measurements in W x m^{-2} were converted to µmoles x m^{-2} x s^{-1} by multiplying by 4.59 , assuming 'cool white fluorescent light'. This to facilitate comparison with Sandrock *et al.* (2014). Black stepwise curve represents the mean for the individual measurements at the five light intensity settings available (0%, 25%, 50%, 75%, and 100%).

Abiotic environmental factors are displayed in Figure 3.11. Neither temperature nor relative humidity could be controlled, though temperature remained stable throughout the study. Relative humidity is rarely deemed as important as temperature when it comes to foraging (Dafni *et al.*, 2005). Bees will also prefer sunlit flowers where radiation helps them keep warm (Dafni *et al.*, 2005). Relative humidity is considered important for the development of *O. bicornis* (van der Steen, 1997; Giejdasz and Wilkaniec, 2002), as is temperature (van der Steen, 1997; Giejdasz and Wilkaniec, 2002; Radmacher and Strohm, 2010, 2011; Giejdasz and Fliszkiewicz, 2016). From these manipulative studies, it seems that development is quite robust to changes in temperature, though less is known of humidity. Figure 3.3 shows only several finished nests, with two fully developed adults. It is therefore speculated here that relative humidity may be quite stable within finished cells.

Relative humidity (Figure 3.11B) was considerably less stable, as well as lower overall than in Sandrock *et al.* (2014). Consequently, there may have been dehydration present in the current study. Non decorated PCR tubes with autoclaved (but non-distilled) water could therefore be trialled in future, such that bees can drink (without drowning) to rehydrate. This if relative humidity cannot be controlled. The lack of fluctuation in temperature is also of concern. *O. bicornis* time their emergence through temperature fluctuations (Beer *et al.*, 2019), and time their emergence to morning hours (10:00-12:00: Holm, 1974; Beer *et al.*, 2019). In the current study, cocoons were taken from 4°C and placed in the cages directly (similar to van der Steen and Ruiter, 1991 who placed cocoons in an incubator at 20°C). This was done with little regard for the time of day. Without temperature fluctuation, *O. bicornis* likely start out stressed post-eclosion, and need to adjust their circadian clock through light cycles (Beer *et al.*, 2019).

On average, light intensity (Figure 3.11C) reached the same levels as it did in Sandrock *et al.* (2014). In their study, they used a ‘sunlight simulation system’, though no further information is provided. Light quality may be important. My initial use of a sodium/halide lamp (S/H; 230-240V ~AC 50 Hz, 1.25 amps; Maxibright; T1-5) left many bees perpetually flying towards the light (phototaxis; Chen *et al.*, 2012). The complexity of natural light has briefly been touched upon before (dominant wavelength, polarisation and intensity; all changing gradually throughout the day). I think there is a case to be made for light being a major factor in getting *O. bicornis* to oviposit. van der Steen (1997) managed 0.9 eggs per active female using daylight from a window supplemented by a 80W light. Sandrock *et al.* (2014) managed roughly 6.2 eggs per female using a sunlight simulation system. Holm (1974) achieved 2.2-3.6 eggs using a cold greenhouse, while reporting 4.6-6.6 eggs in the wild. Fliszkiewicz *et al.* (2015) reported between 3 - 4.8 in the wild. The main outlier is Raw (1972) with ~18 eggs using an insectary (presumably a greenhouse). While matters are further complicated through various other differences between studies — in addition to studies reporting reproductive success in different ways — all successes seem to be consistently associated with the use of natural light (or the simulation thereof). Light used in this study, while matching Sandrock *et al.* (2014) in intensity; lacked UV (one could argue this leaves bees one third colour blind: Figure 3.8C), lacked polarisation (navigation and orientation; Von Frisch, 1954; Mazokhin-Porshniakov, 1969; Wehner, 1984) and differed in composition to natural light (constantly white instead of varying across the visible spectrum: red-blue-red).

A final consideration was available space. Placing individuals in small cages in constant temperature rooms (Table 3.3: T5-6) yielded no activity whatsoever (bees also lacked purchase in these plastic cages). After acquiring a nesting substrate, bees are also known to perform an orientation flight (Holm, 1974) by flying in an expanding figure of eight (O'Toole, 2000). This behaviour was never observed in any of my set-ups. This may again be down to the polarisation of light, but the flight cages used in this study were likely too small as well. Sandrock *et al.* (2014) for instance used climate chambers spanning 4.3 x 2.4 x 1.8 m (18.6 m^3), whereas the largest flight cage utilised here spanned only 0.65 x 0.9 x 0.14 m (0.0819 m^3). A lack of flight space is also a likely stressor.

3.5 Concluding remarks

Raw (1972) netted bees while flying with mud pellets. These bees lost their pellets, but upon release, continued to fly back to the nest and started nest building even though it had nothing to add. After a few seconds the bee would fly out to collect mud again. Holm (1974) also noted empty nest cells occurring under greenhouse conditions. These nest tubes likewise contained only mud partitions. Such nest tubes were not encountered in the wild however. I consistently found unfinished Fabre's thresholds in early trials, and empty nests in later trials (no pollen loafs, but finished septa). These sort of observations are reminiscent of fixed action patterns (Lorenz, 2013). As such, they give the impression that if the right '*trigger*' could be found, oviposition could be elicited. While such single-trigger-threshold models are tempting, the various degrees in which various authors have accomplished *O. bicornis* egg laying argue against the idea that the change in one key variable would make the majority or all females complete full sequences of nesting behaviour.

That being said, some variables may be more important than others. I have personally stressed the importance of light and pollen, and offered various suggestions on these and other factors throughout this chapter. But I would finally like to argue against a 'threshold model', and in favour of a 'holistic model'. Discovering and refining all preferences with regard to the aspects outlined in Table 3.1 and 3.2 may all aid in inducing laboratory egg laying. Another consideration, not previously mentioned, is the vitality of the bees (PC Whittles). Many of the studies mentioned (e.g. Raw, 1972; van der Steen, 1997; Sandrock *et al.*, 2014) sourced their bees from different places.

> *'It is a very ambitious attempt to set up a laboratory culture of a solitary bee. Many researchers tried this before, but to my knowledge, all failed.'*
> *— K. Seidelmann (PC)*

I maintain that establishing *O. bicornis* in a laboratory environment remains possible. Sandrock *et al.* (2014) managed to do so with astounding success. The results here, while not yet allowing a full experimental set-up, are promising. Especially considering that the few females that did lay eggs, did so well past their normal diapause period. This is also why establishing bees in a controlled environment is so appealing, it would allow for experiments outside of the flight season. I also hope I provided a proof of concept for behavioural assays within a controlled setting for solitary bees. Building on the work here, my main recommendations for future endeavours at establishing solitary bees in a controlled environment are:

- The use of a greenhouse if possible. And the establishment of a local nearby population (as done by most research groups), by setting out bees near nesting substrates and abundant floral resources (taking care not to introduce parasites; possibly by eclosing individuals inside the laboratory)[29].
- A saturation of both floral resources, and nesting resources. And a refinement of both (e.g. the use of reeds, and more representative flower mimics; sugar solutions should ideally also not be ignored in this respect).
- The addition of UV to the light used. In combination with *Ranunculus* mimics that reflect UV this may lead to a significant increase in the number of individuals foraging.[30] The polarisation of light may prove more difficult (polarisation filters also reduce light intensity).
- More precarious practices for emerging the bees may be beneficial (fluctuating temperature).

Climate controlled rooms with sunlight simulation are likely not economically viable for many research groups. Hence it is my hope that these recommendations and efforts facilitate the use of *O. bicornis* and other solitary pollinators inside laboratories.

[29] This allows for ecological and behavioural studies, albeit mainly within the flight season, but also provide a backup and easy access to more individuals. Note also that studies in greenhouses are still susceptible to at least some weather conditions (e.g. clouding).

[30] Simply fitting fluorescent lights, without UV filters may easily accomplish this. Though health and safety measures would need to be put in place depending on the level of UV radiation.

Chapter 4 Validation of microsatellite markers in *O. bicornis*

4.1 Summary

In this chapter, I further establish *O. bicornis* as a model species through the development and validation of new microsatellite markers. Microsatellite markers and other genetic resources like them find their use from evolutionary analysis to ecology and conservation. In collaboration with NBAF (NERC Biomolecular analysis facility) Sheffield microsatellite markers were mined from the *O. bicornis* genome (Beadle *et al.*, 2019) and designed, and subsequently tested and validated. Of the initial twenty microsatellites, seventeen were amplifiable and informative. By adding seventeen new markers to six previously published ones (Neumann and Seidelmann, 2006), I extend the base for genetic studies. In this species, in particular to hereafter address whether this species is truly monandrous or whether females display low levels of extra-pair paternity, as this is relevant in directing future research into the evolution of eusociality in this species, as well as having conservation genetic implications. In addition, the markers may be used to assess possible levels of inbreeding in at least commercially and voluntarily managed populations of *O. bicornis*. Finally, in order to use the markers to assess gene flow, monandry and population dynamics of natural populations of *O. bicornis* in future, I attempt to enhance local populations through trap nesting. I discuss how future efforts at trap nesting *O. bicornis* may be made more efficient.

4.2 Introduction

Relatedness, is paramount to inclusive fitness theory (Hamilton, 1964a, b), and consequently the study of the evolution of eusociality (Pernu and Helantera, 2019; section 1.1). An immediate way of estimating relatedness is through the use of microsatellite markers (Queller and Goodnight, 1989; Blouin *et al.*, 1996), and this approach has been applied in numerous studies of social insects. For example, genotyping individuals through microsatellites has facilitated direct tests of inclusive fitness hypotheses (Paxton *et al.*, 1996; Chapman and Crespi, 1998; Paxton *et al.*, 2002). In *A. mellifera* microsatellite markers have further been used to test relatedness (Baudry *et al.*, 1998), mating frequency (Tarpy *et al.*, 2015) and mating range (Jensen *et al.*, 2005), sperm admixture (Franck *et al.*, 1999), investigating worker laid brood (Neumann *et al.*, 1999), and the creation of linkage maps (Solignac *et al.*, 2003;

Solignac *et al.*, 2004; Solignac *et al.*, 2007). Such examples illustrate the broad utility of microsatellite markers, and their implicit value to eusocial research.

Monogamy is considered ancestral to eusocial lineages (Hughes *et al.*, 2008; Boomsma, 2009), precisely because it facilitates higher relatedness and the evolution of helping behaviour (section 1.1 and references therein). *O. bicornis* females are considered to be monogamous, a belief which is grounded in behavioural assays and observations (Seidelmann, 2015, 2014a). This assumed monogamy seems to be predominantly driven by males through: extensive mate guarding (Seidelmann, 1995), an elusive anti-aphrodisiac[31] (Ayasse and Dutzler, 1998; Seidelmann, 2014a; Seidelmann and Rolke, 2019), and a male mating plug (Seidelmann, 2015). These behavioural adaptations make sense in light of a scramble competition polygyny (Seidelmann, 1999b). Given that females use various and spread out resources (i.e. floral, mud, and nest resources), it does not pay off for males to defend territories in order to gain access to females. Instead, males wait for females to emerge at nest sites or search for females at foraging sites (Raw, 1976; Seidelmann, 1999b). Mechanisms such as: a mating plug (Seidelmann, 2015) and induced female unreceptivity (Seidelmann, 2014a) make more sense in light of assuring paternity. The *O. bicornis* mating system has thus been described as a ‘searching for mates’ type scramble competition (Seidelmann, 1999b). Yet it should be noted that Raw (1976) did observe aggressive behaviour among males searching for females by nest sites, even if Seidelmann (1999b) did not. Inconsistencies like these, are perhaps the first indication towards the inadequacy in ascertaining mating systems from behavioural observations.

From the female’s perspective, mating multiply should not increase her reproductive success in a direct and linear manner (Bateman, 1948), this is also predicted to be the case where high maternal investment is present (Arnold and Duvall, 1994). Indirect benefits could be accrued by polyandrous females however (Zeh and Zeh, 2001). Moreover, post-copulatory mechanisms such as a mating plug (Seidelmann, 2015) and mate guarding (Seidelmann, 1995) can be considered costly in terms of both time and energy invested. Hence, if no selection pressure were to be maintained on these mechanisms, one would expect them to erode over evolutionary time. This raises the question of whether low-levels of polyandry occur in wild populations of *O. bicornis*. As stated above, behavioural observations indicate that *O. bicornis* is monandrous

[31] Female unreceptivity is initiated by a male post-copulatory display(Seidelmann, 2014a), yet the application of a male produced anti-aphrodisiac during this display has been subject of debate with contradicting lines of evidence(Ayasse and Dutzler, 1998; Seidelmann and Rolke, 2019). The issue remains unresolved to date.

(Seidelmann, 2014a, 2015), yet behavioural observations of mating frequency have been shown to be unreliable (e.g. the case in birds; Griffith *et al.*, 2002) and it is therefore important to verify mating frequencies using genetic resources (Bretman and Tregenza, 2005), i.e. microsatellite markers.

Neumann and Seidelmann (2006) identified and validated six microsatellite markers to interrogate population structure in *O. bicornis*. These were isolated using an enrichment protocol for partial genomic libraries, and consisted of five dinucleotide repeats and one trinucleotide repeat. Dinucleotide repeats occur more frequently than others (Fan and Chu, 2007), making their use innate and commonplace. Yet, tetranucleotide repeats, for instance, are easier to score given the distance between alleles as they are less prone to the artefacts common in dinucleotide repeats e.g. stutter and slippage. This in turn leads to fewer human errors in genotype calling, which is considered the main source of genotyping errors (Pompanon *et al.*, 2005). For example, during preliminary tests of microsatellite protocols performed by Dr Elizabeth Duncan and Vanessa Barlow (data not shown), many of the markers presented by Neumann and Seidelmann (2006) were difficult to score unambiguously due to their dinucleotide nature. Furthermore, amplifying more loci and more variable loci, is known to increase power more than increasing the number of individuals does (Landguth *et al.*, 2012). For instance, the OruS4 marker (Neumann and Seidelmann, 2006) was found to be uninformative (no variation in our populations) when tested. For this reason, the use of these six microsatellites necessitates incredibly large sample sizes (e.g. Conrad *et al.*, 2018 used 779 individuals in total).

From the recent publication of the *O. bicornis* genome (Beadle *et al.*, 2019) ensues the opportunity of mining and developing additional microsatellite markers from it (Beier *et al.*, 2017). The development of more markers would increase power for inferring population structure (Landguth *et al.*, 2012). This is important, since knowledge on the genetic diversity of solitary bees lags behind that of other pollinators (Packer and Owen, 2001). Moreover, Hymenopterans habitually have brother-sister matings (Packer and Owen, 2001), and *O. bicornis* likewise may not avoid inbreeding (Conrad *et al.*, 2010; Conrad and Ayasse, 2015; Conrad *et al.*, 2018). In honey bees homozygosity at the *csd* locus results in sterile male offspring (Gempe and Beye, 2009). A loss of heterozygosity, as a result of a lack of inbreeding avoidance, could therefore have profound consequences for solitary bees (Zayed and Packer, 2005). Additional microsatellite markers will therefore play a pivotal role in conservation genetics (Zayed, 2009), helping to inform decision making in the face of global pollinator decline (Potts *et al.*, 2010).

Genomic microsatellites could further be used to create linkage maps (Solignac *et al.*, 2004), which in turn could help with further assembly of the genome (Solignac *et al.*, 2007) as the *O. bicornis* genome is currently in 10,223 scaffolds (Beadle et al., 2019) while the karyotype of a closely related species contains 16 chromosomes (*Osmia cornuta*; Armbruster, 1913) and across all bees the number of chromosomes is likely to fall within the range of 6-20 (Crozier, 1977). In this way genomic resources feed into one another. Ultimately, the improvement of genomic resources themselves, helps the field of sociogenomics. High quality genomic resources would further facilitate comparisons of eusocial and solitary Hymenoptera to further uncover the genetic underpinnings of eusociality (Kapheim, 2016). Comparing, for instance, how clusters of genes implicated in control of reproduction in the highly eusocial *A. mellifera* (Duncan *et al.*, 2020) are organised with the *O. bicornis* genome may provide insights into evolutionary co-option during the evolution of eusociality. There further exists the potential to investigate DNA methylation in *O. bicornis* (Strachecka *et al.*, 2017), a mechanism that has been implicated directly in aspects of caste and reproduction *A. mellifera* (Kucharski *et al.*, 2008; Elango *et al.*, 2009; Lockett *et al.*, 2012; Shi *et al.*, 2013; Strachecka *et al.*, 2015; Kilaso *et al.*, 2017) or even *B. terrestris* (Amarasinghe *et al.*, 2014).

In this chapter, I aim to validate and establish informative microsatellite markers to further *O. bicornis* as a model species, with special reference to its potential as a model organism for studying the evolution of eusociality. Specifically, markers were mined, and tested, for future use in determining monogamy (Hughes *et al.*, 2008; Boomsma, 2009) and ascertaining levels of possible inbreeding (Packer and Owen, 2001; Conrad *et al.*, 2010; Conrad and Ayasse, 2015; Conrad *et al.*, 2018). This because inbreeding pertains to extinction proneness and consequently conservation efforts (Zayed and Packer, 2005; Zayed, 2009). Another factor affecting genetic diversity is the mating system. Under monogamy, the effects of inbreeding on extinction proneness could thus be exacerbated (Zayed, 2009). Given the importance of monandry to both relatedness in the evolution of eusociality (Hughes *et al.*, 2008; Hamilton, 1964b) and conservation genetics (Zayed and Packer, 2005; Zayed, 2009) — I aimed to explicitly test monandry in *O. bicornis* using microsatellite markers on commercially available nest tubes (where a nest tube can be regarded as a family unit).

Additionally, many research groups working on *O. bicornis*, have populations established in the surrounding area. While this could be achieved by releasing *O. bicornis* cocoons near provided nest sites (e.g.: Gruber *et al.*, 2011; Persson *et al.*, 2018), this has the potential of introducing parasites and

diseases (or different strains thereof; Krunic *et al.*, 2005; Fliszkiewicz *et al.*, 2012b) to local populations when using a commercial supplier. Additionally, information on gene flow in this species is sparse (Conrad *et al.*, 2018). Hence, preliminary attempts were made to sample the local *O. bicornis* populations through the provision of trap nests (e.g.: Tscharntke *et al.*, 1998; an urban example: Everaars *et al.*, 2011). Through the use of trap nests, I also aimed to ascertain the presence of monogamy and inbreeding in wild populations. This because wild populations may differ from populations maintained for commercial breeding in for instance density, which in turn might affect levels of inbreeding and polyandry.

4.3 Materials and methods

4.3.1 Trap-nesting

Trap-nesting of *O. bicornis* in greater Leeds was attempted (Figure 4.1), during both 2017 and 2018. Fabre's hives with cardboard tubes (Oxford bee company Ltd. ;Figure 4.1 inset) were fastened to wooden stakes impaled into the soil, some 1-1.5m from the ground (Steffan-Dewenter and Schiele, 2008), facing South to South-West. 32 nest traps were spread over as many locations (Figure 4.1 and see Table B.1 for further details). In 2017, these trap nests were placed *in situ* fairly late in the *O. bicornis* flight season (mid-May); in 2018 trap nests were placed *in situ* before both male and female emergence (early April).

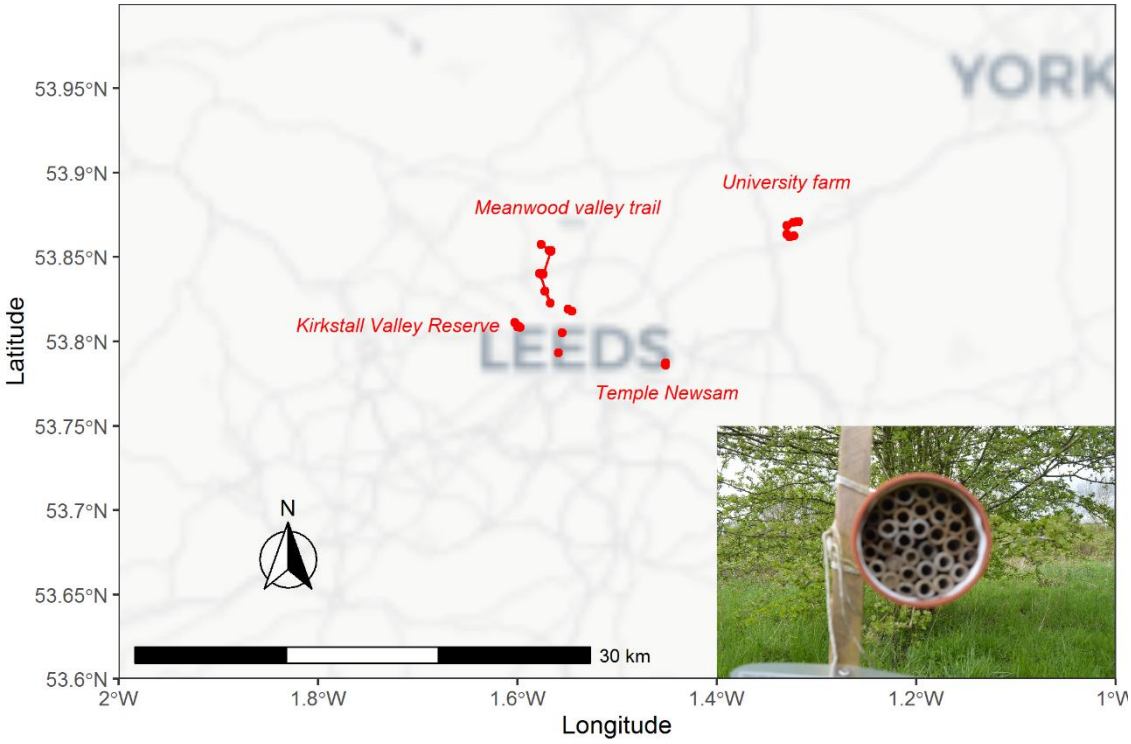

Figure 4.1: Trap-nest locations. Sites in the city of Leeds consisted of the canalside , the University of Leeds campus , and Sugarwell Hill Park — points not annotated on map. Sites in the greater Leeds area consisted of Kirkstall Valley Reserve , the Meanwood Valley trail , Temple Newsam and the Leeds University farm. Inset: Fabre's hive with cardboard tubes (Oxford bee company Ltd.).

4.3.2 Commercial nest tubes

Intact nest tubes were provided by Dr Schubert plant breeding (Landsberg, Germany; commercial breeder) from two localities in Germany 100 km apart (G1 and G2), totalling 24 nest tubes, containing 210 individuals. Further nest tubes of *O. bicornis* were provided by MasonBees Ltd. (Shropshire, UK; guardian scheme), from North Shropshire (NS) and Surrey (Su; ~ 240 km apart) totalling 20 tubes, containing 166 individuals. The use of intact nest tubes (Figure 4.2) should facilitate easy reconstruction of paternal and maternal genotypes. Since males are haploid representations of the mother's genotype. Consequently, any nest usurpations by another female (Raw, 1972) can be inferred from the genotypes of haploid sons directly. Furthermore, to address the question of female polyandry, it is sufficient to detect more than a single father to a nest. Given that maternal genotypes can be inferred unambiguously (Figure 4.2) in our design; our power of inference with regard to detecting polyandry should increase linearly with both the number of informative markers used and the total number of nests used.

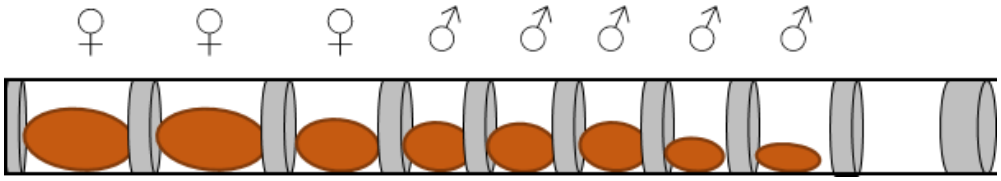

Figure 4.2: The nest tube as a family unit. An *O. bicornis* nest tube, with females (from fertilised eggs) positioned at the back and males (from unfertilised eggs) positioned at the front — was considered a family unit. In cases where an unfertilized male egg is laid in amongst females or vice versa; fertilisation errors (Raw and O'Toole, 1979) or fights and usurpation of nests (Raw, 1972; Strohm *et al.*, 2002) may have occurred. This was a common feature, with 12 out of 24 nest tubes showing either males interspersed in between females or vice versa. In 10 out of 12 cases, the mismatch could be explained by only a single individual (male or female) disrupting the female-to-male order within the nest tube.

4.3.3 DNA extraction

DNA extractions were performed using hot sodium hydroxide and tris (HotSHOT; Truett *et al.*, 2000). An individual's leg was removed using tweezers, which were flamed and rinsed with 10% bleach in between use, to prevent contamination. The leg was placed in a thermal cycling tube and 75 µl of HotSHOT alkaline lysis buffer (25 mM NaOH, 0.2 M EDTA, pH 12) was added. Samples were incubated at 95°C for 30 minutes and cooled to 4°C for three minutes. 75 µl of HotSHOT neutralisation buffer (40 mM Tris-HCl, pH 5) was added to neutralise pH. Samples were stored at -20°C and used within 3 months. Occasionally, no whole bees were found in one or several cells of a nest tube. In these cases larvae were affected by chalkbrood (*Ascosphaera* spp.; Krunic *et al.*, 2005), cocoons contained larvae of *Monodontomerus obscurus* (Krunic *et al.*, 2005), or an empty cocoon was found where an individual had already emerged and escaped.

In the case of chalkbrood (Krunic *et al.*, 2005), an ammonium acetate DNA extraction was performed. The sample was placed in a 1.5 ml microcentrifuge tube, and 250 µl DIGSOL buffer (20 mmol/L edetic acid [EDTA], 50 mmol/L Tris [pH 7.5], 0.4 mol/L NaCl, 0.5% sodium dodecyl sulfate [SDS]; Lagisz *et al.*, 2010) and 10 µl of 10 mg /ml proteinase K (Thermo Scientific) were added. Sample was ground using a sterile pestle, and incubated at 55°C for three hours (rotating). 300 µl 4M ammonium acetate was added, and proteins were subsequently precipitated by vortexing the solution several times over a period of fifteen minutes. Solution was centrifuged for ten minutes at 13,000 rpm, and supernatant was transferred to a fresh 1.5ml microcentrifuge tube. 1 ml of a 100% ethanol was added to precipitate DNA (inverted several

times), and centrifuged for ten minutes at 13,000 rpm. The solution was decanted off, and the pellet was washed with 500 µl of 70% ethanol, and centrifuged for five minutes at 13,000 rpm. Ethanol was again decanted and pellet was airdried. The dried pellet was resuspended in 50µl low Tris-EDTA buffer (low TE; 10 mM Tris-HCl and 0.1 mM EDTA). DNA was quantified using Qubit® (Invitrogen life technologies), and diluted to 10 ng/µl. In the case of empty cocoons, an attempt was made to obtain residual DNA from the cocoon itself. Any *M. obscurus* larvae present were removed from the cocoon using sterile technique, and soaking the left over cocoon overnight at -20°C in 250µl TE (1M Tris, 0.5M EDTA; pH 8).

4.3.4 PCR — Cytochrome oxidase subunit I

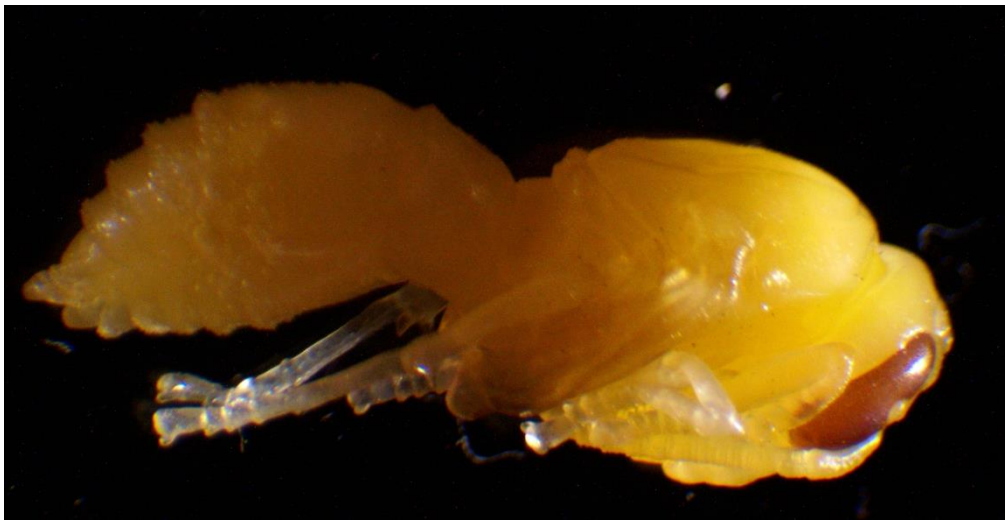

Figure 4.3: Unidentifiable trap nested individual. Example of a pupal individual. Underdeveloped samples could not be identified to species level using conventional methods.

To identify trap-nested but underdeveloped individuals (prepupal or pupal; Figure 4.3) to species level, the cytochrome oxidase subunit I (COI; Folmer *et al.*, 1994) was amplified from ~1ng of HotSHOT extracted DNA (Truett *et al.*, 2000) using the PCR conditions (Table 4.1) and primers:

LCO1490: 5'-ggtcaacaaatcataaagatattgg-3'

HC02198: 5'-taaacttcagggtgaccaaaaaatca-3',

as described in Folmer *et al.* (1994). Samples were subsequently sent for sequencing to Eurofins Genomics.

Table 4.1: Thermocycling conditions for COI. (Folmer *et al.*, 1994)

Stage		°C	Time
Initial denaturation		94°C	3 minutes
x35	Denaturation	94°C	1 minute
	Annealing	40°C	1 minute
	Extension	72°C	1.5 minutes
Final extension		72°C	7 minutes

4.3.1 Microsatellites

Microsatellites were mined from the *O. bicornis* genome (Beadle *et al.*, 2019; accession number: SRP065762;) using MIcroSAtellite (Thiel *et al.*, 2003) by Dr Kathryn Mayer (University of Sheffield). Di-, tri-, and tetra-nucleotide repeats were mined, leaving 20-50 bases on either side of the repeat region to design primers in. Sequences were selected with a preference for tetranucleotide repeats. These are often most informative (personal communication: Dr Deborah Dawson), due to lower mutation and slippage rates than dinucleotide repeats (Fan and Chu, 2007), alongside facilitating easier genotype calling by minimising problems associated with stutter and human error (Pompanon *et al.*, 2005). Primers were further designed by Dr Gavin Horsburgh (University of Sheffield; Table 4.2). Sequences were scanned by eye, avoiding poly(N) sequences and composite repeats. Primers were designed using Primer3 (v. 0.4.0; Untergasser *et al.*, 2007; Untergasser *et al.*, 2012) at an optimum of 60°C T_m, a maximum difference of Tm of 0.5°C, a maximum poly(N) of three, a CG clamp, and using Schildkraut and Lifson's (1965) original salt correction formula. These primer design thresholds or conditions were relaxed, only when no appropriate primers could be found.

Table 4.2: 20 Microsatellite markers tested. Microsatellite markers mined from the *O. bicornis* genome by Dr Kathryn Mayer, alongside their forward (F) and reverse (R) primer sequences as designed by Dr Gavin Horsburgh. Tm = melting temperature, and Motif = motif of the repeat domain.

Marker	F/R	Primer sequence	Tm	Motif
Obic1	F	CGGTTTATGGCAGGTAAACG	60.37	$(ag)_{14}$
Obic1	R	GTAGCAGCAGCCGGTGTATC	60.83	$(ag)_{14}$
Obic113	F	CTGCCCTCTCGTCTCTTCC	60.08	$(ccag)_{7}$
Obic113	R	AATTCGGGTTGAAACCTGTG	59.83	$(ccag)_{7}$
Obic1176	F	ACGCTTGTCGCTTTCAG	60.14	$(tgta)_{8}$
Obic1176	R	TTCTCGAACAGATGTCCTTGG	60.24	$(tgta)_{8}$
Obic1181	F	CTCGGGAATCCACCTTATTG	59.38	$(cttt)_{13}$
Obic1181	R	TGCCTAGCGAAAGAGGGTAG	59.61	$(cttt)_{13}$
Obic1206	F	CCAACCTTCCCACACCTAAC	59.3	$(acct)_{9}$
Obic1206	R	AACAGGACAAAGGAGCGAAG	59.47	$(acct)_{9}$
Obic1238	F	ACAATTTGTAGGGTGGACACG	59.77	$(agca)_{13}$
Obic1238	R	GCGATTCAACCTCCTTTCAC	59.68	$(agca)_{13}$
Obic1252	F	CCTTCCTATGTCGCTGCTG	59.56	$(tttc)_{17}$
Obic1252	R	TCCAAGTTCCTGTACCAATGTG	59.89	$(tttc)_{17}$
Obic1344	F	CTCAACGGTTTGCAGGTTTC	60.67	$(ttcc)_{9}$
Obic1344	R	GCATCGTAGATCTGTAAGCTTGTG	60.33	$(ttcc)_{9}$
Obic1374	F	CTATCCGGCACTCTTTCTCG	59.97	$(gttc)_{9}$
Obic1374	R	AAACGCGGAATGAGATATGC	60.07	$(gttc)_{9}$
Obic168	F	AGCCACGTTGAAGTTGTTGC	61.28	$(ttc)_{10}$
Obic168	R	GGGTTTCTCCGTTCTGCTG	60.79	$(ttc)_{10}$
Obic220	F	CTGCATCACCTACGCAACTG	60.47	$(cgca)_{8}$
Obic220	R	AACGCGCCAAGTAGAATCTG	60.41	$(cgca)_{8}$
Obic415	F	GAATGGGCAACGTCTATTTACAG	59.91	$(caga)_{8}$
Obic415	R	ATCCTTTGTTGCCGTTTGTC	59.98	$(caga)_{8}$
Obic428	F	GGGTAAAGGGTTAGGGAACTG	58.88	$(tggc)_{6}$
Obic428	R	AGCAAGGGTGGTAGTGAAGG	59.21	$(tggc)_{6}$
Obic450	F	TTGCCTTTCGAAATCAAGC	58.98	$(gaag)_{6}$
Obic450	R	CGACAGATCGAAACGTCATC	59.25	$(gaag)_{6}$
Obic52	F	GGCACCCAAACCATCAAC	59.74	$(ac)_{19}$
Obic52	R	CGATCTCGTGTTCACGGTAG	59.31	$(ac)_{19}$
Obic629	F	CTGCTTCGGCCTCTTTCTAC	59.22	$(cttt)_{12}$
Obic629	R	AAGTCGGTTCTTCGCATACC	59.2	$(cttt)_{12}$
Obic73	F	CCAATACCTCCCTCTTCTCCTC	60.44	$(tcc)_{14}$
Obic73	R	CCCACGTTCTGCCATTACTC	60.52	$(tcc)_{14}$
Obic740	F	AGTACGCGTCACGACAAAGAG	60.5	$(aagg)_{17}$
Obic740	R	GTACAACCGGCCATCGTATC	60.22	$(aagg)_{17}$
Obic77	F	GATCTCGTGTTCACGGTAGG	58.16	$(gt)_{19}$
Obic77	R	CTGCAGTTTCCTGGATCG	57.82	$(gt)_{19}$
Obic95	F	TTTAAGGAAACAGCCAGCAG	58.17	$(ggaa)_{9}$
Obic95	R	TTCATGAAGTATAAGAGGAAACGAC	58	$(ggaa)_{9}$

4.3.2 PCR — Microsatellite

Microsatellite work was performed at the NERC Biomolecular Analysis Facility – Sheffield (NBAF-S). 0.5-20 ng of DNA template[32] was placed in 96-well PCR plate wells (Agilent). DNA was airdried in the uncovered PCR plate in an open thermocycler at 50°C for 30 minutes[33]. 2µl PCR-reactions were used, containing: 1x Multiplex PCR Master Mix (QIAGEN) and 0.2µM primer mix — containing fluorophore-labeled forward primer(s) (6-FAM and HEX, Sigma-Aldrich; NED, ThermoFisher Scientific) and unlabeled reverse primer(s) in low TE. 10µl of mineral oil was placed on top of each 2µl PCR reaction to prevent evaporation. Table 4.3 displays the PCR thermocycling conditions used. A T_m of 57°C was used for all markers, regardless of calculated T_m (Table 4.2), and was sufficiently low to accommodate all primers (57.82 - 61.28°C).

Table 4.3: Microsatellite marker thermocycling conditions.

Stage		°C	Time
Initial denaturation		95°C	15 minutes
x45	Denaturation	95°C	30 seconds
	Annealing	57°C	1.5 minutes
	Extension	72°C	1.5 minutes
Final extension		60°C	30 minutes

4.3.3 Genotyping

1µl of 1:160 PCR amplified product with marker (section 4.3.2) was placed in a semi-skirted 96-well plate (Agilent), and 9µl of Hi-Di™ formamide (ThermoFisher Scientific) containing GeneScan 500 ROX dye Size Standard (Applied Biosystems) was added. Samples were then denatured at 95°C for three minutes and placed on ice for three minutes immediately after. Plates were then transferred and run on an ABI 3730 48-capillary DNA analyzer (Applied Biosystems).

[32] HotSHOT extraction and TE-soaking yielded low amounts of DNA.

[33] This facilitates standardizing PCR reaction volumes for large numbers of samples, in addition to evaporating volatile contaminants (e.g. possible residual ethanol from ammonium acetate extractions).

4.3.4 Preliminary analysis

Genotypes were called using GeneMapper Software (v 3.7; Applied biosystems). Allele frequencies, null allele frequencies, and expected and observed heterozygosity were estimated using Cervus (v 3.0.7; Marshall *et al.*, 1998; Kalinowski *et al.*, 2007); Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested using GENEPOP (v 4.7; Raymond, 1995; Rousset, 2008) and related individuals were identified using MLRelate (v 1.0; Kalinowski *et al.*, 2006). Multiplex Manager (v 1.2; Holleley and Geerts, 2009) and AutoDimer (v 1.0; Vallone and Butler, 2004) were used to check for overlaps in allelic ranges and primer dimerization during multiplex design.

4.4 Results

4.4.1 Fieldwork

Nest trapping in the greater Leeds area yielded 31 finished (end capped with mud) nest tubes in 2017, and 24 tubes in 2018 for 14 localities in total. These localities primarily comprised of acid heathland and grassland, surrounded by woodland (for further details on the localities, see Appendix B). Nest traps were collected at the end of September when individuals have presumably fully developed into imagines. After collection from the field, nest tubes were placed at -20°C for storage. Leafcutting species could easily be distinguished by their leaf encased cells and were disregarded. Upon opening of the nest tubes, some contained larvae in cells that possessed mud walls like *O. bicornis*. While collection at the end of September should suffice for larvae to have developed to imagines, development could still technically be underway for offspring laid very late in the season (e.g. Fig. 7 in Seidelmann and Rolke, 2019).

To verify whether these larvae could indeed have been *O. bicornis* from late in the previous season, DNA of one larva was HotSHOT extracted, PCR-amplified for COI and sent for sequencing. Nucleotide to nucleotide BLAST (NCBI; Altschul *et al.*, 1990; Madden, 2013) generated a 99.84% identity to the COI of *Ancistrocerus trifasciatus* (three-banded mason-wasp; accession: JN934287.1). This wasp retains a flight season from June to August[34], starting around the time the *O. bicornis* flight season terminates. These underdeveloped samples all originated from the same localities, and with the exception of two pupal samples (Figure 4.3) they all constituted larvae which are put under the

[34] BWARS; https://www.bwars.com/wasp/vespidae/eumeninae/ancistrocerus-trifasciatus; accessed: 11/03/2020; last updated: 1998.

common denominator of early development here (regardless of instar). Fliszkiewicz *et al.* (2012a) suggests that larval development should have concluded by September. Given the cost of sequencing; the remaining samples were assumed to be the same species and disregarded. This left a single nest tube (2018; Meanwood Valley Trail; grassland surrounded by woodland) from the trap nesting efforts, containing clear *O. bicornis* cocoons which were identifiable upon opening of the pupal cases.

4.4.2 Validation of extraction method

The allelic dropout of specific alleles due to suboptimal PCR conditions, or due to mutations in the primer binding site are commonly referred to as null alleles (Selkoe and Toonen, 2006). The null allele frequency (F_{null}) is important, since the failure to amplify can make a heterozygote appear to be a homozygote. Null alleles may be detected through statistical approaches (Selkoe and Toonen, 2006). Yet, failure to amplify may also occur due to poor DNA quality, hence it is important to exclude poor DNA quality prior to F_{null} analysis. To test whether the HotSHOT extraction method led to an increase in allelic dropout, it was compared to the ammonium acetate extraction method (side by side) for seven females. In only 1.59% (2/126) of cases was allelic dropout detected for the HotSHOT method of extraction. Not only was the detected dropout low, but in 3.17% (4/126) of cases there was allelic drop-out using the ammonium-acetate extraction method. This illustrates that allelic dropout occurs even from high quality DNA (Soulsbury *et al.*, 2007). Hence, given the presumed stochastic nature of allelic dropout (Navidi *et al.*, 1992; Taberlet *et al.*, 1996); I argue that the extraction method is unlikely to have posed a problem here.

4.4.3 Marker validation

Markers were expected to work, since they were not only mined from the *O. bicornis* genome, but also because this genome was sequenced from a single male originating from the same German supplier (Dr Schubert Plant Breeding; Beadle *et al.*, 2019). Preliminary tests of the HotSHOT extraction method using previously published markers (Neumann and Seidelmann, 2006) were performed by Dr Elizabeth Duncan and Vanessa Barlow at the University of Leeds (data not shown). The twenty mined microsatellite markers (Table 4.2) were first tested in singleplex (either Hex or 6FAM) on several expendable samples (both female and male individuals). Of all the markers, only Obic1344 ($[ttcc]_9$) failed to amplify, this may have been due to the fact that the reverse primer was situated immediately adjacent the repeat region.

Next, using preliminary allelic ranges, duplexes were created using Multiplex Manager, and primer dimerisation was further checked against using AutoDimer. Females from all localities were genotyped in duplex (Germany1 = 14, Germany2 = 11, MeanwoodValleyTrail11 = 1, NorthShropshire = 8, Surrey = 8). The genotypes of Obic77 and Obic52 overlapped each other completely. BLASTing all markers against one another revealed that they were duplicates (100% identity) rather than linked markers. Marker Obic52 was dropped, and Obic77 was retained as it performed better. The seventeen remaining markers preliminarily demonstrate 4.22 alleles on average, and possess an average expected heterozygosity of 0.62.

Given the distances between localities, there exists the possibility of population substructure (e.g. through isolation by distance: Conrad *et al.*, 2018; which may be reinforced through assortative mating with local males: Conrad and Ayasse, 2015). Substructure, or the presence of subpopulation may skew certain measures. For instance, the heterozygosity statistic reported earlier (0.62) might be largely driven by a single population or a subset of the markers, distorting the overall picture. Hence, for marker validation, localities were considered as subpopulations. Another consideration is gametic or linkage disequilibrium (LD). This occurs when two loci are more likely to be passed on together than would be expected by chance. This can arise when loci are present on the same chromosome, or when they are under joint selective pressure (Selkoe and Toonen, 2006). Hence markers/loci were checked for LD. I also checked whether markers/loci are within Hardy-Weinberg equilibrium (HWE). I.e. whether the observed genotypes frequencies are as would be expected in a population with: random mating, no mutation, no genetic drift and no migration (Selkoe and Toonen, 2006). This is important, as deviations from HWE (e.g. assortative mating, which is associated with a certain marker) would overestimate the power of the markers and confound results in a naive analysis. As with the possibility of population substructure, care must also be taken with regard to relatedness. Incorporating related individuals in these preliminary analyses will inflate/overestimate measures of LD and underestimate measures of heterozygosity (inflating deviations from HWE). This is because related individuals are disproportionately more likely to share alleles, making it appear as if certain alleles are linked (LD) when they are not, and making it appear as if there is less heterozygosity than expected (deviation from HWE). Even though a single female was chosen from each nest tube/family unit (Figure 4.2), relatedness of females within a locality was assessed using MLrelate (using all seventeen markers). A single individual was removed from each related pair.

Only half-siblings were found (Tables C.1 to C.4), preliminarily indicating that all commercially acquired nest tubes stem from different mothers.

Naively testing all markers together, across the meta population, indicated no significant LD for the 136 pairwise marker comparisons (Table C.5). Testing per locality, only two out of the 449 pairwise marker comparisons showed a significant LD (Table C.6), likewise indicating that LD is not a problem for the markers. In a similar vein, only 7 out of 66 instances showed a significant deviation from HWE (Table C.7). Given that null alleles can lower observed heterozygosity, they can affect tests for deviations from HWE (Selkoe and Toonen, 2006). F_{null} could only be tested for one German subpopulation, due to low sample sizes (Table C.8). Only six out of the seventeen markers showed an estimated null allele frequency greater than 10% in this locality (Table C.8). Finally, in only eight of 68 instances (seventeen markers by four localities) did the expected (H_e) and observed (H_o) heterozygosity differ by more than 20% (Table C.8; ‘rule of thumb’, personal communication Dr D. Dawson).

The eventual low sample sizes (total = 33; with the subpopulations Germany1 = 10, Germany2 = 9, North Shropshire = 7, and Surrey = 7),which followed here from testing only unrelated individuals by their subpopulations/localities, led to a decrease in power. Yet, p-values (LD and HW tests) were not adjusted for multiple testing. Not correcting for multiple testing inflates the chance of finding a significant result when in reality there is none[35]. Since the paradigm here is reversed — i.e. significant results for LD and HW tests are ‘unwanted’ — not correcting for multiple testing is the most conservative approach. Additionally, significant LD and HW tests, as well as heterozygosity and F_{null} estimates did not occur consistently: across both localities and markers. Deviations for markers generally occurred only once within one locality. Finally, as stated previously, to address the main question of female polyandry, no accurate estimates of parentage or underlying population structure are required. It simply suffices to detect more than a single father to a nest. Therefore, with the low number of significant deviations overall, in conjunction to the considerations outlined, the remaining seventeen markers were deemed adequate to perform parentage analysis in future.

[35] I did not correct for multiple testing here since the paradigm is reversed — i.e the “desired” outcome of a test in this case would be a non-significant result. In combination with the already low sample sizes this would reduce power massively. Additionally, it is unclear what the “family” of the family wise error rate should be in this case (e.g does one correct for multiple testing per marker, per subpopulation or both?).

4.4.4 Multiplex design

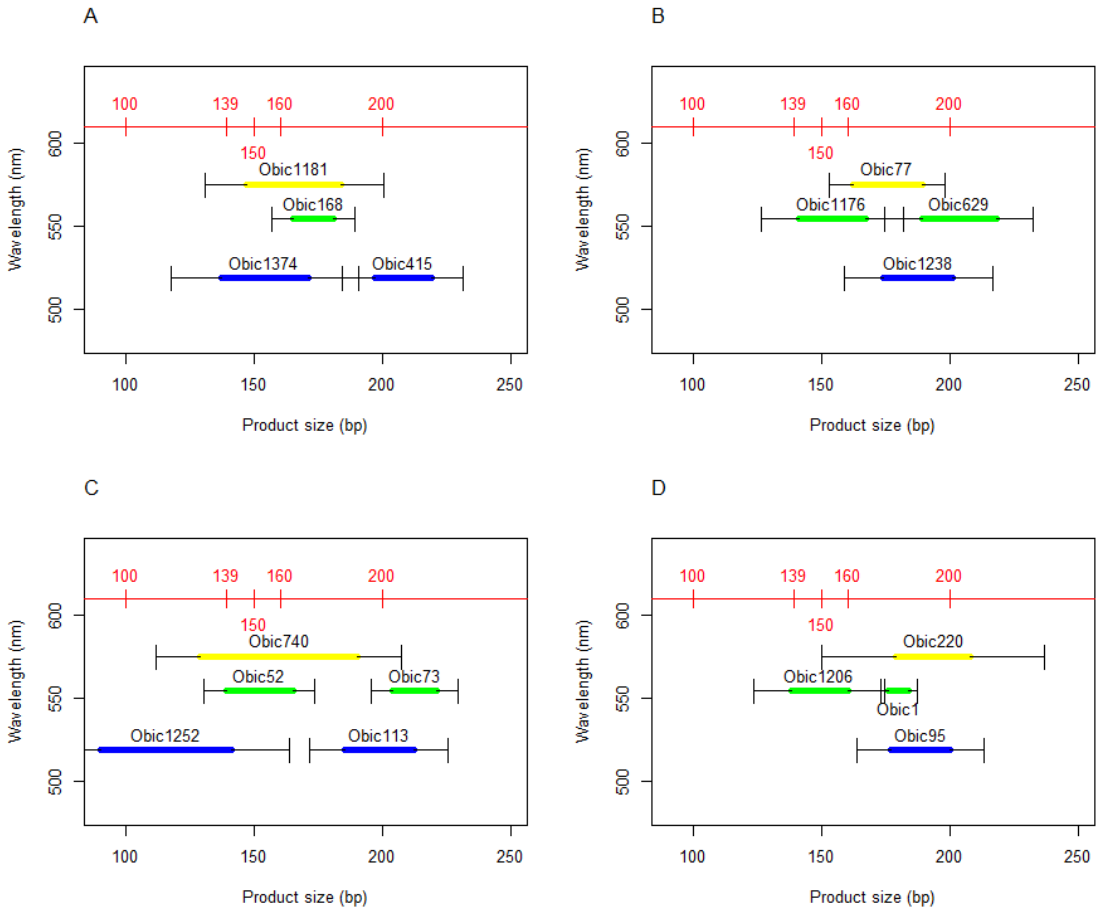

Figure 4.4: Designed and validated multiplexes. A-D) Multiplexes A through to D respectively. Coloured bands represent allelic ranges of the respective markers, with: yellow = NED labelled forward primers, green = HEX labelled forward primers, and blue = 6FAM labelled forward primers. Red = Rox sizing ladder. Since full allelic ranges are yet unknown, multiplexes were designed manually using Multiplex manager. Error bars displayed, therefore, reflect a subjective index:
(allelic range / number of observed alleles) x repeat size,
roughly doubling the existing (observed) allelic range by appending an expected allelic range (based on the observed data; ignoring expected and observed heterozygosity parameters). Given the further use of families (nest tubes) and consequently related individuals, this conservative estimate is unlikely to be reached, let alone exceeded.

Finally, Figure 4.4 presents the final four designed multiplexes incorporating the seventeen validated markers. These were designed manually in Multiplex Manager. The allelic range of a marker may expand still, since when more individuals within a population are genotyped, the chance of hitherto unidentified alleles appearing increases. The largest number of polymorphisms are found genotyping the first five to twenty individuals, after which novel allele discovery starts levelling off (Hale *et al.*, 2012). Thirty three unrelated females

were tested here, but this was done over four subpopulations (disregarding the Leeds trap nest).Hence, given the low sample sizes of each subpopulation (Germany1 = 10, Germany2 = 9, North Shropshire = 7, and Surrey = 7), one could still expect novel alleles to be found (Hale *et al.*, 2012). Regardless of this consideration, the allelic ranges are not expected to expand by much where related individuals are concerned (nest tubes are family members; Figure 4.2). For further details on the criteria used to estimate allelic range expansion, see the caption of Figure 4.4. Multiplexes, similar to the extraction method (section 4.4.2), were likewise investigated for possible increases in allelic dropout. Since primer interactions may still occur in spite of performing checks using bioinformatic tools (Multiplex Manager, and AutoDimer). Of 40 unrelated females tested across the four multiplexes: 1.9% (13/672[36]) genotype calls were found with a loss of heterozygosity, countermanded by a 3.1% (21/672) gain in heterozygosity across genotyping calls. A further 3.4% (23/672) of PCR amplifications failed completely, but these were chiefly concentrated along two samples (9 of 23 failures) and one marker (12 of 23 failures; marker Obic 1176), which may need its primer concentrations optimised within its multiplex (multiplex B).

4.5 Discussion

4.5.1 Trap nesting

Trap nesting resulted in only one *O. bicornis* female creating a nest over the two year sampling period. Trap nesting is generally intended to study species diversity and abundance, community structure and trophic interactions, and species ecology (Krombein, 1967; Gathmann *et al.*, 1994; Tscharntke *et al.*, 1998; Steffan-Dewenter and Schiele, 2004; Staab *et al.*, 2018). It is rarely used to trap specific species. Studies showing specific *O. bicornis* trap nesting are performed: where they are already abundant or have long been established (Yoon *et al.*, 2015; e.g.: Giejdasz *et al.*, 2016; Seidelmann *et al.*, 2016; Coudrain *et al.*, 2016), or nesting tubes and sites are seeded with cocoons (e.g.: Everaars *et al.*, 2011; Fliszkiewicz *et al.*, 2015), or both (Steffan-Dewenter and Schiele, 2004).

Urban trap nest colonisation of *O. bicornis* has been demonstrated previously (Everaars *et al.*, 2011; Yoon *et al.*, 2015), and should be achievable. Several factors are known to affect the nesting choice of *O. bicornis*, the main

[36] Two wells in a 96-well plate, had their sizing ladder fail. Resulting in two multiplexes for two samples that could not be scored. Bringing the total number of genotype calls down to 672 from 680 (i.e. 17 markers x 40 females).

factors are the type of nest trap used (Wilkaniec and Giejdasz, 2003), its dimensions (Seidelmann *et al.*, 2016), and the surrounding forage and environment (Everaars *et al.*, 2011; Persson *et al.*, 2018), and sun exposure (Everaars *et al.*, 2011). Contrary to popular belief, nest orientation (Yoon *et al.*, 2015) and height (Everaars *et al.*, 2011)[37], may not matter much, at least in this species. A main reason, for the very limited success in trap nesting *O. bicornis* here, is that there may be a prevailing low population size based on longitude (based on recordings from BWARS; data not shown). Yet very few *O. bicornis* were spotted overall. Furthermore, given that 43.7% of trap nests did yield occupation by other species, and that trap nesting sites were chosen strategically (personal communication, Dr Thomas Dally), the trap nesting method should in theory work.

The establishment of successful trap nesting may be a numbers game. More trap nests, further spread out across the Leeds area may be beneficial. Yet, while trap nests are a 'passive method', they are still relatively labour intensive (Kessler *et al.*, 2011). Hence, for non-ecology research groups, more directed efforts to improve the trap nesting of *O. bicornis* may be considered. For instance, the use of more sheltered traps to avoid moist and fungal growth (wet cardboard tubes were a frequent problem in this study; MacIvor, 2017; Staab *et al.*, 2018), more sunlit locations (Everaars *et al.*, 2011), and the use of reeds (100% acceptance rate: Wilkaniec and Giejdasz, 2003, do note that cardboard tubes as used here were not tested in their study). Furthermore, the factor of time may be important. Populations may need to be enhanced consistently over several years. Another indication of the importance of time, is the preference of aged material, presumably due to natural nesting cavities being located in dead plant material (Staab *et al.*, 2018). Hence nest tubes should likely be left out for several years (while still regularly checking up on them; Staab *et al.*, 2018), further necessitating durable material and moist avoidance. Alternately, Staab *et al.* (2018) suggest the use of *Phragmites australis* stems, that are several months aged (as cavity nesting Hymenoptera avoid freshly harvested material).

4.5.2 Microsatellites

Even though the use of single nucleotide polymorphisms (SNP) is on the rise as a molecular genotyping tool, microsatellite markers have repeatedly proven their value, particularly with regard to relatedness (Morin *et al.*, 2004;

[37] Note that while Everaars *et al.* (2011) found no effect of height through their citizen science approach, height was not explicitly measured, and the "minimum height requirement" of ~1.5m may still apply.

Städele and Vigilant, 2016; Flanagan and Jones, 2019) — owing to their faster mutation rate and higher levels of polymorphism. Presented here was a preliminary analysis aimed at marker validation. Due to external factors (Covid 19; Lancet, 2020) genotyping the samples from the commercial population in full was not possible as of yet, and subsequently nor was testing monandry in these samples.

I tested and validated twenty newly mined and designed microsatellite markers for use in *O. bicornis*. I showed that seventeen of these markers, are robust under both low and high quality DNA extraction, as well as robust in a multiplex design. These seventeen markers preliminarily demonstrate 4.22 alleles on average and possess an average expected heterozygosity of 0.62. This seems to be somewhat in line with Neumann and Seidelmann (2006), who detected on average 4.18 alleles and an expected heterozygosity of 0.51 for their six microsatellite markers. However, these estimates may be deceptive, as it should be noted that the markers presented here were validated and tested on roughly 40 individuals over four subpopulations (~ 10 per subpopulation). Whereas the six microsatellite markers in Neumann and Seidelmann (2006) were tested on 224 individuals over 10 subpopulations (~ 22 individuals per subpopulation). This is important, since Hale *et al.* (2012) showed that:

> *'The accuracy and precision of mean HE (across loci) increased with increasing sample size from 5 to 20 individuals, but increasing sample size beyond 20 individuals appeared to have little impact on the precision or accuracy of mean HE.'*

Therefore, testing more individuals (across more subpopulations) might show that the seventeen markers here are more informative than currently estimated. In addition to an approximate 10% higher expected heterozygosity in these markers, only two of the markers presented here are dinucleotide repeats. The predominant use of tetranucleotide repeats (13 tetranucleotide; 2 trinucleotide) should further reduce human error in genotype calling (Pompanon *et al.*, 2005), which is crucial when one considers that error rates of even 0.01 per allele can have far-reaching consequences on a study (Hoffman and Amos, 2005). In addition, the number of microsatellite markers validated here is more than double those of Neumann and Seidelmann (2006), with further implications to power in for instance gene flow inference (Landguth *et al.*, 2012).

These microsatellites, will be used in future to test female monogamy in this species, as it relates to the evolution of eusociality (Hughes *et al.*, 2008; Boomsma, 2009). While the supplied nest tubes may not accurately represent circumstances in the wild (commercial breeder and managed population); females are still freely mated. For the German populations in particular, large

aggregations of *O. bicornis* — as maintained by breeders — should yield higher male-male competition. This in turn, increases the probability of detecting female polyandry should it exist. Additionally, the lack of inbreeding avoidance in Hymenoptera, creates the possibility of inbreeding (Packer and Owen, 2001). In *O. bicornis* specifically, females are known to mate with males that are more closely related (Conrad *et al.*, 2010). Along with the relevance of inbreeding to the evolution of eusociality (Hamilton, 1964b), inbreeding is known to reduce fitness (Henter, 2003) and increases the odds of extinctions under haplodiploid sex determination (Zayed and Packer, 2005). Hence levels of inbreeding will also be investigated in future, using these validated microsatellite markers.

Finally, these markers could be used for further resolving outstanding questions of geneflow in *O. bicornis*. For instance, Conrad *et al.* (2018) reported 'only a weakly positive, non-significant trend' for their test of isolation by distance in *O. bicornis*, using the six microsatellites developed by Neumann and Seidelmann (2006). They indicated that a low sample size might be the cause of their results. I would argue that the microsatellite markers presented here would greatly increase the power of inference. Additionally, the markers could be used to test for any effects of the 'commercial movements' of *O. bicornis* across Europe, on gene flow (Conrad *et al.*, 2018). On a similar note, assessing differences in genetic diversity between natural and commercially maintained populations (along with associated fitness traits; Henter, 2003), could inform future breeding practices. Natural populations can be assessed through a combination of trap nesting (4.5.1), and non-lethal sampling (tarsal removal: Holehouse *et al.*, 2003; clipping of wingtip: Châline *et al.*, 2004). Overall, these markers are a useful resource for evolutionary, ecological and conservational studies in *O. bicornis* and related species.

Chapter 5 Ovarian microstructure, oogenesis and mating

5.1 Summary

The reproductive ground plan hypothesis (RGPH) posits that reproductive constraint evolved through the co-option of traits controlling reproduction and maternal care in ancestrally solitary species. One way of assessing hypotheses like the RGPH, is through cross-species comparisons. Given the role of mating to activate or accelerate oogenesis in many insect species, it can be seen as a controlling facet of reproduction. Mating status is therefore a likely candidate for co-option into QMP-mediated reproductive constraint. In order to investigate the potential co-option of mating status into reproductive constraint — and in order to facilitate further cross-species comparison — I present a microstructural study of the *O. bicornis* ovary in this chapter. I hypothesised that young, virgin *O. bicornis* females' ovaries would resemble the quiescent ovaries of queenright *A. mellifera* workers. I compare key stages of oogenesis to those of *A. mellifera* workers, and study oogenesis with regard to age and mating status. I show that *O. bicornis* females eclose with primed and active ovaries, with the rate of oogenesis increasing over time, but that mating does not affect the reproductive physiology of *O. bicornis* in any discernible manner. I then go on to conclude that QMP-mediated adult reproductive constraint in the honey bee worker is unlikely to have been derived from mating status.

5.2 Introduction

The reproductive ground plan hypothesis (RGPH; Amdam *et al.*, 2006) posits that the reproductive division of labour originated from a decoupling of maternal behaviour (non-reproductive worker) and reproductive status (reproductive queen). Consequently, ancestral mechanisms that controlled reproduction in solitary individuals in response to environmental stimuli — further referred to as reproductive control— are thought to have been de-coupled from these environmental factors and co-opted into the social environment (Amdam *et al.*, 2006), where ultimately a queen reproductively inhibits workers — further referred to as reproductive constraint. This social environment is mainly signalled through queen pheromones (Winston, 1991).

Within the social hymenoptera, queen pheromones are thought to be derived from cuticular hydrocarbons (CHC; Van Oystaeyen *et al.*, 2014; Holman, 2018). CHCs serve pleiotropic roles in insect communication. They

signal mating status, species recognition, colonial and/or kin recognition (Oi *et al.*, 2015). Honey bee queen mandibular pheromone (QMP) on the other hand, was likely not derived from CHCs (Van Oystaeyen *et al.*, 2014; Lovegrove *et al.*, 2019), although a redundant set of queen pheromones has also been found in *A. mellifera* (Princen *et al.*, 2019a). The current paradigm suggests that queen pheromones act as conserved honest[38] queen fecundity signals (Van Oystaeyen *et al.*, 2014; Oi *et al.*, 2015). These honest fecundity signals are thought to be: derived from fertility cues produced as by-products of ovarian development[39] , derived from contact sex pheromones, and/or derived from oviposition deterring pheromones (Oi *et al.*, 2015). In the species of study, *O. bicornis*, female fecundity is thought to be signalled through CHCs (Seidelmann, 2014a; Seidelmann and Rolke, 2019).

Fecundity and insemination are closely linked in Hymenopteran queens. Queen pheromones are known to change significantly with mating status (*A. mellifera*: Slessor *et al.*, 1990; *Leptothorax gredleri*: Oppelt and Heinze, 2009). In advanced social species, mating is even necessary for queen ovary activation (*Melipona quadrifasciata anthidioides*: Martins and Serrão, 2004a; de Souza *et al.*, 2007; Tanaka *et al.*, 2009; *A. mellifera*: Tanaka and Hartfelder, 2004; Tanaka *et al.*, 2006; Nino *et al.*, 2013). The effects of mating have also been shown to be important for the chemical profile of mandibular glands and QMP in *A. mellifera* (Plettner *et al.*, 1997; Richard *et al.*, 2007) and CHC profiles in *Bombus terrestris* (Jansen *et al.*, 2016). Furthermore, *A. mellifera* workers lack spermatheca (Winston, 1991; with the exception of the queen-like laying workers of *A. mellifera capensis*: Anderson, 1963; Phiancharoen *et al.*, 2010). Indeed, a lack of mating and spermatheca in workers can also be considered a facet of reproductive constraint (Khila and Abouheif, 2010).

Given the above links of mating status with: fecundity, ovary activation, CHC (or QMP in the case of *A. mellifera* queens), and reproductive constraint (non-reproductives often lacking spermatheca; Bourke, 1999) — it is plausible for mating status to have been a precursor to QMP-mediated reproductive constraint. As such, mating status may have had an ancestral role in reproductive control in solitary bees (as it does in many diplo-diploid insects), and may have been co-opted into QMP-mediated reproductive constraint.

In this chapter, I present a microstructural study of the *O. bicornis* ovary, in order to facilitate further reproductive cross-species comparisons between *A. mellifera* and *O. bicornis* in further chapters. Additionally, I hypothesised that

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[38] Honest signalling *sensu* Zahavi (1975).

[39] Ovarian development causes changes in the CHC of many arthropods, including solitary insects.

young, virgin *O. bicornis* females' ovaries would resemble the quiescent ovaries of queenright *A. mellifera* workers. This, since the ovaries of hibernating *O. bicornis* are presumably in stasis over winter, and would require activation upon female eclosion. I further hypothesised that oogenesis would remain inactive, would not achieve full capacity over time, or would proceed at a slower pace in females that retained their virginity as opposed to females that were mated. In summary, I hypothesised that virginity would have an overall negative effect on oogenesis in *O. bicornis*.

5.3 Materials and methods

5.3.1 Husbandry and experimental design

For a more exhaustive overview of *O. bicornis* husbandry see Chapter 3. Briefly, bees were kept at 21-23˚C with a 18:6 h light: dark cycle. Bees were supplied with: makeshift flowers and catkins with dusted with ground pollen, 50% sucrose solution (filter sterilised; 0.22 µm; Millipore), additional fondant paste (Candipolline Gold), Fabre's hives (Oxford bee company), and mud for nest building (70% Fuller's earth by Intra Laboratories and 30% white silica sand by Cristobalite). Bees were fed *ad libitum* throughout the study.

Females were hatched in isolation in individual plastic containers in the dark, and subsequently housed according to treatment for three days (mesh cage; 60 x 60 x 90 cm). The mated group was kept in a in a 3:1 ratio (i.e. 9 females and 3 males; Fliszkiewicz *et al.*, 2013), the unmated group contained 12 females and no males. Two one-hour observations were performed on the same day to observe attempts at mating. Additionally, mating status was confirmed upon dissection of the females by visual examination of the spermathecae. Mating plugs were found rarely in our laboratory set-up (these regress within a day: Seidelmann, 1995). Three days after the introduction of males, mated females were marked red (Uni Posca marker) on the thorax, and both mated and virgin females were subsequently transferred to a larger cage (65 x 90 x 140 cm). Females were dissected for their ovaries at eight different time points (Figure 5.1).

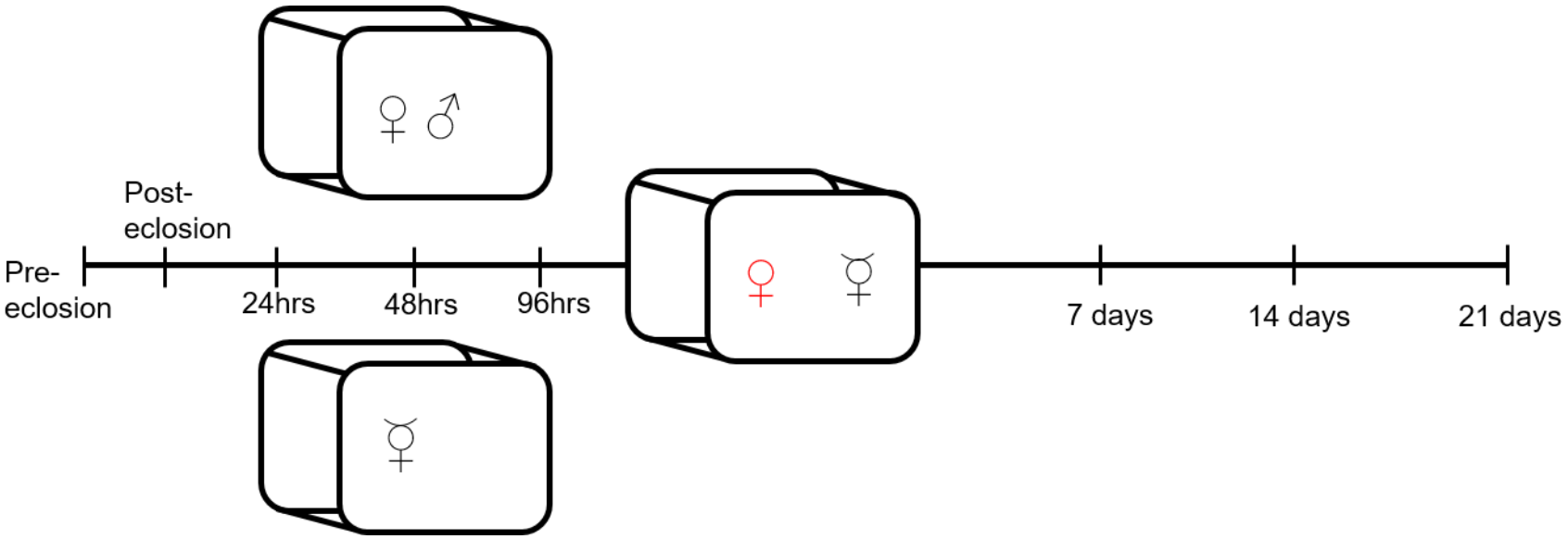

Figure 5.1: Overview experimental design *O. bicornis*. Females were dissected pre-eclosion, and within 24 hours post-eclosion. Further females were divided up into two cages (mated and virgin) for three days, with females being dissected on each day (24hrs, 48hrs, and 96hrs timepoints). After three days, the remaining females were placed together in a larger cage, with mated females being marked red on the thorax. These were then dissected after 7, 14 and 21 days. Note that in a field experiment, the median survival time of *O. bicornis* females was 30 days (inferred from figure in Felicioli *et al.*, 2018).

A. mellifera mellifera workers were kept according to standard practices in British National hives at the University of Leeds, School of Biology, Research apiary. Colonies were assessed weekly for egg-laying, queen cells, food stores and parasites. Queenless workers were obtained by placing frames of brood and adult bees into a standard polystyrene nucleus box. Foraging bees typically return to the parent colony, leaving the transferred frames with nurse bees and emerging workers only. These typically activate their ovaries after 2-4 weeks in the absence of a queen (Duncan *et al.*, 2016). A queenless hive was considered reproductively active once 30% of dissected bees showed stage 3 ovaries (Duncan *et al.*, 2016). Classification of ovary state followed the modified Hess scale as in Duncan *et al.* (2016). These four ovary states were taken to represent ovaries activating over time.

5.3.2 Staining and microscopy

Microdissection, fixation and staining followed the protocol outlined in Chapter 2 (sections 2.2 and 2.10). With the exception of phalloidin (actin) and DAPI (nuclear counterstain) staining, which followed a modified protocol for IHC (section 2.10). Tissue was fixed for only ten minutes and permeabilised for only 90 minutes, after which counterstains were applied immediately (i.e. omitting all blocking and washing steps required for antibody staining). Confocal imaging was performed on the following day using a Zeiss LSM 880 upright (2 PMTs) using a 405 nm diode laser (DAPI) and a 488 nm argon laser (phalloidin). Images were taken at x10 (EC Plan-Neofluor 10x/0.30) or at 20x for germarial and terminal filament detail (Plan-Apochromat 20x/0.8). Images were acquired and processed using Zen 2.3. Processing involved stitching image tiles (normalised cross correlation coefficient = 0.9), maximal intensity projections of z-stacks, and cropping of images. Z-stacks varied in thickness from 15 to 238 µm, with thickness averaging 100 µm for images at 10× magnification and 36 µm for images at 20× magnification. Only informative slices were used for maximal intensity projections. Whether confocal images presented are maximum intensity projections or single slices (i.e. ‘optical section’) is indicated in the figure captions. Several *O. bicornis* females, considered outside of the above experimental design, were dissected for IHC staining. IHC staining of phospho-histone H3 (pHH3) was used as a marker of cell division (Hendzel *et al.*, 1997), with the germarium and terminal filament as regions of particular interest (Tanaka and Hartfelder, 2004). Ovarian nomenclature follows Büning (1994).

5.3.3 Morphological measurements

Measurements of *O. bicornis* ovarioles were taken dependent on tissue sample quality (3-6 intact ovarioles per individual). Specifically, the transition from terminal filament to germarium proved particularly fragile, leading to the terminal filament regularly breaking off while removing the intima. To test for quantitative differences between treatments in ovarian dynamics, I used ImageJ to measure ovariole traits. The lengths of the terminal filament, the germarium and vitellarium were measured to investigate egg limitation and ovariole growth. The total number of oocytes, number of globular yellow bodies (i.e. corpora lutea that consist of degenerating postovulatory follicle cells; Büning, 1994) and number of mature oocytes (equivalent to stage 7 and 8 oocytes in Wilson *et al.*, 2011) were counted to investigate the onset of oogenesis. Mature oocytes were (generally) not mounted on slides, and hence were not part of vitellarium length measurements. Rate of oogenesis was approximated here, by first measuring longitudinal and transverse sections of individual oocytes, and subsequently calculating their volume as a prolate spheroid (similar to Cane, 2016):

$$V_{prolate\ spheroid} = \frac{4\pi}{3}\ a^2\ c \qquad \text{Equation 5.1}$$

With *a* the polar radius and *c* the equatorial radius. By fitting these into a model (Appendix D), '*oogenesis rate within an ovariole'* was approximated. Additionally, the number of cells in the terminal filament and the number of cells until the first discernible oocyte in the germarium were counted. This was done in ImageJ using the DAPI counterstain, and was semi-automated (Papadopulos *et al.*, 2007) to limit observer bias. Thresholding, and watershedding were performed manually, after which cells were counted automatically.

5.3.4 Statistical analysis

For detailed information on statistical analysis performed along with all results, see Appendix D. Briefly: statistical analysis was carried out using R 3.5.1 (R Core Team, 2016). Linear mixed models and generalised linear mixed models were made using *lme4* (Bates *et al.*, 2015). Assumptions were investigated following Zuur *et al.* (2010) and model tests were performed using *lmerTest* for linear mixed models (Kuznetsova *et al.*, 2016), or through log likelihood-ratio tests for generalised linear mixed models (Bates *et al.*, 2015). Dependent variables were modelled with time (days) and an individual's weight as covariates, treatment (mated or virgin) as a fixed effect, and individual as a random effect (and random slopes for oocyte maturation estimates). Degrees of freedom presented throughout the text and in Table D.2 are Satterthwaite approximations.

5.4 Results

5.4.1 Overview of the *O. bicornis* ovary

O. bicornis consistently possesses three ovarioles (Figure 5.2A) for each of its two ovaries (Figure 5.2B; as in other *Osmia*, Maeta and Kurihara, 1971). In between the ovarioles and the intima, corpora lutea Figure 5.2C; Büning (1994) are shed from the mature egg (Figure 5.2D) as it leaves the lateral oviduct (Figure 5.2B). These corpora lutea accumulate beside the ovarioles. For the mature egg to pass into the median oviduct, it needs to pass by the epithelial plug (Figure 5.2B), which consists of a population of cells that undergo autolysis (Velthuis, 1970). The mature egg can then be fertilised in the median oviduct, when sperm is released from the spermatheca (Figure 5.2B). A male mating plug may also be present in the median oviduct shortly after mating (Figure 5.2B; Seidelmann, 2015).

As in *A. mellifera*, the *O. bicornis* ovary is polytrophic and meroistic in nature, where oocytes alternate with nurse cell chambers (Figure 5.2E). A nurse cell chamber consists of up to 37 nurse cells (Figure 5.2F) and is in direct contact with the developing oocyte. The individual ovarioles can further be subdivided into: the terminal filament, the germarium which is the region of the ovary where the nurse cells (trophocytes) and oocytes are specified, and the vitellarium which contains nurse cell clusters and maturing oocytes covered in a follicular epithelium (Figure 5.2G).

The nurse cells, along with the follicle cells, deposit RNAs and protein into the developing oocyte, these RNAs are essential for maturation of the oocyte and early development of the embryo. The nurse cell chamber is lined with actin bundles (Figure 5.3A), presumably to facilitate cytoplasmic streaming (Gutzeit and Koppa, 1982; Gutzeit, 1986a). Cytoplasmic streaming occurs when the adjacent oocyte is almost fully mature. The actin bundles of the nurse cell chamber will contract, which allows for the nurse cells to dump their remaining cytoplasmic content into the oocyte via the increased number of ring canals, and through the intermediate cell (Figure 5.3B, white arrow). The depleted nurse cells will remain connected to the next maturing oocyte via the follicular stalk (Figure 5.3B), while they degrade (Figure 5.3C). The follicle cells lining the maturing oocyte meanwhile build actin fibres once choriogenesis is complete (Zhang, 1992). These fibres are thought to facilitate ovulation (Zhang, 1992) and will involute along with the follicle cells, leaving corpora lutea in the ovariole.

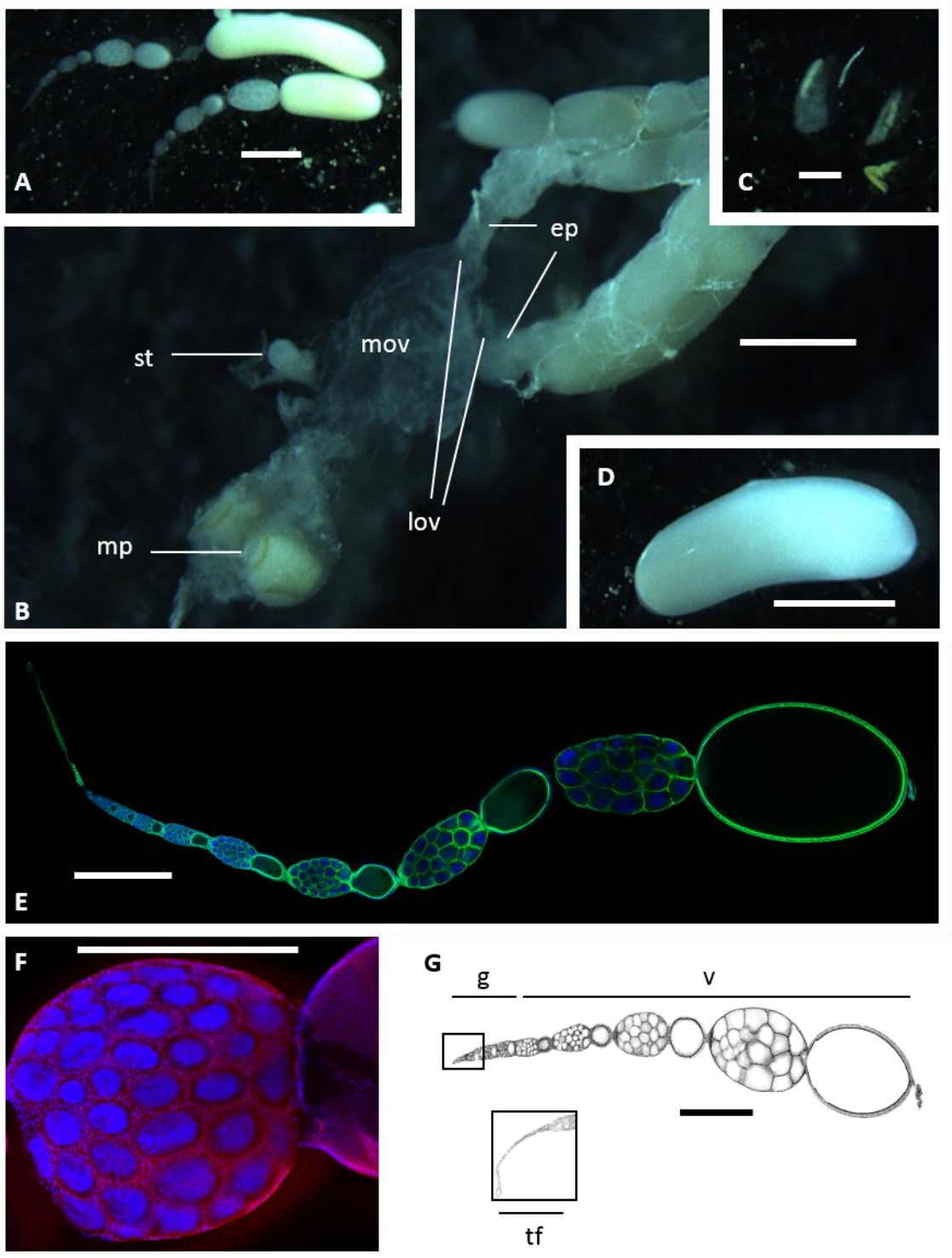

Figure 5.2: Overview of the *O. bicornis* ovary.A) Two ovarioles with maturing terminal oocytes (scalebar = 750µm). B) Ovaries with accessory structures (ep = epithelial plug, lov = lateral oviducts, mov = median oviduct, mp = mating plug, st = spermatheca; scalebar = 500µm). C) Corpora lutea (yellow bodies; scalebar = 500µm). D) Mature egg (scalebar = 1.5 mm). E) Maximum intensity projection of an ovariole, stained for nuclei (DAPI; blue) and actin (phalloidin; green), with scalebar = 500µm. F) Optical section of a squashed nurse cell chamber (with 38 nurse cells) stained with Tubulin E7 (red) and DAPI (blue; scalebar = 500 µm). G) Ovariole overview (g = germarium, tf = terminal filament, and v = vitellarium; scalebar = 500 µm).

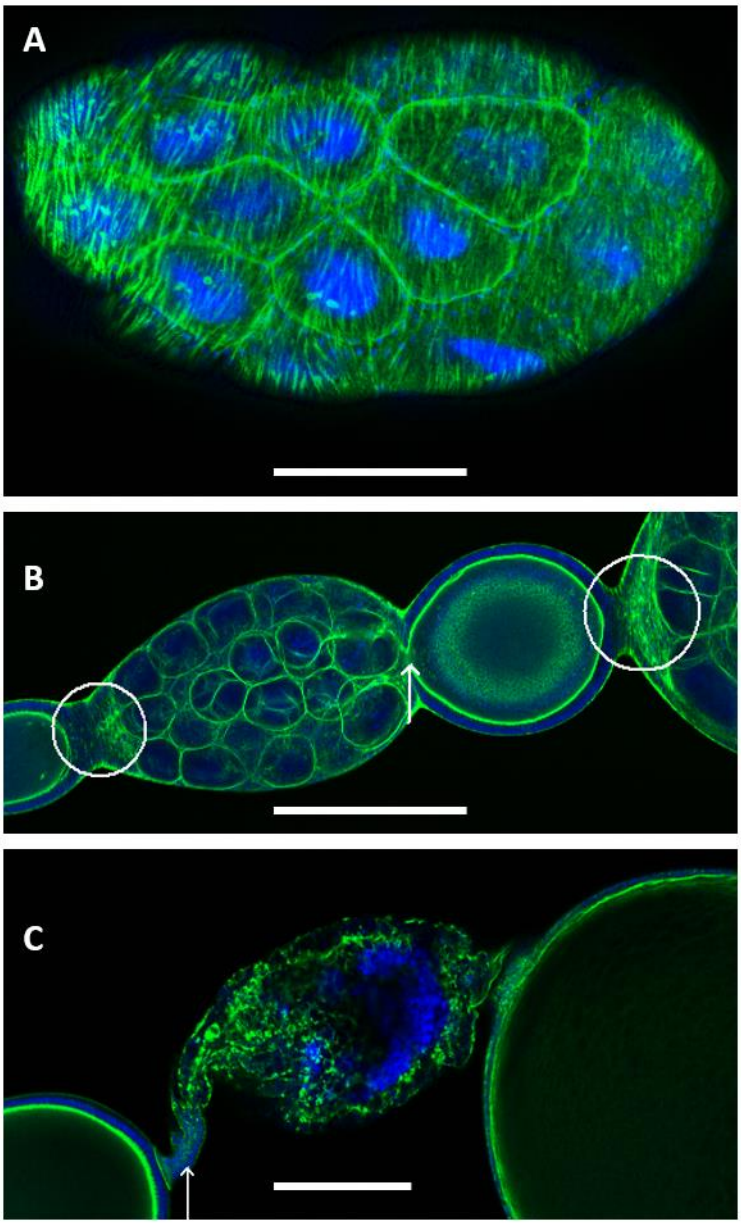

Figure 5.3: Actin bundles in *O. bicornis* nurse cells. A) Nurse cell chamber of a late stage oocyte, showing transversely oriented actin bundles and actin rings at nurse cell nuclei. B) Follicular stalks separate oocyte from following nurse cell chamber (white circles), white arrow shows the intermediate cell (i.e. the nurse cell opening up into the oocyte). Odd pattern inside the right-hand oocyte is an artefact of the maximal intensity projection, projecting a z-plane section of the outside of the oocyte into the middle of it. C) Degenerating nurse cells of a mature oocyte, retaining the follicular stalk to the prior oocyte (white arrow). All ovarioles are DAPI (blue) and phalloidin (green) stained, and all scale bars are 200 µm.

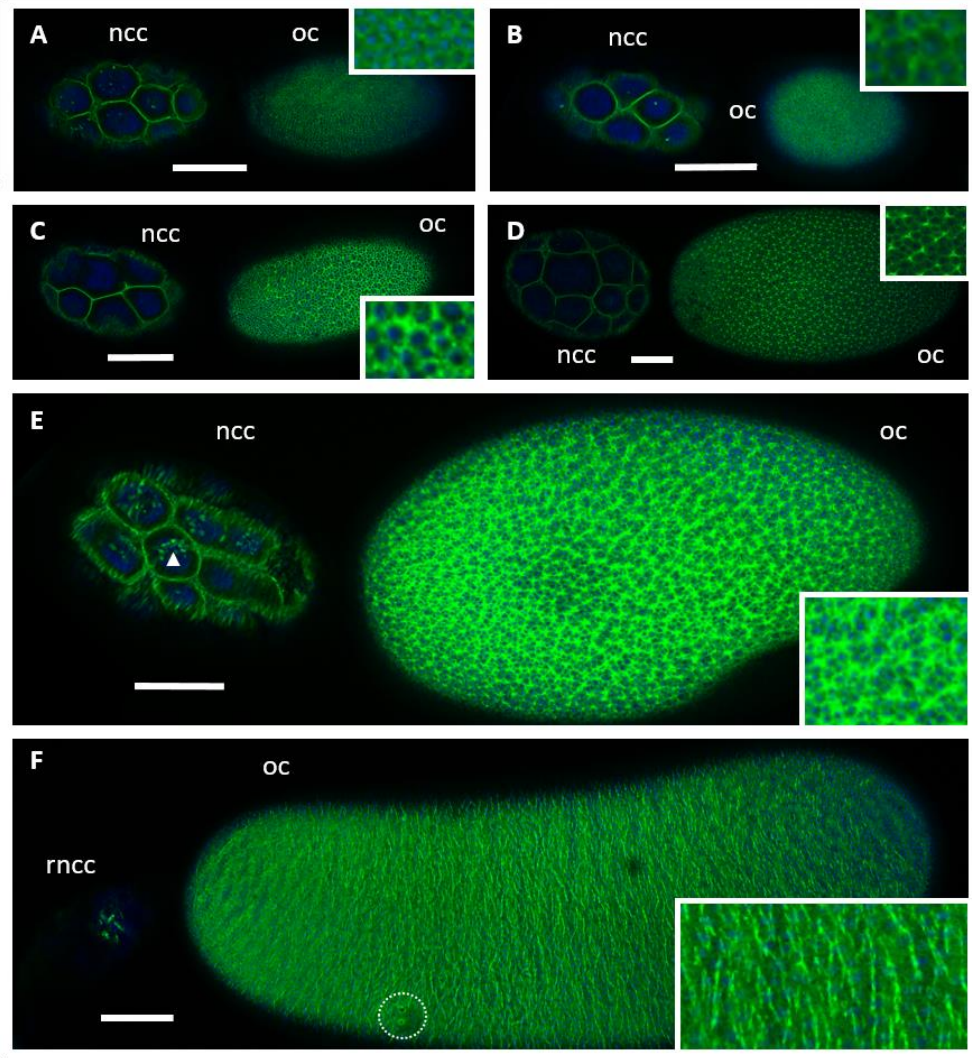

Figure 5.4: Oocyte F-actin during oogenesis in *O. bicornis*. A-D) Follicular actin during patency and oocyte growth. During patency, spaces between follicular cells are made, creating channels so that vitellogenins can pass directly through, into the oocyte (Nation, 2008). At this stage, the actin network appears to form a dense raster (insets of A-C) around the population of epithelial follicle cells. Upon oocyte growth and elongation (D), the space between the follicle cells increases further, and the actin network surrounding the follicle cells breaks up — showing characteristic triangular shapes of actin in the process. E) Following choriogenesis during late stage oogenesis, follicle cells start synthesising actin fibres, showing a star-shaped appearance during the initial stage of synthesis. F) Following full oogenesis, the mature oocyte is surrounded by stress fibres (see inset for detail). These fibres are thought to facilitate ovulation from the ovariole into the oviduct, after which the fibres and follicle cells will involute, and remain in the ovariole as corpora lutea (Zhang, 1992). Phalloidin (green) and DAPI (blue) stained, all scale bars = 200 µm. Insets = digital magnifications, dashed circle = aeropyle., ncc = nurse cell chamber, oc = oocyte, and rncc = regressing nurse cell chamber.

5.4.2 Microstructural comparison of ovarioles

The *O. bicornis* terminal filament (Figure 5.5A,C) lacks the characteristic stack of coin organisation present in *A. mellifera* (Figure 5.5B,D) and other insects (Büning, 1994). In *A. mellifera*, putative germline stem cell nests are thought to be interspersed between these coin shaped cells (white circle Figure 5.5B,D; Tanaka and Hartfelder, 2004), which seemingly funnel out into the germarium from the terminal filament (although the exact way that these cells would enter the germarium is unknown; Tanaka and Hartfelder, 2004). *O. bicornis* only possesses cell nuclei resembling those of the interspersed clusters of putative germline stem cells in its actin enriched terminal filament (Figure 5.5A,C), with a distinct transverse septum marking the boundary between the terminal filament and germarium. Presumptive germline stem cells then vacate the terminal filament (red arrows, Figure 5.5C) to immediately form a cystocyte cluster beyond the transverse septum (white arrow, Figure 5.5A).

The germarial cystocyte clusters contain the presumptive oocyte and a set of sister cells which are destined to become nurse cells. In *O. bicornis*, the cystocyte cluster (white circle, Figure 5.5E) will lose its dense clustering (white arrow, Figure 5.5E), until ring canals are visible when oocyte and nurse cells have been specified (white line, Figure 5.5E). In *A. mellifera* the presumptive oocyte and nurse cells are connected by a distinct polyfusome (white arrows in Figure 5.5F). Cells in this cystocyte cluster undergo successive rounds of cell-division followed by incomplete cytokinesis while the cluster migrates posteriorly. The fusome connects the cells of the cystocyte cluster acting as an intracellular bridge. In *D. melanogaster*, the fusome has been shown to contribute to oocyte specification and microtubule polarisation when it divides assymmetrically (Greenbaum *et al.*, 2011). Following specification of the oocyte from the cystocyte cluster, the fusome will break up, giving rise to individual ring canals which act as stable intracellular connections facilitating the flow of RNA and protein from the nurse cells to the developing oocyte (white circle in Figure 5.5F). In *O. bicornis* the polyfusome is either lacking, or too transitive to be observed. Instead, of a slow progression from polyfusome to cystocyte cluster as is the case in *A. mellifera*; germaria were often found already containing a cystocyte cluster immediately following the transverse septum (Figure 5.5A,E,G). This cluster will often already possess ring canals (e.g. white circle in Figure 5.5G).

Overall, the *O. bicornis* germarium is much shorter than that of *A. mellifera*, and there are generally only a few cystocyte clusters visible in the germarium before oocytes are specified and readily discernible (Figure 5.5G). Once the oocyte is formed, rod-like actin elements can be detected in the ooplasm around the nuclear envelope of the oocyte nucleus (Figure 5.5G). The cystocyte clusters arising in the *O. bicornis* germarium (Figure 5.5E,G) lack the characteristic comet-like arrangement as is the case in *A. mellifera* (Figure 5.6H; Tanaka and Hartfelder, 2004).

Mitosis was present from the vitellarium onwards (Figure 5.5) in follicle cells in both *A. mellifera* and *O. bicornis*. Cell division is known to occur in the terminal filament and at the base of the germarial region in *A mellifera* (BrdU-staining: Tanaka and Hartfelder, 2004). It is thought that the number of germline stem cells is maintained through mitosis (i.e. germline stem cell niche, *sensu Drosophila*; Song *et al.*, 2007), whereas subsequent meiosis will initiate cell differentiation and give rise to oocyte and nurse cells. The pHH_3 IHC staining used here did not capture mitosis in the terminal filament, nor the germarial region (Figure 5.6A). This may be due to the transient nature of cell divisions in conjunction with fixing samples, as is likewise evident from the lack of germarial and terminal filament staining in *A. mellifera* samples (Figure 5.6B). Mitosis within those regions can therefore not be excluded for *O. bicornis*. Note that the non-punctate staining at the base of the *O. bicornis* germarium (Figure 5.6A) is likely an artefact (antibody trapping) as it could also be seen in the control (Figure 5.6B).

Given the description of processes above, the ovariole of both *A. mellifera* and *O. bicornis* can both be viewed as a *conveyor-belt*. Germline stem cells are transportedfrom the terminal filament into the germarium. In the germarium, germline stem cells arise as cystocyte clusters. As this cluster is transported into the vitellarium, cells differentiate to oocyte and nurse cells, where they start to undergo vitellogenesis.

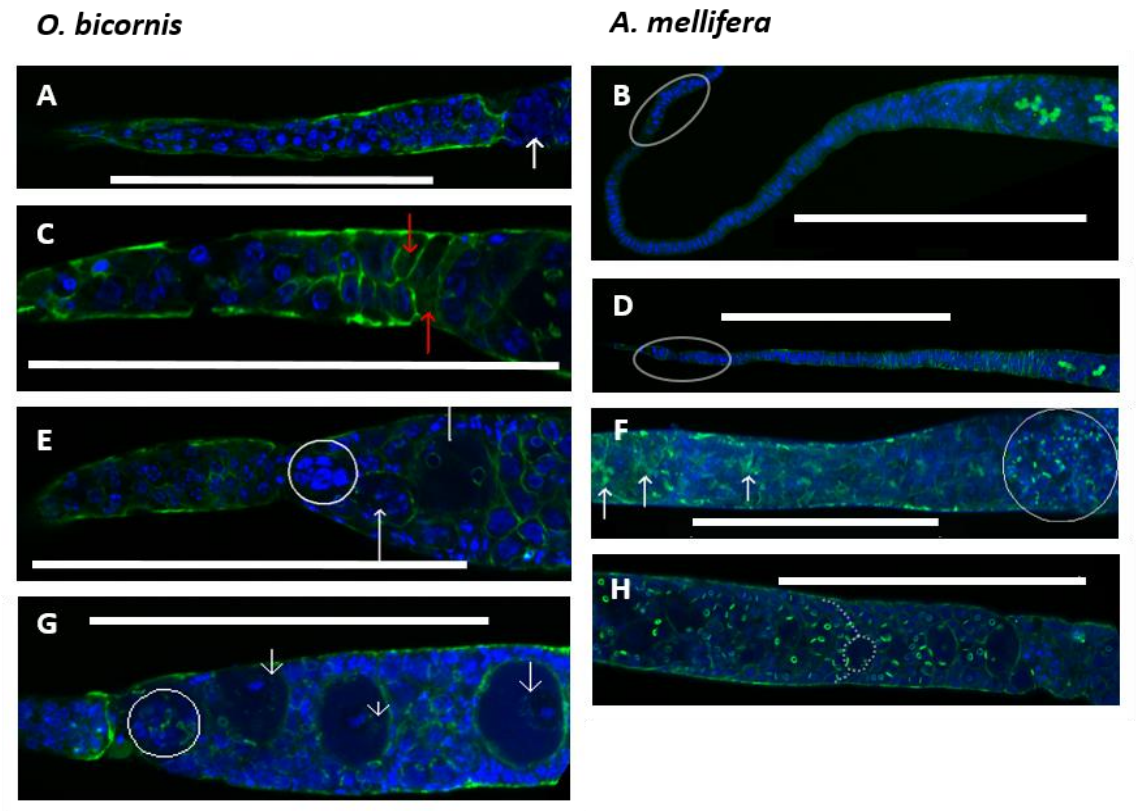

Figure 5.5: Terminal filament and germarial microstructure. (A-G) Maximum intensity projections and (H) optical section, with DAPI (blue) and phalloidin (green). Scale bars; (A-G) = 200 µm and (H) = 100µm. A) *O. bicornis* terminal filament with white arrow indicating a cystocyte cluster exiting the terminal filament into the germarium across the transverse septum. B) *A. mellifera* terminal filament, with distinct stack of coin organisation funneling out into the germarium, followed by the first polyfusome structures. White circle indicates clustered presumptive germline stemcells (Tanaka and Hartfelder, 2004). C) Terminal filament with transverse septum of *O. bicornis*, with red arrows indicating where presumptive germline stem cells vacated the terminal filament. D) *A. mellifera* terminal filament, with distinct stack of coin organisation funneling out into the germarium, followed by the first polyfusome structures. White circle indicates clustered presumptive germline stemcells (Tanaka and Hartfelder, 2004). E) *O. bicornis* germarium, with white circle showing first cystocyte cluster, losing its dense clustering (white arrow) until ring canals are visible when oocyte and nurse cells have been specified (white line). F) Polyfusomes (white arrows) connecting cystocyte clusters progressing along the germarium and dissipating into individual ring canals (white circle) connecting nurse cells and oocyte. G) Further detail of the *O. bicornis* germarium, containing cystocyte cluster (white circle) and rod like actin around the nuclear envelope (white arrows). H) Further detail of the *A. mellifera* germarium, showing typical comet-like appearance of the nurse cells (shape outlined by dashed lines; Tanaka and Hartfelder, 2004).

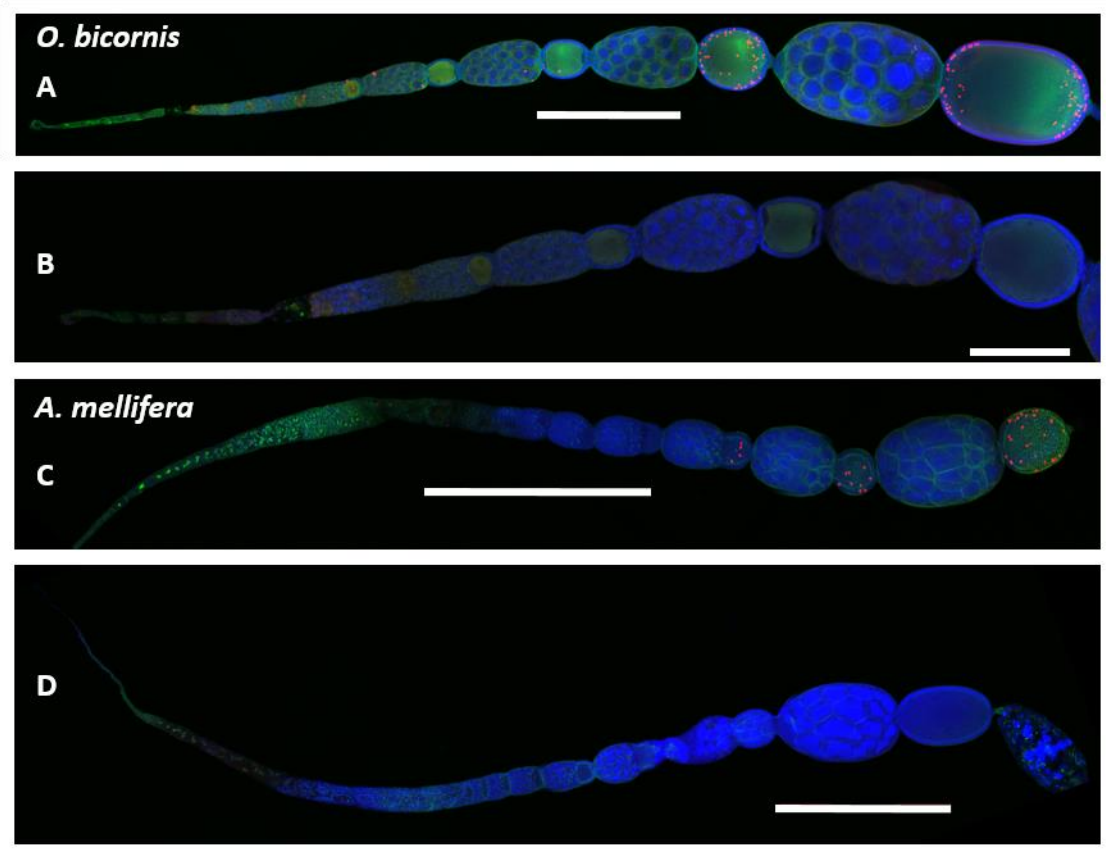

Figure 5.6: pHH$_3$ in *O. bicornis* and *A. mellifera*. Maximum intensity projections with DAPI (blue), phalloidin (green), and pHH$_3$ (red). Scale bars (A-C) = 200 µm and (D) = 500µm. A) *O. bicornis* ovary shows punctate pHH$_3$ staining, marking dividing follicle cells that line the fast growing oocytes. For the terminal oocyte, these seem to be focused around the anterior and posterior pole of the oocyte. More frequent divisions of follicle cells around the poles of the oocyte may be related to oocyte elongation. Occasional staining of follicle cells on nurse cell chambers is also observed. Staining of germline cells was witnessed in neither the terminal filament, nor the germarium. B) Control staining for *O. bicornis* (secondary antibody only) showing only background staining. C) The same pattern is observed in *A. mellifera* as was with *O. bicornis* (A); with dividing follicle cells along the oocytes. D) Control staining for *O. bicornis* (secondary antibody only) showing little to no background staining.

5.4.3 Oogenesis in *O. bicornis*

Unlike honey bee queens which eclose with oocytes arrested in previtellogenic development (Tanaka and Hartfelder, 2004), *O. bicornis* eclose with both pre- and post-vitellogenic oocytes (stage 2 ovaries) and the first fully mature oocytes (Figure 5.2D) can be detected 96 hours after eclosion (Figure 5.7; stage 3 ovaries). Corpora lutea (Figure 5.2C), which consist of post-ovulatory follicle cells (Büning, 1994), start accumulating soon after (Figure 5.7). Examining the number of oocytes in both virgin and mated females for 21 days post-eclosion, revealed that the number of oocytes per ovariole decreased significantly over time in *O. bicornis* ($\chi^2_{1,5}$ = 9.414, p = 0.009; Figure 5.7). This also translated into a decrease in both length of the vitellarium and total ovariole length over time (Figure D.5). However, the vitellarium disproportionately determines total length (Figure D.2) and drives the effects in total ovariole length.

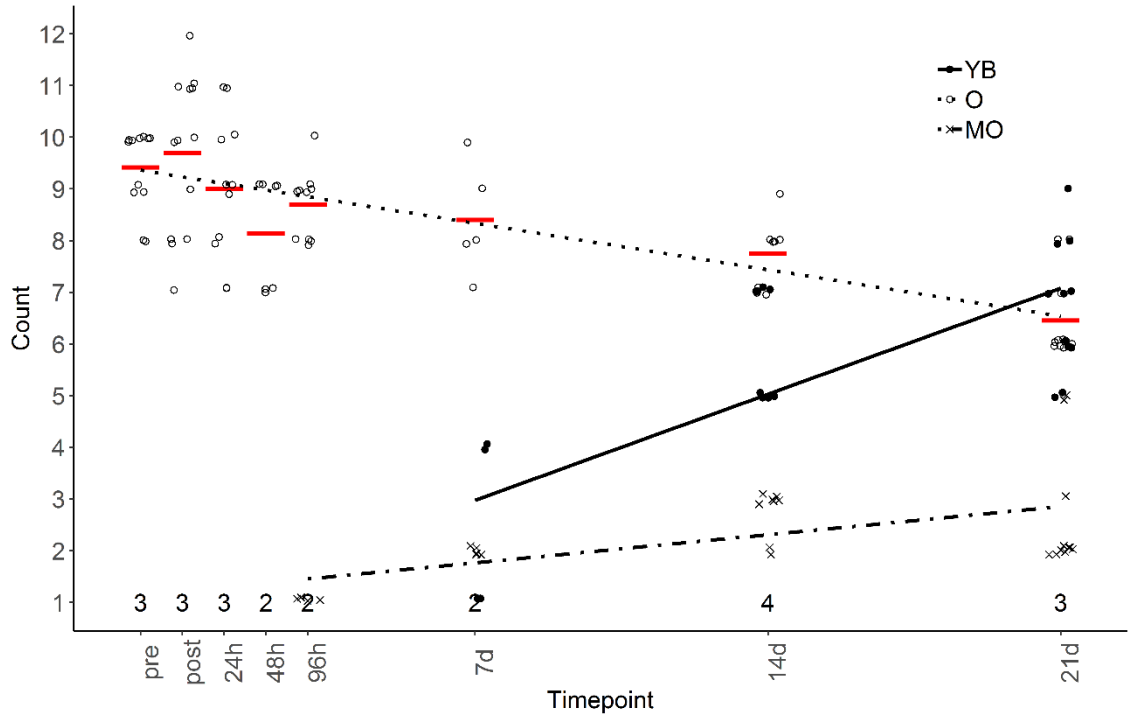

Figure 5.7: Overview of ovarian dynamics in *O. bicornis*. Counts of oocytes, mature oocytes and accumulated corpora lutea over time. YB = yellow bodies per ovary (corporae lutea), O = oocytes per ovariole, and MO = mature oocytes per individual. Points are jittered, slopes represent linear regressions, red bars represent mean number of oocytes per ovariole (pre = pre-eclosion; post = post-eclosion). The number of oocytes in ovarioles decreased over time, while yellow bodies accumulated in the ovaries.

A significant interaction between time and mating status was found for vitellarium length ($F_{1,29}$ = 4.882, p = 0.035). Yet, many data points for the intermediate time points in the mated group are absent (due to poor sample quality; see Figure D.5). Hence, only the overall decrease over time was considered reliable (vitellarium: $F_{1,28}$ = 10.49, p = 0.003). This decrease over time, the absence of a clear polyfusome in *O. bicornis*, the apparent absence of cell division in the terminal filament (Figure 5.6A), and the ambiguity surrounding the existence of a germline stem cell niche in the Hymenoptera in general (Büning, 1994); allows for the possibility of egg limitation and reproductive senescence in this synovigenic species (Rosenheim, 1996). Yet no significant decrease could be found in the number of cell nuclei over time (terminal filament: $\chi^2_{1,4}$ = 0.004, p = 0.949; and early germarium: $\chi^2_{1,4}$ = 1.423, p = 0.233; Figure D.6). Nor did the terminal filament, or the germarium vary significantly in length over time (terminal filament: $F_{1,21}$ = 0.762, p = 0.392; and germarium: $F_{1,26}$ = 0.104, p = 0.750) which may be consistent with the presence of a germline stem cell niche in this species.

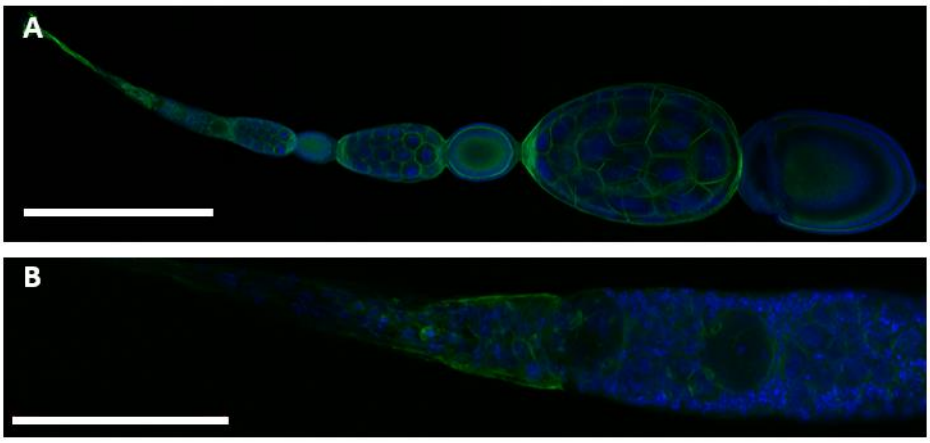

Figure 5.8: Ovarioles of wild mated and free living *O. bicornis*. The ovarioles of free living females showed no structural differences toward the lab reared *O. bicornis* females used throughout this study (e.g. Figure 5.2E; and Figure 5.5 A,C,E and G). (A-B) Maximum intensity projections with DAPI (blue) and phalloidin (green). A) Overview of a free-living *O. bicornis* ovarioles (scale bar = 500µm). B) Terminal filament and germarial microstructure of a free-living *O. bicornis* female (scale bar = 100µm).

5.4.4 Effect of mating on oogenesis in *O. bicornis*

Firstly, I investigated whether lab reared *O. bicornis* were representative of those reared in the wild, and could find no structural nor microstructural differences between the ovarioles of either (Figure 5.8). To address the hypothesis that reproductive constraint evolved from ancestral control of reproduction in response to mating status I compared the stages of ovary activation of queenless workers (QLW; Figure 5.9) with those of both virgin and mated *O. bicornis* females (Figure 5.10). Activating ovaries of queenless *A. mellifera* workers (Figure 5.10) showed a previtellogenic block on oogenesis at the posterior germarium (QLW 0 — Figure 5.10A; as was shown in Tanaka and Hartfelder, 2004), which once removed, allowed vitellogenesis to proceed (QLW 1-3 — Figure 5.9B,C,D). Such a block on oogenesis was not present at any point in time, for neither mated nor virgin *O. bicornis* (Figure 5.10). Instead, *O. bicornis* females eclosed from hibernation with primed ovarioles (Figure 5.10A) in a presumed stasis. Nor was there any discernable structural difference between ovarioles of hibernating, mated, and virgin females (Figure 5.10).

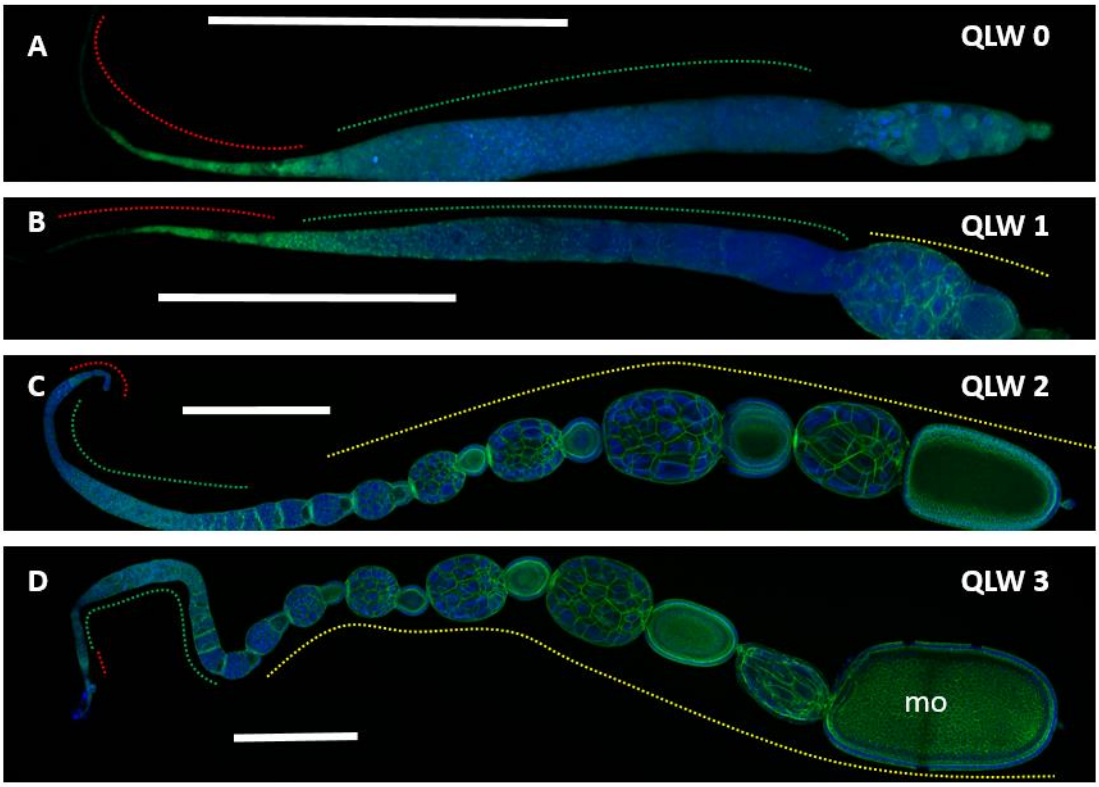

Figure 5.9: Ovary activation of queenless *A. mellifera* workers. (A-D) maximum intensity projections of the four different states of ovary activation in *A. mellifera* workers. Ovarioles stained with DAPI (blue staining) and phalloidin (green staining). mo = mature oocyte, and dashed lines indicate region of the ovariole, with: red = terminal filament, green = germarium, and yellow = vitellarium. The states of ovary activation (QLW 0 - 3) follow Duncan *et al.* (2016). A) QLW 0 showing no vitellarium and resembling queenright worker ovarioles with disorganisation at the posterior of the germarium. B) QLW 1 showing the beginnings of a vitellarium. C) QLW 2 showing a full vitellarium but lacking a mature oocyte, and (D) QLW 3 possessing a mature oocyte. All scale bars are 500 µm and indicate the increase in size of the ovarioles. Odd patterns inside the oocytes are an artefact of the maximal intensity projection; caused by projecting one or several z-plane sections of the outside of the oocyte into the inside of the oocyte. This is due to the orientation of the ovariole on the slide.

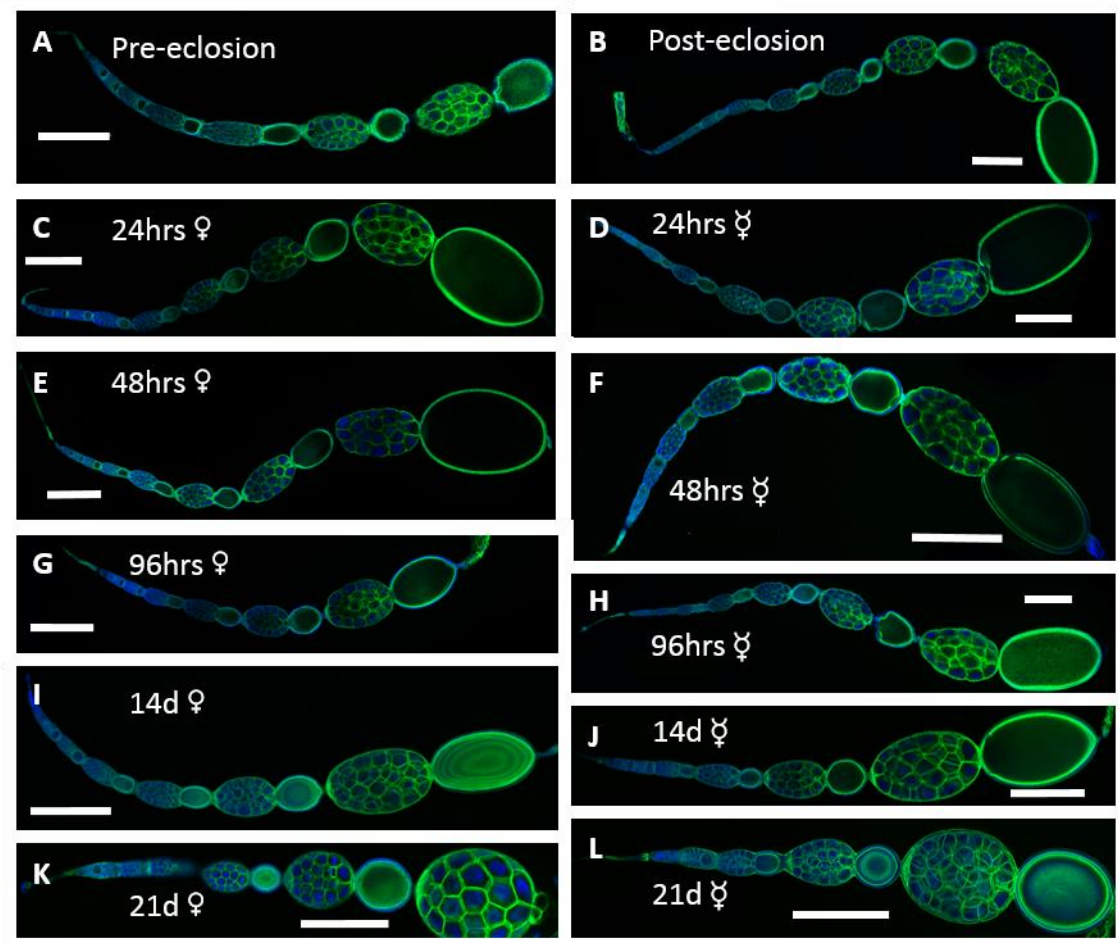

Figure 5.10: Mated and virgin *O. bicornis* ovarioles. (A-L) Maximum intensity projections of DAPI (blue) and phalloidin (green) stained ovarioles of *O. bicornis* females from all timepoints across the experiment, apart from day 7[40]. The first row (A and B) shows pre- and post-eclosion ovarioles, all rows beneath that (C-L) contain images pertaining to mated females (♀) on the left hand side, and ovarioles of virgin females (☿) on the right hand side. Timepoints are denoted in figure. All scale bars are 500 µm, with ovarioles varying in length across and within individuals. Odd patterns inside the oocytes are an artefact of the maximal intensity projection, projecting one or several z-plane sections of the outside of the oocyte into the inside of the oocyte. This is due to the orientation of the ovariole on the slide.

[40] No publication quality images were obtained for this timepoint.

When quantifying the rate of oogenesis in *O. bicornis*, likewise no effect of mating status on the rate of reproduction over time was found (interaction: $F_{1,22} = 1.052$, $p = 0.316$; Figure 5.11), nor a difference with regard to mating separately ($F_{1,20} = 0.555$, $p = 0.465$). However, the rate of oogenesis did increase significantly over time in both treatments ($F_{1,22} = 26.36$, $p < 0.001$; Figure 5.11). This suggests that oogenesis initiates and accelerates regardless of mating status, even once oocyte stores generated prior to eclosion were depleted. In fact, no differences were found between mated and unmated females for any of the measured variables, nor did the weight of the female correlate to any of the measurements taken (Table D.2). Suggesting that mating status has no effect on oogenesis in this solitary bee, and does not arrest oogenesis as has been seen in some social species (Tanaka *et al.*, 2006; de Souza *et al.*, 2007).

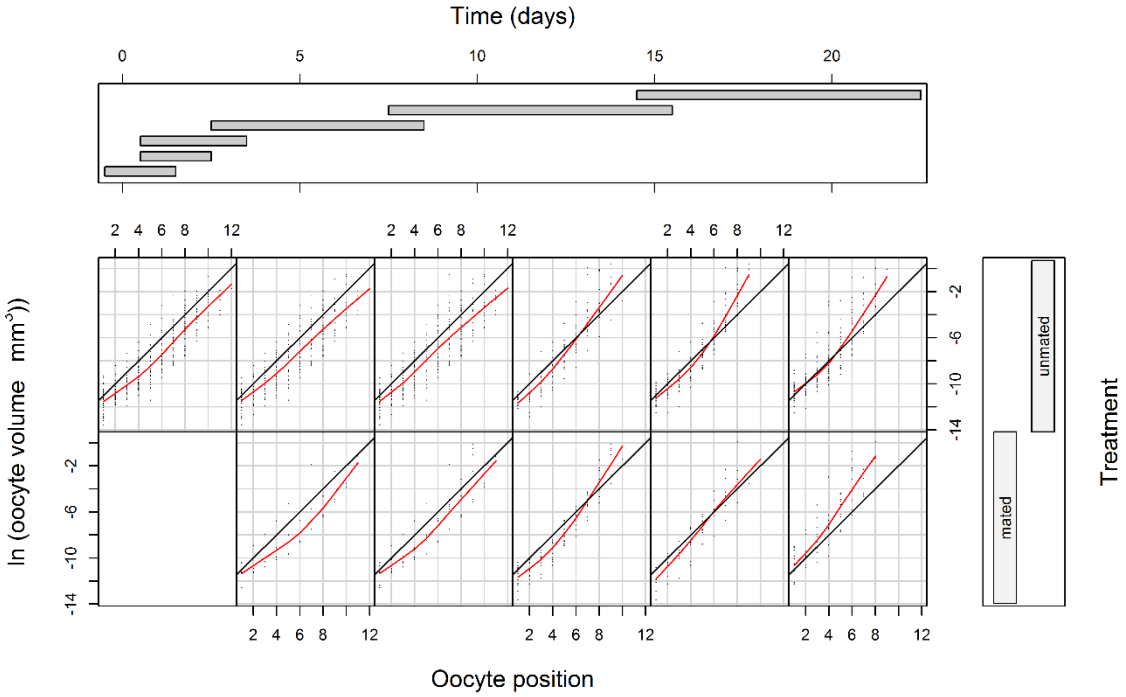

Figure 5.11: The rate of oogenesis in *O. bicornis*. The approximated rate of oocyte maturation (red slopes) increased over time (left to right), and did not differ significantly across mating status (virgin top row and mated bottom row). Points may overlap and mask one another. Red lines represent LOWESS smoothing, black lines are constant (intercept = -14 and coefficient = 1) to facilitate comparison. Horizontal bars in top panel represent overlap of time points data used for each plot.

5.5 Discussion

5.5.1 The ovarian microstructure of *O. bicornis*

The ovaries of *O. bicornis* follow the general architecture of polytrophic meroistic ovaries, as other Hymenopterans (Büning, 1994). Yet *O. bicornis* females differed markedly in microstructure from *A. mellifera* workers. The terminal filament in particular, lacks the typical 'stack-of-coins' cells prevalent in *A. mellifera* (Tanaka and Hartfelder, 2004), *Melipona quadrifasciata* (Tanaka *et al.*, 2009) and other insects (Büning, 1994). These cells may have germ line stem cells interspersed between them (Tanaka and Hartfelder, 2004; first suggested by Gutzeit *et al.*, 1993). Tanaka and Hartfelder (2004) also found terminal filament cells to be mitotically active (BrdU labelling), in spite of their apparent paucity in organelles. Another striking difference is the apparent lack of a polyfusome in the germarium of *O. bicornis* (although it may be transiently present and therefore hard to detect). The polyfusome generally marks the cystocyte cluster. Not only that, but the asymmetrical breakup of the polyfusome helps specify which cells of the cystocyte cluster will become nurse cells and which will become the oocyte (Greenbaum *et al.*, 2011). The lack of a distinct polyfusome, in conjunction with the immediate appearance of cystocyte clusters with ring canals behind the transverse septum; all argue the case that germline stem cells may indeed be originating from the terminal filament, rather than a germline stem cell niche being maintained in the germarium as is the case in *Drosophila melanogaster* (Lin *et al.*, 1994; Song *et al.*, 2007). Germarial oocytes also possessed rod-like actin associated with the nuclear envelope. Speculatively, these may be remnant components of the microtubule mitotic spindle, or they might possess a role in localisation or anchorage within the oocyte. E.g.: the localisation of maternal determinants or a role in the anterior-dorsal localisation and anchorage of the oocyte nucleus itself (Büning, 1994 p. 135).

Furthermore, early oocyte clusters did not possess the typical comet-like appearance of the trophocytes as it does *A. mellifera* (Tanaka and Hartfelder, 2004), a trait that is less pronounced and polarised yet still present in *M. quadrifasciata* (Tanaka *et al.*, 2009) and has likewise not been found in other solitary bees (Martins and Serrão, 2004b). These traits may be due to the higher rates of egg production in social species; where the longer germarium represents more cystocyte clusters being made and maintained, and the comet-like appearance of these clusters implies a compacting of the egg-conveyer belt.

The higher rates of egg production are particularly apparent in the vitellarium, where *A. mellifera* strikingly possesses more oocytes than does the solitary *O. bicornis* (compare Figure 5.9 with Figure 5.10). Finally, with respect to the vitellarium: the number of nurse cells did not adhere to the *'$(2^n - 1)$ rule'* (Büning, 1994) in *O. bicornis* (e.g. 37 nurse cells in Figure 5.2F). *A. mellifera* likewise does not adhere to this rule (possessing between 47 and 60 nurse cells; Büning, 1994). This indicates that mitosis is not always performed by all cystocyte descendants. The nutritive chamber also contained actin bundles orientated transversally, as found in other species (Gutzeit, 1986b, 1990, 1991; Fleig *et al.*, 1991; Jablonska and Kisiel, 2002). These bundles are thought to have originated from the follicle cells, and contract the nurse cells prior to their apoptosis and degradation to initiate cytoplasmic streaming (Gutzeit and Koppa, 1982) of the remaining nurse cell content into the oocyte (Gutzeit, 1986a). This function has been questioned in *A. mellifera* (Gutzeit *et al.*, 1993). Gutzeit *et al.* (1993) suggested the mechanical function in *A. mellifera* to be to '*increase the stiffness of the basal side of the cells and/or increase the cells' adhesiveness to the basement membrane*'. It seems reasonable to suggest that the apparent loss of the cytoplasmic streaming function in *A. mellifera* may be due to both the smaller egg size (fewer nutrients need to be pumped into the oocyte) and the higher rate of egg laying (i.e. removing cytoplasmic streaming from oogenesis speeds up oogenesis). The difference in both egg size and egg laying rate, might in turn be attributable to the different selective pressures experienced by social and solitary species. In any case, here, the occurrence of distinct actin rings around the nurse cell nuclei and the pronounced nature of actin bundles during late stage oogenesis, argue in favour of a contractile function and cytoplasm streaming for *O. bicornis*.

5.5.2 Mating and oogenesis

In insects, mating is known to affect reproductive physiology in a variety of ways. In diplo-diploid insects: mating plugs, seminal proteins, sex peptides, and other male accessory gland products often accelerate if not outright activate oogenesis and other aspects of ovarian physiology (Gillott and Friedel, 1977; Gillott, 2003; Colonello and Hartfelder, 2005; Avila *et al.*, 2011). Under the haplo-diploidy system, mating is not strictly necessary for females to be reproductive. Yet the requirement of mating is still seen in many Hymenoptera. In virgin *A. mellifera* queens, oogenesis is blocked at the initial stages of vitellogenesis, just as it is in reproductively constrained workers (Tanaka *et al.*, 2006). Virgin queens of the eusocial *Melipona quadrifasciata anthidioides* likewise show degenerated ovarioles (de Souza *et al.*, 2007). In the primitively eusocial wasp *Ropalidia marginata*, mating is not necessary for ovary

activation, and a virgin queen can hold a nest, yet she will show more resorbing oocytes and lay fewer eggs (Shukla *et al.*, 2013). Finally, in the parasitoid wasp *D. rapae*, mating delay negatively affects female reproductive output which is restored after mating (Kant *et al.*, 2013). Across the Hymenoptera, the effects of mating seemingly vary in queens and females along their level of social complexity. I therefore hypothesised that mating status may have had an ancestral role in reproductive control, and subsequently may have been co-opted into reproductive constraint in eusocial insects like the honey bee.

Yet, I could not detect any response in the ovary with regard to mating in solitary *O. bicornis*. Even after enough time had transpired for the initial oocyte stores to be depleted, *O. bicornis* showed no quantitative differences in oogenesis with regard to mating status. Likewise, no microstructural differences could be discerned between virgin and mated females, in contrast to virgin *A. mellifera* queens (Patricio and Cruz-Landim, 2002; Tanaka and Hartfelder, 2004) and virgin *Melipona quadrifasciata* queens (de Souza *et al.*, 2007). Indicating that mating status does not control reproduction in this solitary bee. Egg laying was not measured in this study due to constraints in experimental design (see provisional constraints on lab-rearing in Chapter 3). Hence virgin females might yet show lower egg laying rates and higher rates of oocyte resorption as is the case in *D. rapea* (Kant *et al.*, 2013)[41]. Regardless, mating status seemed to play no part in the onset nor rate of ovary activation in the solitary bee *O. bicornis*. This stands in opposition to the situation in *A. mellifera* queens, but is consistent with workers which cannot mate because they lack spermatheca (which is in itself a form of reproductive constraint: Khila and Abouheif, 2010). I therefore propose that the lack of dependence on mating likely resembles the ancestral solitary state, and future work might further ascertain this in other solitary Hymenoptera (e.g. for the Apidae: *Eulaema nigrita* and *Euglossa cordata*, Woodard *et al.*, 2011; and *Habropoda laboriosa*; Kapheim *et al.*, 2015b).

Consequently, the dependence on mating seen in queens of some species may have evolved as a consequence of increased colony size, where the queen-worker conflict has shifted towards brood composition (Bourke, 1999). A point which is further corroborated by the fact that workers under a mated queens show lower ovary activation than do workers raised under unmated queens (Peso *et al.*, 2013). Overall, my data indicates that the mechanisms underlying QMP-mediated adult reproductive constraint (Duncan

[41] Egg degeneration and resorption occur naturally in *Osmia* species (Maeta and Kurihara, 1971).

et al., 2016) were unlikely to have been co-opted from mechanisms underlying mating status.

However, there are some important considerations with regard to the life history traits of *O. bicornis* that need to be taken into account. *O. bicornis* females have a limited flight season (May-June; Vleugel, 1952; Raw, 1972), during which they are able to lay up to 27 eggs (Raw, 1972). To maximize reproductive output, females prime oocytes during autumn and early spring (but not during winter months; Wasielewski *et al.*, 2011a). Females then mature these primed oocytes shortly after eclosing and accelerate their rate of oogenesis (Figure 5.11). Even with primed oocytes, it takes seven days or fewer (given the time points used in this experiment) for corpora lutea to be present and indicate fully matured eggs[42]. This is in line with van der Steen (1997) who described a one to two week pre- oviposition period. Similarly, Cane (2016) found that *O. californica* required a diet of daily pollen for the oocytes to start swelling and maturing further, this over a period of ten days. *O. bicornis* likely follows this anautogenuous strategy as well. Consequently, the number of oocytes per ovariole dropped over time, as the primed oocytes were depleted and the maturation rate started increasing steadily over time. Such life-history constraints on oogenesis and flight season might make it unlikely for oogenesis to be slowed or arrested.

A further life-history consideration is that little selective pressure may have been maintained on female *O. bicornis* to acquire a mate. In aculeate Hymenoptera there is a general asymmetry to female and male investment in offspring (Seidelmann, 1999b; Ayasse *et al.*, 2001). Females tend to invest heavily in offspring (nest building, provisioning, etc.), while males contribute little to nothing. This asymmetry leads to higher levels of both inter- and intrasexual selection (i.e. female choice and male-male competition respectively). Given the intense male-male competition over highly valuable females, males hatch up to two weeks prior to females. Males then lie in wait for emerging females or seek them out at feeding sites (Raw, 1976; Seidelmann, 1999b; Ayasse and Dutzler, 1998). This ’race for females’ (Seidelmann, 1999b) makes it unlikely for females to end up without a mate. That virgin females become unattractive and unreceptive to males through their CHC after only three days corroborates this lack of selective pressure further (Seidelmann, 2014a). This type of initial and brief receptivity is common in Hymenopterans and Dipterans (Ringo, 1996).

[42] Corpora lutea are post-ovulatory follicle cells (Büning, 1994). Additionally, corpora lutea are known to produce ecdysone to help maintain a high rate of oogenesis in *D. melanogaster* (Deady *et al.*, 2015).

The dispensability of mating to ovary activation in conjunction with virgin unattractiveness after three days (Seidelmann, 2014b) in this species is inconsistent with the hypothesis that queen pheromones may have evolved from sex pheromones (Oi *et al.*, 2015). However, changes in the CHC-profile of female *O. bicornis*, include a marked transition towards longer chained C_{27}-alkanes (Seidelmann and Rolke, 2019). This linear alkane stops workers in the common wasp (*Vespula vulgaris*) and the desert ant (*Cataglyphis iberica*) from reproducing (Van Oystaeyen *et al.*, 2014). Furthermore, both the onset of unattractiveness and the shift towards the C_{27}-alkane coincides with the timing of maturing oocytes appearing in our study. These observations are in line with another hypothesis outlined by Oi *et al.* (2015): queen pheromones could be derived from the fertility cues that are the by-products of ovary development.

Chapter 6 Notch signalling

6.1 Summary

Insect oogenesis is generally thought to be concomitant with environmental conditions (food/protein availability, hibernation, nest availability, and/or mating). Under the RGPH, conserved signalling pathways that link environmental conditions to the ovarian response thereto, are predicted to have been recruited into the social environment of eusocial species over evolutionary time. Tackling the question of reproductive constraint from the perspective of genetic co-option, allows functionally demonstrating how it may have arisen over evolutionary time. Plastic ovary activation in *A. mellifera* is, at least partially, regulated through germarial Notch signalling in the ovary. This well conserved signalling pathway was likely co-opted into this function over evolutionary time. By comparing germarial Notch signalling of *A. mellifera* with that of the related solitary *Osmia bicornis*, its ancestral role in the control of reproduction may be elucidated. Through the use of *in situ* hybridisation, I show that germarial Notch signalling is reversed in the *O. bicornis* ovary with respect to *A. mellifera*; in that active Notch signalling is associated with active oogenesis in *O. bicornis*, and the Notch inhibitor Numb is concurrently not-expressed in the germarium. Yet the expression patterns of the Notch ligands (*Serrate* and *Delta*) are comparable to those of *A. mellifera*.

I subsequently attempt to repress germarial oocyte specification and early oogenesis, in order to establish a direct functional link between germarial Notch signalling and active reproduction in *O. bicornis*. To do this I used an inhibitor of Notch signalling through feeding, secondly by limiting protein and carbohydrate depletion, and finally by treating with QMP directly (through feeding, topical application and injection) in *O. bicornis*. None of these approaches were able to inhibit or limit oogenesis in *O. bicornis*.

Given that *O. bicornis* possess a limited flight season (May-June) in which to mass provision for eggs; terminating early oogenesis might be a physiological impossibility in this species, as it could preclude future reproduction entirely. Alternately, activated ovaries may not be susceptible to repression in Hymenoptera. In order to test the latter hypothesis, I subjected adult *A. mellifera* workers with activated ovaries to QMP. This likewise did not repress early oogenesis within the constraints of the experimental design. I posit that ovary activation may not be reversible once oogenesis is underway, and that early oogenesis is irreversible once underway.

6.2 Introduction

One of the key questions in sociogenomics remains: '*Are conserved genetic pathways repeatedly recruited into functional roles associated with social traits?*' (Kapheim, 2016). QMP represses honey bee worker ovary development (Hoover *et al.*, 2003), and it does so through the highly conserved Notch signalling pathway (Duncan and Dearden, 2010; Duncan *et al.*, 2016). Notch signalling essentially provides communication between two neighbouring cells (Bray, 2006). A Notch ligand (either *Delta* or *Serrate* in insects) based on the membrane of one cell, binds to the Notch receptor on an adjacent cell. This leads to two proteolytic cleavage events (first by an ADAM-family metalloprotease and subsequently by γ-secretase) that release the Notch intracellular domain (NICD), which migrates to the nucleus (Bray, 2006). In the nucleus it interacts with various transcription factors, and releases co-repressors, and regulates the expression of target genes (typically upregulating the enhancer of split genes: Bray, 2006). Notch signalling is active in many developmental processes (cell-fate determination: Artavanis-Tsakonas *et al.*, 1999; Guruharsha *et al.*, 2012). Various feedback mechanisms regulate Notch signalling (e.g. the Notch inhibitor Numb; Bray, 2006; Guruharsha *et al.*, 2012), even *cis*-binding of Notch ligands with the Notch receptor (cis = both ligand and receptor are on same cell; Sprinzak *et al.*, 2010; Sprinzak *et al.*, 2011).

Duncan *et al.* (2016) showed that in queenright *A. mellifera* workers, Notch signalling is active in the germarium — i.e. the same region of the ovary where oogenesis is repressed in queenright workers (Tanaka and Hartfelder, 2004). They then went on to show that in the absence of QMP, this signalling is reversed completely. Finally, when workers were subjected to QMP in addition to an inhibitor of Notch signalling (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; DAPT), these (essentially queenright) workers managed to activate their ovaries. This categorically implicated germarial Notch signalling in regulating QMP-mediated adult reproductive constraint in the *A. mellifera* worker. Further investigating how this mechanism may be controlled, Duncan *et al.* (2016) looked for differential expression of the Notch ligands *Delta* and *Serrate*. They found no evidence in the expression patterns of the ligands within the germarium, indicating that the ligands were not responsible for differentially regulating Notch signalling in the QMP-context. Instead, Duncan *et al.* (2016) found that the Notch receptor itself, rather than its ligands, was being degraded. The degradation of the Notch receptor further overlapped both spatially and temporally with the expression of *Numb*, a known inhibitor of Notch signalling (Bray, 2006). Hence, Duncan *et al.* (2016) posited a model implicating the Notch inhibitor Numb, in differentiating between inactive and

active oogenesis (seemingly under a threshold model, since relative *Numb* expression levelled off at the first sign of ovary activation).

That the Notch signalling pathway is an important mediator of adult worker reproductive constraint may be unsurprising. Notch has been known to have a role in *D. melanogaster* oogenesis for some time (Xu *et al.*, 1992). In the germarium of *D. melanogaster*, the Notch signalling pathway controls germline stem cell niche formation and maintenance (Song *et al.*, 2007). As such, germarial Notch signalling is also known to respond to diet (Bonfini *et al.*, 2015), likely through the insulin pathway (Hsu and Drummond-Barbosa, 2011). Insulin signalling is broadly implicated in insect reproduction (Badisco *et al.*, 2013), and is also known to have both priming and regulating roles in *A. mellifera* (DoL: Ament *et al.*, 2008; caste determination: Wheeler *et al.*, 2006; de Azevedo and Hartfelder, 2008; Mutti *et al.*, 2011; Wolschin *et al.*, 2011; Wheeler *et al.*, 2014). The environmental responsiveness of germarial Notch signalling (Bonfini *et al.*, 2015) through insulin signalling (Hsu and Drummond-Barbosa, 2011) in *D. melanogaster*, taken together with its role in reproductive constraint in *A. mellifera* (Duncan *et al.*, 2016), could tie germarial Notch signalling into the broader RGPH hypothesis (Amdam *et al.*, 2006). Note that the molecular co-option of germarial Notch signalling would imply a role reversal over evolutionary time, since active germarial Notch signalling is associated with active oogenesis in *D. melanogaster* (Song *et al.*, 2007), whereas it is associated with inactive oogenesis in *A. mellifera* (Duncan *et al.*, 2016). Such a role reversal is plausible given the divergence time between *A. mellifera* and *D. melanogaster* (330 my; Misof *et al.*, 2014).

Another mechanism often associated with *A. mellifera* worker sterility is the occurrence of programmed cell death (apoptosis) in the ovary, both reducing the number of ovarioles in worker destined larvae (Tanaka *et al.*, 2006; Hartfelder and Steinbruck, 1997), in aging workers (Ronai *et al.*, 2017), as well as regulating adult reproductive constraint directly (Tanaka and Hartfelder, 2004; Ronai *et al.*, 2015; Ronai *et al.*, 2016a; Duncan *et al.*, 2016). With regard to the role of apoptosis in adult reproductive constraint, it may simply imply that germ cells and oocytes are not kept in a form of stasis, but rather that cell proliferation and differentiation continuously proceed but fail to pass a certain ‘checkpoint’ (Pritchett *et al.*, 2009). Nevertheless, Notch signalling has also been implicated in autophagy in the ovary *(D. melanogaster*: Barth *et al.*, 2011; Barth *et al.*, 2012), and is known to regulate proliferation, differentiation and apoptosis depending on the cellular context (Miele and Osborne, 1999; Schwanbeck *et al.*, 2011). Strikingly, Notch regulated apoptosis has been shown to operate through Numb (in *D. melanogaster* neuronal cells: Lundell *et*

al., 2003), the Notch inhibitor proposed by Duncan *et al.* (2016) to regulate adult reproductive constraint. Because Notch signalling is influenced by many other pathways and components, we are cautioned against describing it as a simple linear model, and urged to regard it as a network (Artavanis-Tsakonas *et al.*, 1999). Yet, when the multiple modulators of Notch signalling are concatenated and regarded as cellular context, Notch might be considered as a 'master switch' for cell-fate (Miele and Osborne, 1999).

It is clear that multiple pathways may be at work in the honey bee ovary with regard to reproductive constraint. But given its status as a master switch, Notch signalling may very well be at the centre of a network that ties these mechanisms together. Investigating proximate mechanisms may inform us of ultimate causes, or succinctly put: *'phenotype is the link between cause and consequence*' (paraphrased from: Kapheim, 2019). Consequently, investigating whether germarial Notch signalling controls oogenesis in the related solitary bee *O. bicornis*, and with regard to what environmental cues it does so (e.g. diet), can provide clues to what underlying mechanisms were co-opted to confer reproductive constraint in response to QMP(e.g. parental manipulation: Ronai *et al.*, 2016b). Hence, in this chapter, I investigated germarial Notch signalling in the *O. bicornis* ovary in relation to oogenesis. To this end I attempted to repress early oogenesis in this solitary bee. I hypothesised that germarial Notch signalling would mimic the situation found in *A. mellifera* (Duncan *et al.*, 2016), with active oogenesis being associated with inactive germarial Notch signalling. I further hypothesised that germarial Notch signalling would respond to dietary cues (as in *D. melanogaster*: Bonfini *et al.*, 2015), as expected under the RGPH where non-reproductives are thought to forage and feed mainly on nectar (Dunn and Richards, 2003; Amdam *et al.*, 2006).

6.3 Materials and methods

6.3.1 ISH

O. bicornis genes were initially identified by reciprocal BLAST (Altschul *et al.*, 1990) analysis of a *de novo* transcriptome assembly generated by Dr E.J. Duncan from publically available RNA-seq data from adult female *O. bicornis* (SRA accession numbers SRR2895245 and SRR2895246) using Trinity (Grabherr *et al.*, 2011; Haas *et al.*, 2013) and default parameters. Sequences were confirmed by RT-PCR (Jens Van Eeckhoven) and through bioinformatic comparison with the *O. bicornis* genome (Beadle *et al.*, 2019) when it was published (performed by Dr E.J. Duncan). Orthology assignments were made based on phylogenetic analysis (Figure 6.1A) and by comparison of genomic architecture of the E(spl)-C in *O. bicornis* with other holometabolous insects (Duncan and Dearden, 2010; Figure 6.1B).

Presence of Notch associated genes in *O. bicornis* ovaries was checked using RNA extraction (section 2.3), cDNA synthesis (section 2.4) and subsequent RT-PCR (section 2.6). PCR products were subsequently cloned and sent for sequencing to verify the respective genes (section 2.7). Cloned products were subsequently reverse transcribed (section 2.8) to generate ISH probes of the Notch associated genes. ISH followed the overall protocol presented in section 2.9, which I adapted and optimised for use in *O. bicornis*, and originated from work carried out in *A. mellifera* (Dearden *et al.*, 2009c). Images presented are representative, and sense probes were used as negative controls. Primers used to PCR genes, to subsequently clone, are presented in Table 6.1. ISH related work (i.e. certain RNA probe extractions) was carried out with assistance from Dr E. J. Duncan.

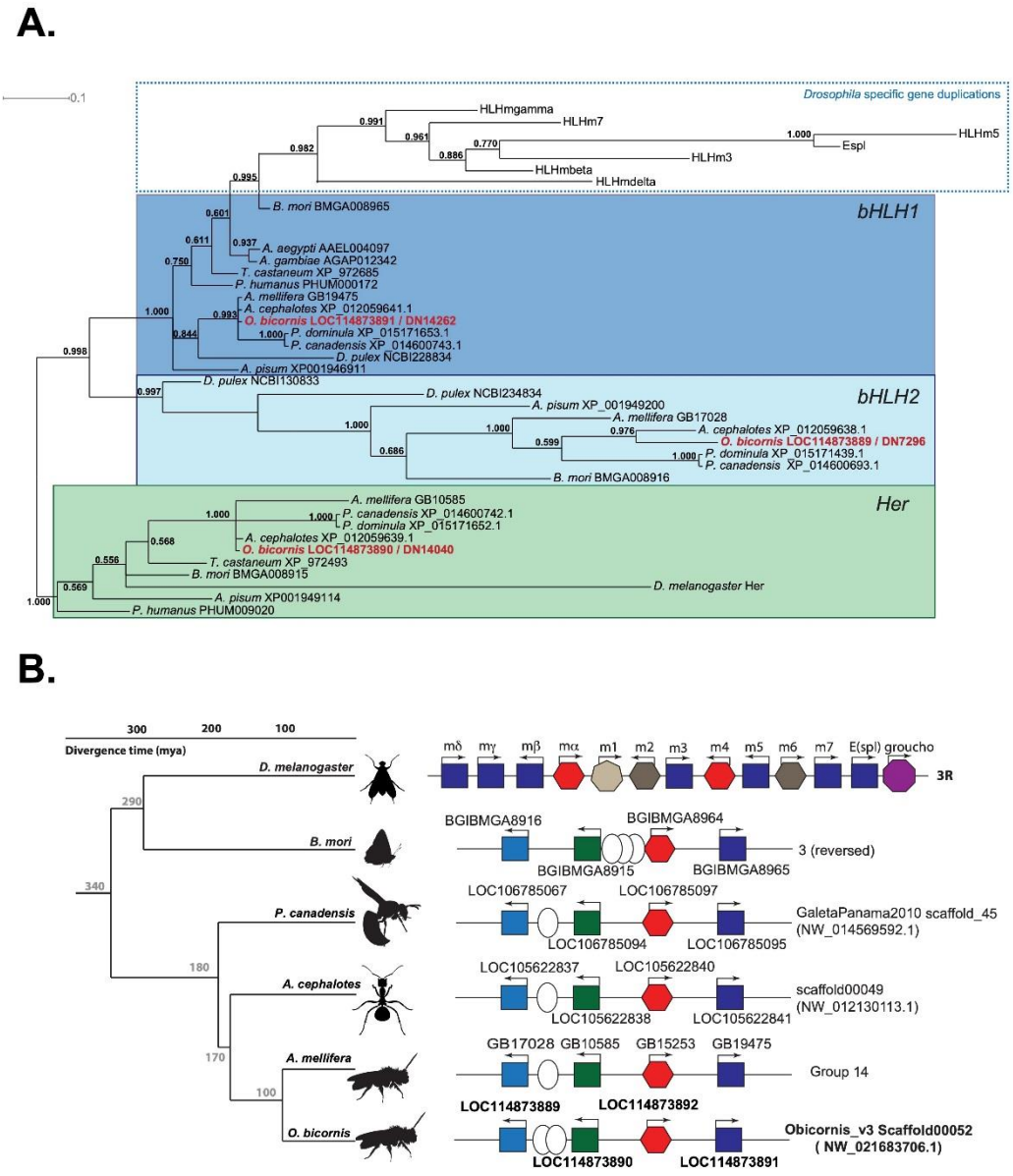

Figure 6.1: Identification and orthology of *Ob_E(spl)-C* genes. A) Bayesian phylogeny bHLH and orange domains of E(spl)-C bHLHs and HER-like bHLH proteins from sequenced arthropod genomes. As previously described (Duncan and Dearden, 2010) phylogenetic analysis resolves three clades: (1) a large clade with representatives from all insect genomes, including all *Drosophila* E(spl)-C genes designated E(spl)-C bHLH-1 (dark blue), (2) A clade with a smaller number of members designated E(spl)-C bHLH2 (light blue), (3) The final clade contains representatives from insects and includes *Drosophila* Her; we designate this clade Her (green). bHLH and orange domains were identified using HMMER (Prakash *et al.*, 2017) using the relevant pfam motifs (HLH: PF00010, Hairy_orange: PF07527). Sequences were aligned using Clustal Omega (Sievers and Higgins, 2014) and phylogenetic relationships were reconstructed using the Jones model (Jones *et al.*, 1992) which was found to be the most appropriate after preliminary investigations using mixed models. The

first 25% of trees were discarded as burn-in and the remaining trees summarized and visualized using Dendroscope (Huson and Scornavacca, 2012). B) Genomic architecture of the arthropod E(spl)-C complexes, data for *Drosophila*, *B. mori* and *A. mellifera* taken from (Duncan and Dearden, 2010). Phylogenetic relationships between the species is indicated by the dendrogram on the right hand side. Divergence times are based on Misof *et al.* (2014) and Peters *et al.* (2017). bHLH genes are represented as squares, bearded class genes as hexagons and intervening genes in arthropod E(spl) complexes with no similarity to *Drosophila* E(spl)-C genes are shown as ovals. Order of genes within the E(spl)-C is highly conserved in insects (Duncan and Dearden, 2010) and confirms the orthology assignment based on phylogeny (shown in A). Genes are color coded according to orthology assignment: E(spl)-C bHLH2-derived sequences = light blue; E(spl)-C bHLH1 sequences = dark blue; Her-derived sequences = green and Tom/Ocho/bearded-like sequences = red. Figure provided by Dr E. J. Duncan.

Table 6.1: Oligonucleotide sequences used to clone *O. bicornis* Notch related genes for *in situ* hybridisation probes. F = forward or 5' primer; R = reverse or 3' primer; T_m = melting temperature (Untergasser *et al.*, 2007); PS = product size in base pairs.

Gene	Primer	Sequence	T_m	PS
BHLH14262	F	CAGATGCACGAGCAAATGAT	59.8	746
	R	GTCTCCAGATCGGCTCGTT	60.4	
BHLH7296	F	ACGACGTGCACGAATAAACA	60.2	678
	R	GGTCGCCACATAGGATCAGT	60.0	
Delta	F	ATTTGTCGAAGCACGTAGCA	59.5	850
	R	TCGTGCCTGTAATGATCGTC	59.7	
Her	F	GGGTCTCCAGACAGCGTTAG	59.9	604
	R	GGTCGGTGGTATGGAGTACG	60.3	
Neuralised	F	CTGAGGAGTGGACGAGGAAG	60.0	1046
	R	GTGACGTTCATTTCGGTGTG	60.0	
Numb	F	GCATGCAAGTATGCGAAGAA	60.0	772
	R	CGTGAAAGCTGCTGACACAT	60.1	
Serrate	F	TTCTGCAACGGCACTTGTAA	60.4	968
	R	GAACCTGTCACCCTGCAACT	60.2	

6.3.2 Repressing oogenesis in *O. bicornis*

Bees were kept at 21-23˚C with a 18:6 h light:dark cycle and housed according to treatment in flight cages (mesh cage; 60 x 60 x 90 cm). Bees were supplied with: makeshift flowers and catkins with dusted with ground pollen, 50% sucrose solution (filter sterilised; 0.22 µm; Millipore) and Fabre's hives (Oxford bee company). No further nest building material was provided, since oogenesis would be investigated directly, and egg laying is intermittent and unreliable (Chapter 3). Three treatments to arrest oogenesis were tried over different trials: Firstly, DAPT, an inhibitor of γ-secretase in the Notch signalling

pathway (Geling *et al.*, 2002), was added to the 50% sucrose solution on two consecutive trials. Secondly, a treatment group was deprived of ground pollen (protein) and given a lower concentration of sucrose solution in another trial (a 10% sucrose solution instead of 50%; i.e. 0.1038 mg/µl and 0.6148 mf/µl respectively: Dafni *et al.*, 2005). Sucrose solutions were provided *ad libitum* in cages, and refreshed and measured daily, to provide an estimate of uptake per bee per cage. Finally, QMP was administered in four more consecutive trials; twice through oral exposure (mixed in with sucrose solution; QMP-trials 1 and 2), and through both injection and topical application (QMP-trial 3), and repeated topical administration (QMP-trial 4). Sample sizes are displayed in Table 6.2.

DAPT is highly soluble in DMSO (dimethyl sulfoxide), and somewhat less soluble in EtOH. Furthermore its various manufacturers disagree on the solubility in water, from being insoluble to <2.12 mg/mL (Adooq bioscience, Selleckchem, Sigmaaldrich, APExBIO, Alfa Aesar). Dissolution with DMSO was avoided (given its high toxicity), DAPT (Cell guidance systems) was first diluted to 50mM in EtOH, and subsequently provided to bees in a 1mM concentration (Duncan *et al.*, 2016) in the sucrose solution. Given the presence of both multiple solutes and multiple solvents — DAPT and sucrose, and EtOH and water respectively — these interact and change each other's solubility (often in non-linear ways: Stumm and Morgan, 2012). Hence I did not manage to dissolve DAPT, and it stayed emulsion (as in Williams *et al.*, 2012). QMP (Intko Supply Ltd, Canada) was likewise dissolved in EtOH and subsequently diluted into the sucrose solution for the feeding assays (QMP-trial 1: 0.050 eq/µl and QMP-trial 2: 0.017 eq/µl), and similarly experienced solubility issues. For QMP-trial 3, bees were sedated for at least 30 minutes on ice. Subsequently bees were either: injected in between two sternal plates (between S3 and S4, or S4 and S5) with 10.69 µl of 0.145 eq QMP / µl EtOH using a Nanoliter 2010 injector (World Precision Instruments), or exposed to 3µl of 1 eq QMP / µl acetone atop the thorax. For QMP-trial 4, 3µl of 3 eq QMP / µl acetone were applied topically over the course of 10 days (acetone is known to increase the penetrance of pesticides: e.g. Kerkut and Gilbert, 1985; and is commonly used for the topical application of methoprene: O'Donnell and Jeanne, 1993). All controls were exposed to the equivalent amount of solvent in the relevant method of administration. Trials lasted ten days on average, since ovaries are fully active by this time (see Chapter 5).

6.3.3 Repressing oogenesis in queenless *A. mellifera*

Workers were housed together in cages (10cm x 10 cm x 5.5 cm; larger bee study cage, Small-Life Supplies; 55-115 workers per cage) in an incubator (Incu-160S, SciQuip), in total darkness at 35°C with relative humidity averaging 40%. Distilled water was provided for thermoregulation and homeostatic balance, in a 15 ml polypropylene tube from the cage ceiling. 2-4 g of complete bee food (CBF: 20 g pollen, 52 g sucrose, 18.8 g brewer's yeast, and 9.2 g lactalbumin; all ground and made to a paste using a minimal amount of honey: Duncan *et al.*, 2016) was provided in food caps on the side of the cage, and refreshed daily. A microscope slide dotted with either 10 µl of 0.1 eq QMP/µl EtOH (i.e. 1 eq of QMP) or 10 µl EtOH was placed in the centre of the cage and replaced each day.

For details on overall honey bee husbandry, see section 2.1.1. Queenless workers were taken from a queenless hive. A hive was considered to have reproductively active workers once worker laid eggs were detected, and a sufficient amount of brood had emerged over a sufficient amount of time.Methods for maintaining *A. mellifera* workers caged in the laboratory are well known (Williams *et al.*, 2013). Adults can even survive for extended periods in cages inside incubators (survival over 60 days has been reported: Evans *et al.*, 2009), yet they do so best when fed on a carbohydrate source exclusively (Pirk *et al.*, 2010; Paoli *et al.*, 2014). The provision of a protein source enables higher ovary activation, while simultaneously increasing mortality (Pirk *et al.*, 2010; Paoli *et al.*, 2014). This is because workers do not defecate inside the hive (a situation the cages inside incubators simulate), and the presence of pollen or other protein will accelerate mortality by constipation. Given that worker ovary activation and the duration until activation are both seasonal (Velthuis, 1970; Hoover *et al.*, 2006), exposing adult reproductive workers to QMP therefore afforded several experimental difficulties. For these reasons: food intake per cage was measured daily, through weighing of the CBF caps, and deceased honey bees were removed and recorded daily.

Ovaries were scored based on a modified Hess scale (Hess, 1942), identical to Duncan *et al.* (2016). Briefly, stage 0 ovaries resembled queenright worker ovaries, stage 1 ovaries possessed signs of cell differentiation (typically constrictions visible), stage 2 ovaries contained clearly defined oocytes with deposited yolk, and stage 3 ovaries possessed at least on fully mature oocyte. Workers from queenless hives were considered reproductively active when at least 30% of workers possessed stage 2 and stage 3 ovaries. Caged workers consisted of either: non-age matched workers captured at random from a queenless hive and randomly attributed to cages (resulting in a homogenous

age-distribution in cages; Williams *et al.*, 2013), or newly emerged workers that were age marked and placed back into the queenless hive until reproductively active (age-matched within cage and across treatment; between 18 and 21 days old). Newly emerged workers were also trialled, to see if workers could activate their ovaries on time (within 5 days), to be able to survive another 5 days of QMP-treatment, given that caged honey bee workers generally did not survive past 10-14 days. Ovaries of all trials were scored blind, by the most experienced observer (Rosemary Knapp).

6.3.4 Staining and microscopy

At the end of each trial, *O. bicornis* females were sedated (at least 30 minutes on ice), weighed, ovaries were dissected out, individualised, and fixed (1:1 heptane: 4% formaldehyde) nutating for 1hr at RT. Samples were subsequently washed twice (PTx for 5 minutes), stained with 1 µl of 5 mg/ml DAPI for 10 minutes at RT in darkness, washed twice more (PTx for 5 minutes), cleared in glycerol, and mounted. Roughly half of all samples were imaged by means of a slidescanner (AxioScan Z.1 Slidescanner; search and imaging algorithm designed and optimised by Dr Sally Boxall), the remainder of samples were imaged or scored by widefield microscopy (Axioplan Universal, Zeiss) under a mercury bulb. Possible phenotypes (Figure 6.2) were tracked and recorded, and the number of oocytes in the germarium was counted. The germarium was considered to end when clearly defined follicle cells surrounded the oocyte, and the oocyte was followed by a clear nutrient chamber with well-developed nurse cells.

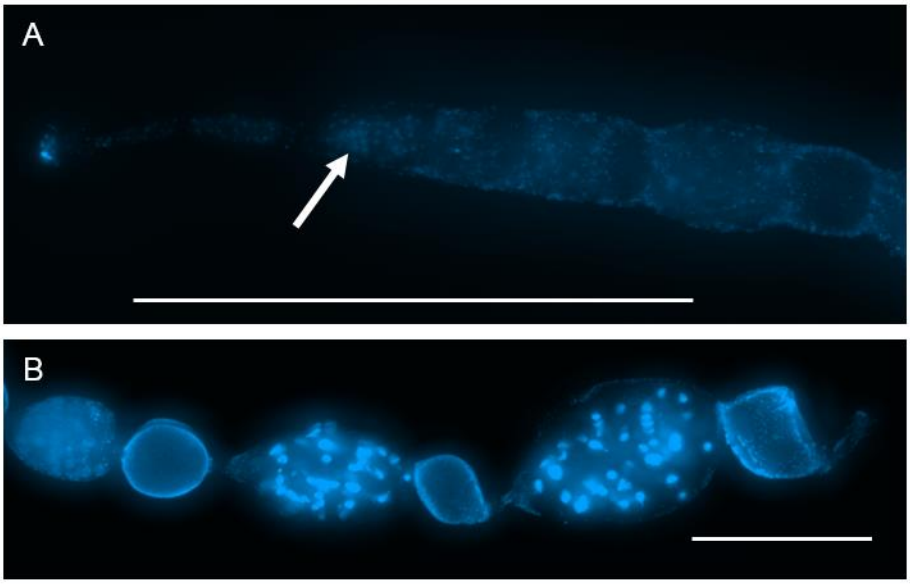

Figure 6.2: Phenotypes in trials. Extended depth of focus (EDF) images DAPI stained *O. bicornis* ovarioles of trials (as obtained by the slidescanner). A) Germarial cluster of cells (white arrow) at the base of the terminal filament exhibited more condensed and numerous nuclei than the cystocyte clusters described in Chapter 5. Note that the difference with Chapter 5 may also have appeared as an artefact stemming from the use of EDF over maximum intensity projection (MIP) with the slidescanner (MIP would require and inordinate amount of computer memory and computing time). Under wide field microscopy with a mercury bulb source, these cells seemed to resemble the more condensed follicle cells generally found interspersed in the germarium. B) Condensed nurse cell nuclei in late stage nutrient chambers. This phenotype resembles the situation of nurse cells prior to the cytoplasmic streaming associated with oocyte maturation (described in Chapter 5), but occurred in association with non-mature oocytes or non-terminal oocytes. Image represents the most obvious and distinct example, but nutrient chambers with even only a single condensed nurse cell nucleus were counted as having this phenotype. Scale bars = 500µm.

6.3.5 Statistical analysis

Statistical analysis was carried out using R 3.5.1 (R Core Team, 2016). Linear mixed models and generalised linear mixed models were made using *lme4* (Bates *et al.*, 2015). Assumptions were investigated following Zuur *et al.* (2010) and model tests were performed using ANOVA, or through model comparisons using log likelihood-ratio tests for generalised linear mixed models (without stepwise reduction of the model, except in the case of interaction terms, see: Engqvist, 2005; Whittingham *et al.*, 2006; Mundry and Nunn, 2008; Forstmeier and Schielzeth, 2011; Bates *et al.*, 2015). Food uptake for *O. bicornis* was modelled for each trial separately, to estimate the amount of treatment chemical consumed, and check for differences between treatments and controls. Linear models, using estimates of food per bee per day as a

response variable were made for each trial. Dependent variables were treatment, day and their interaction; ANOVA was performed. Food intake in *A. mellifera* trials was likewise modelled, jointly, to check if treatments differed significantly in food uptake, and whether differences in survival could be related to differences in food consumption. A linear mixed effects model (lme4; Bates *et al.*, 2015) was constructed with estimated food consumption per bee per hour as the response variable, and treatment and age category (and their interaction) as explanatory variables. Random slopes (food consumption over time) were constructed per cage (substantially improving model fit: AIC score and distribution of residuals). ANOVA was carried out using *lmerTest* (Kuznetsova *et al.*, 2016; Luke, 2017), and degrees of freedom represent Satterthwaite approximations. Survival of caged *A. mellifera* workers — while illustrated through Kaplan-Meier survival probability curves and Cox proportional hazard ratios — was modelled using generalised linear mixed models with a logit link function (i.e. binomial distribution; Bates *et al.*, 2015). This because individual deaths are not independent within cages (pseudoreplication: Pirk *et al.*, 2013). Consequently, proportional survival per cage was used as the response variable, with random slopes constructed for survival over time by cage identity, and treatment and age categories (and their interaction) as fixed effects.

For germarial oocyte counts in *O. bicornis*, generalised linear models with a log link function were constructed. Treatment and bee weight were treated as fixed factors, and bee identity nested in treatment was considered the random factor. For QMP-trial 1, statistical power was inflated, since individual bees were not kept separated and ovaries were pooled (standard general linear model). For QMP-trial 3 the method of administration (topical or injection) along with its interaction with bee weight were also included within the model. Distinct phenotypes (Figure 6.2) were rare (i.e. highly zero inflated), hence Fisher's exact test was used to compare treatments. Finally, *A. mellifera* workers' ovary scores were modelled using mixed effects ordinal regression (Christensen, 2015). Cage identity, nested within age matching category (non-age matched, or aged matched; 18-21 days old), was considered the random effect. Days until dissection also varied among cages (due to sudden mass deaths in cages), but was not modelled to avoid overfitting the model, and any variance attributable to it are assumed to co-vary with cage identity. Treatment and age matching (and their interaction) were set as fixed effects. Data on newly emerged workers (since they were not yet reproductively active workers) were omitted from the above model, and analysed separately using one-sided Fisher's exact tests.

6.4 Results

I firstly attempted to assay Notch signalling in the ovary directly through immunohistochemistry (IHC). Under active Notch signalling, the antibody raised against *D. melanogaster* NICD (C17.9C6, Developmental Studies Hybridoma Bank) should locate to the nucleus, rather than to the cell membrane as is the case in the absence of active Notch signalling. While this antibody cross reacts with *A. mellifera* NICD (Wilson *et al.*, 2011; Duncan *et al.*, 2016), preliminary data (IHC; data not shown) suggested that it does not cross react with *O. bicornis* NICD, as did the alignment of the NICD epitope in *D. melanogaster* and *A. mellifera* to that of *O. bicornis* (Appendix E). Since raising an antibody against *O. bicornis* NICD was outside of the scope of the current study, I used ISH to look at the expression patterns of the relevant Notch responsive genes instead (E(spl)-genes; Duncan *et al.*, 2016).

While ISH can be used in any species, it first still needs to be optimised and validated for this species and tissue, since this technique has not yet been carried out before in *O. bicornis*. Existing protocols of *A. mellifera* were adapted (Dearden *et al.*, 2009a; Dearden *et al.*, 2009c). The adaptations on the protocol consisted of: removing the intima upon dissection of tissue, extending the tissue fixation time to one hour, extending pre-hybridisation to at least four hours, and extending antibody-blocking to at least two hours. *Neuralised* was opted for as a positive control. This because *Neuralised* has a distinct stripe pattern, which seems to be conserved from *A. mellifera* (Duncan *et al.*, 2016) to *Nasonia vitripennis* (fig. 5a in Pers *et al.*, 2016 shows the residual and dissipitating stripe pattern in a *Nasonia* embryo, and the striped pattern also appears in oocytes — personal communication Dr Jeremy Lynch). Furthermore, Neuralised is known to be a mediator of Notch signalling, where it activates the ligands (Serrate and Delta), as well as marks them for endocytosis (Bray, 2006). Figure 6.3 corroborates the pattern in late stage oocytes in *O. bicornis* by showing a distinct striped enrichment (Figure 6.3A white dashed oval; and Figure 6.3C), as well as the more pronounced expression in nurse cells located posteriorly in the nutrient chamber (Figure 6.3A black brace, and magnified in Figure 6.3C; Duncan *et al.*, 2016), validating the technique for further use in the *O. bicornis* ovary.

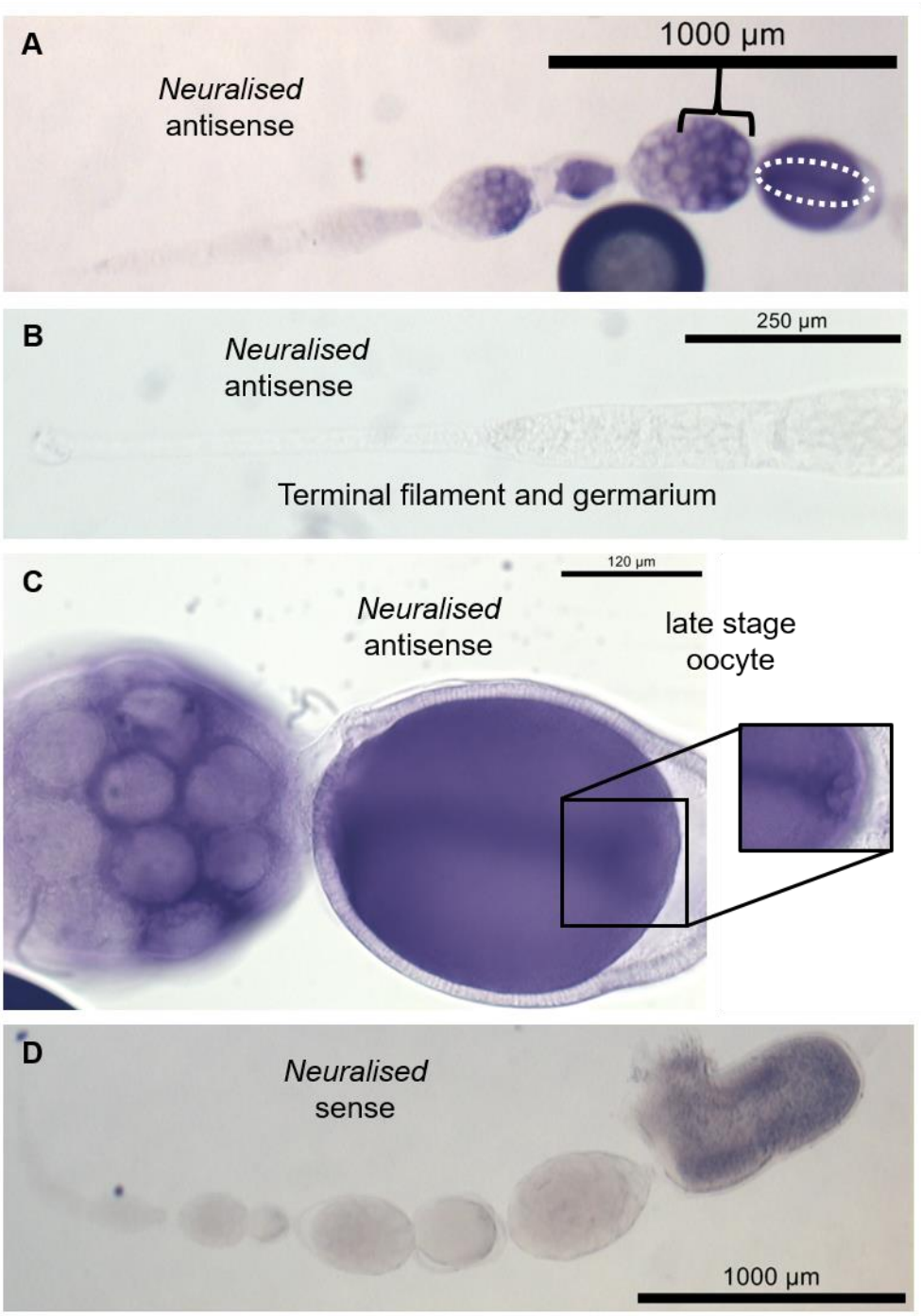

Figure 6.3: Ovarian expression of *Ob_Neuralised*. *Neuralised* as a positive control for establishing ISH in the *O. bicornis* ovariole. A) Antisense staining. Note the enriched stripe pattern in the late stage oocyte (white dashed oval) and expression in the posterior nurse cells only (black brace). B) Sense staining was clear in both the terminal filament and germarium. C) Antisense stainingfurther further showed enrichment around the oocyte nucleus (inset).D) Sense staining was clear throughout the vitellarium, apart from some staining artifact in the terminal oocyte. The specific and distinct staining pattern of *Neuralised* matches that of *A. mellifera* (Duncan *et al.*, 2016) and *N. vitripennis* (Pers *et al.*, 2016).

6.4.1 Germarial Notch signalling in *O. bicornis*

Figure 6.4A-F shows the expression patterns with sense controls of the E(spl)-genes (*Ob_BHLH7296*, *Ob_BHLH14262*, and *Ob_Her*) in *O. bicornis* undergoing active oogenesis, and their expression patterns indicate active Notch signalling in the germarial region of active ovaries. This is in direct contrast to the situation in *A. mellifera* (Duncan *et al.*, 2016), where queenless workers as well as actively laying queens show inactive germarial Notch signalling. The situation in *O. bicornis*, is more in line with the situation in *D. melanogaster* where active germarial Notch signalling is required for the maintenance of oogenesis and the germline stem cell niche (Xu *et al.*, 1992; Song *et al.*, 2007). Duncan *et al.* (2016) also postulated a model suggesting *Numb*, an inhibitor of Notch (Bray, 2006), might regulate germarial Notch signalling in its social-reproductive context. I found *Numb* expression to be absent in the germarium of the active *O. bicornis* ovariole (Figure 6.4G), likewise counter to expectation. Taken together, these results imply that a reversal in germarial Notch signalling function did not occur after the Diptera (*D. melanogaster*) and Hymenoptera (*A. mellifera*) diverged (330 mya; Misof *et al.*, 2014). Indeed, the functional role reversal of germarial Notch occurred much later; at least some time after the Megachilidae (*O. bicornis*) and Apidae (*A. mellifera*) split (95 mya; Peters *et al.*, 2017).

Also note that *Ob_BHLH7296* and *Ob_Her* (corresponding to *Am_BHLH2* and *Am_Her* respectively; Figure 6.4A and E) showed the most distinct staining as in Duncan *et al.* (2016), where they also predicted ovary state most accurately. Given the role of Notch signalling in maintaining the germline stem cell niche (Song *et al.*, 2007), I further point out that the expression of these two genes extends into the terminal filament (Figure 6.4), lending further weight to the hypothesis of a germline stem cell niche within the terminal filament (see Chapter 5; as proposed by Tanaka and Hartfelder, 2004 in the honey bee). The *O. bicornis* expression patterns for the Notch ligands *Serrate* (Figure 6.5A-B) and *Delta* (Figure 6.5C-D) mirror their counterparts in queenright *A. mellifera* (i.e. under active Notch signalling; Duncan *et al.*, 2016). In both *O. bicornis* and *A. mellifera*, *Delta* is transcribed in the germarium but not the terminal filament (Figure 6.5C and inset; Duncan *et al.*, 2016). *Serrate* expression in *O. bicornis* is absent in both the terminal filament and the germarium, and only starts occuring in the vitellarium (Figure 6.5A), as is the case in queenright *A. mellifera* workers (Duncan *et al.*, 2016).

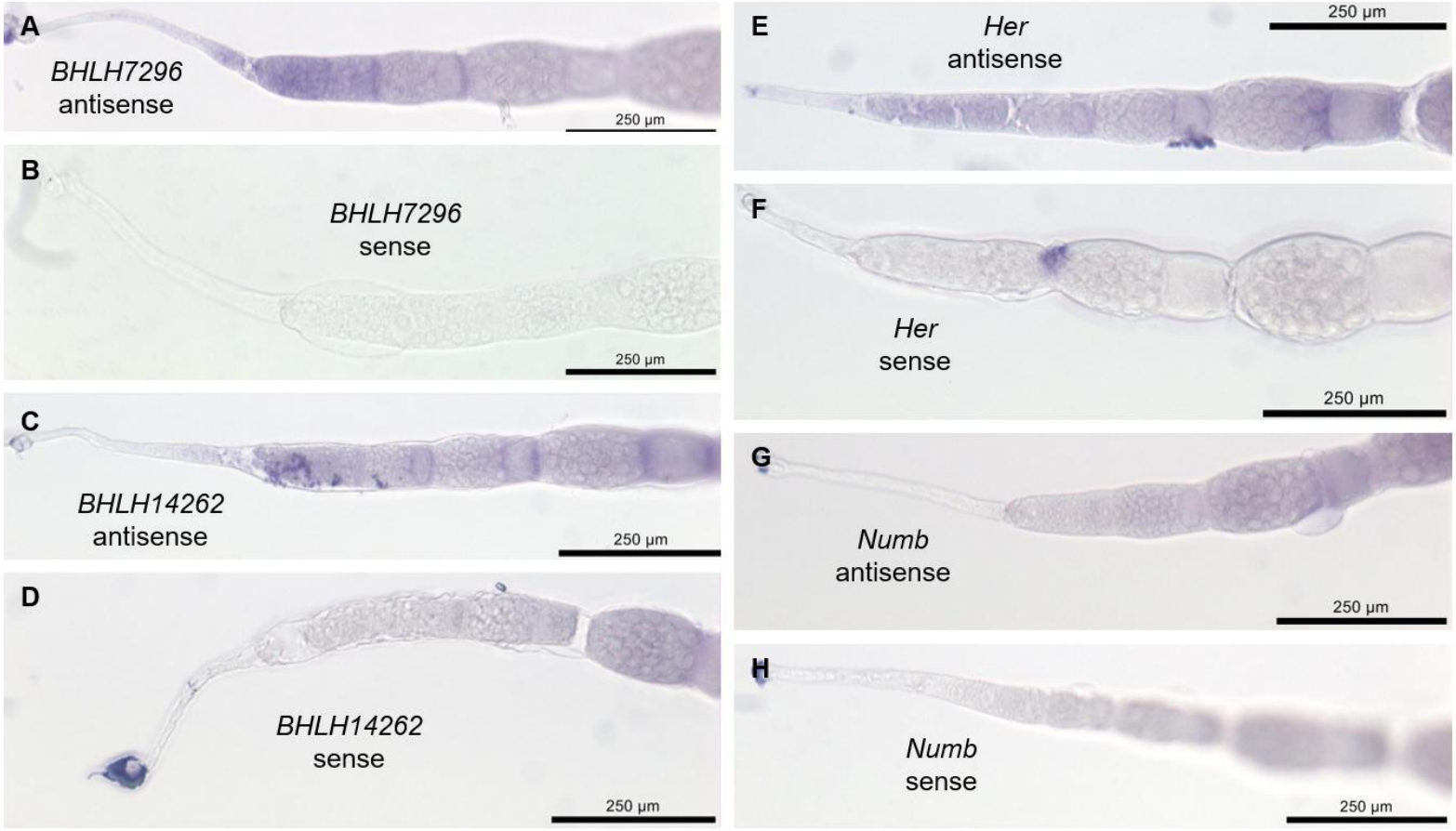

Figure 6.4: Notch signalling in the active *O. bicornis* ovary. *In situ* hybridisation of *O. bicornis* germaria; indicating gene expression regions for the Notch targeted E(spl)-genes. A) Antisense staining of *Ob_BHLH7296* (representing *Am_BHLH2*). B) Non-staining sense-control of *Ob_BHLH7296*. C) Antisense staining of *Ob_BHLH14262* (representing *Am_BHLH1*). D) Sense-control of *Ob_BHLH14262.* E) Antisense staining of *Ob_Her*. F) Sense-control staining of *Ob_Her*. G) Antisense staining of the Notch inhibitor *Numb* is clear, as is its sense control (H)..

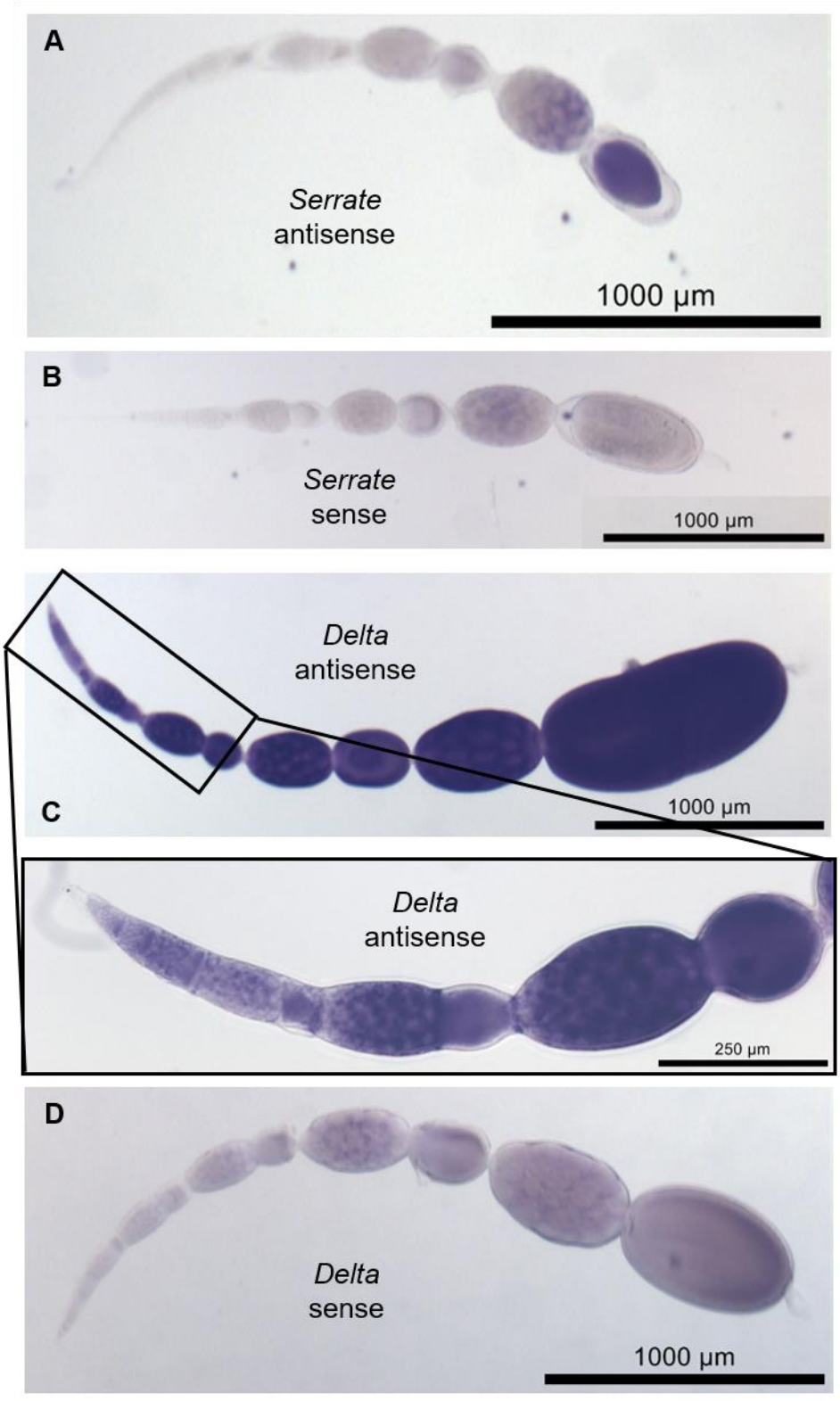

Figure 6.5: Ovarian expression of Notch ligands in *O. bicornis*. A) *Serrate* expression in the *O. bicornis* ovary. *Serrate* expression occurs earlier on in stage 1 oocytes in *A. mellifera* (Wilson *et al.*, 2011; Duncan *et al.*, 2016). B) *Ob_Serrate* sense control. C) *Ob_Delta* antisense staining, with magnified germarial region in inset. *O. bicornis Delta* is expressed ubiquitously, as it is in the *A. mellifera* ovary (Duncan *et al.*, 2016). D) *Ob_Delta* sense control.

6.4.2 Repressing oogenesis in *O. bicornis*

Given that: germarial Notch signalling represses oogenesis in the socio-reproductive context in *A. mellifera* (Duncan *et al.*, 2016), that this repressive effect can be overcome by the Notch specific inhibitor DAPT (Duncan *et al.*, 2016), and that a reversed situation of Notch signalling is present in *O. bicornis* (active oogenesis ~ active Notch signalling; 6.4.1) — I attempted to repress oogenesis in *O. bicornis* by feeding them known amounts of that same Notch inhibitor used in the Duncan *et al.* study; DAPT. This to ascertain directly whether Notch signalling also serves a regulatory role in the related solitary bee *O. bicornis*, and whether it is also required to maintain early oogenesis and the germline stem cell niche as it does in *D. melanogaster* (Xu *et al.*, 1992; Song *et al.*, 2007) and as suggested by the ISH experiments.

Duncan *et al.* (2016) successfully fed *A. mellifera* workers DAPT, hence a similar approach was used here by mixing DAPT in with 50% sucrose solution. For DAPT trial 1, solution uptake did not differ significantly between DAPT-treated and control cages over time (interaction term: $F_{1,12} = 0.083$, $p = 0.778$), nor over time ($F_{1,12} = 0.342$, $p = 0.569$), but DAPT treated sucrose solution was taken up significantly more on average ($F_{1,12} = 9.495$, $p = 0.009$; Figure 6.6A). For the second DAPT feeding trial, which used the same concentrations (1mM DAPT), no significant differences were found across the board (interaction: $F_{1,16} = 0.683$, $p = 0.421$; time: $F_{1,16} = 3.775$, $p = 0.070$; and treatment: $F_{1,16} = 0.987$, $p = 0.335$, Figure 6.6B).

These first feeding trials also contained evaporation controls, to check whether sucrose solution was actually being taken up. When these data points were included in the models, there was a consistent effect of treatment (treatment; DAPT trial 1: $F_{2,18} = 36.76$, $p < 0.001$; and DAPT trial 2: $F_{2,24} = 21.61$, $p < 0.001$), with both DAPT treated and control groups being consistently higher than the loss by evaporation alone (Figure 6.6A & B). The evaporation control also provided a more accurate estimate of possible uptake of the solute in these early feeding trials, and clearly illustrated that the majority of the apparent variance in daily uptake was due to differences in ‘evaporation rate’ over time (i.e. there were no significant time and treatment interactions for uptake, DAPT trial 1: $F_{2,18} = 1.618$, $p = 0.226$; DAPT trial 2: $F_{2,18} = 1.848$, $p = 0.179$; see also covariance of treatment and control with the evaporation control over time in Figure 6.6A & B). The daily variation in evaporation rate was likely due to fluctuations in relative humidity (Figure 3.11B), in addition to refreshment of feed and measurements of uptake not always occurring at consistent times of day.

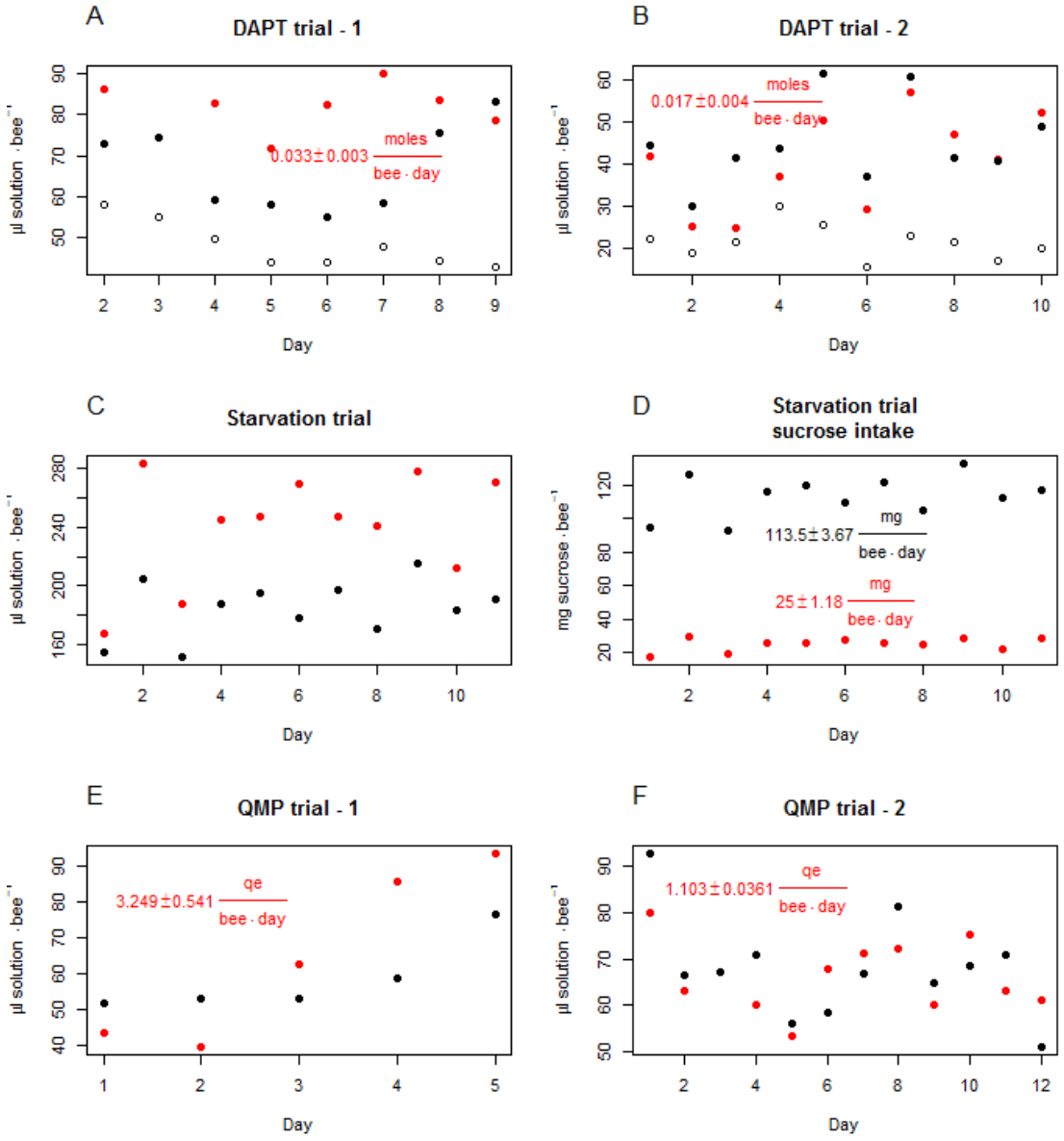

Figure 6.6: Estimated sucrose solution intake in trials. Treatments = red, controls = black. A-B) estimated intake of sucrose solution mixed with: DAPT dissolved in EtOH (red) or solvent only (black) during DAPT trials 1 and 2 (different concentrations were used across trials). Open circles show covariation of the evaporation controls, indicating that evaporation is the main underlying cause of variation across days. C) Estimated sucrose solution uptake during the starvation trial shows a higher uptake of 10% (red) sucrose solution than 50% (black) sucrose solution. D) Estimated sucrose uptake was consistently higher for the 50% (black) control group than the 10% (red) treatment group. Showing that females presented 10% sucrose solution could not compensate sucrose uptake by drinking more solution overall (see C). E-F) estimated intake of sucrose solution mixed with: QMP dissolved in EtOH (red) or solvent only (black) during QMP trials 1 and 2 (different concentrations were used across trials).

Notch signalling does not only maintain the germline stem cell niche in *D. melanogaster* (Song *et al.*, 2007), it is also known to be under reversible dietary control (both protein and carbohydrate: Hsu and Drummond-Barbosa, 2011; Bonfini *et al.*, 2015), and dietary components have long been known to affect its reproduction (Bownes *et al.*, 1988; Badisco *et al.*, 2013). Protein is implicitly required for the production of vitellogenin. Consequently, an adult pollen diet is essential to egg maturation in, at least one *Osmia* species (*Osmia californica*: Cane, 2016). Furthermore, with respect to the RGPH (Amdam *et al.*, 2006), insulin signalling is thought to have been co-opted into division of labour in honey bees (Ament *et al.*, 2008), and is also considered an important mediator of caste differentiation (Wheeler *et al.*, 2006; de Azevedo and Hartfelder, 2008; Mutti *et al.*, 2011; Wolschin *et al.*, 2011; Wheeler *et al.*, 2014). Hence, in order to try and repress oogenesis with respect to this context, a treatment group was denied pollen, and fed a 10% sucrose solution, as opposed to the control group with access to free to pollen and a 50% sucrose solution.

While there were no differences in uptake of solution with treatment over time (interaction term: $F_{1,18}$= 0.336, p = 0.569; Figure 6.6C), nor differences over time alone ($F_{1,18}$= 3.053, p = 0.098; Figure 6.6C), bees fed 10% sucrose solution did attempt to compensate total sucrose intake by drinking more solution overall ($F_{1,18}$= 20.73, p < 0.001; Figure 6.6C). When subsequently estimating actual sucrose intake, again no effects of treatment over time (interaction term: $F_{1,18}$= 0.619, p = 0.442; Figure 6.6D) nor time were found ($F_{1,18}$= 2.510, p = 0.131; Figure 6.6D). But bees fed 10% solution proved unable to compensate their sucrose intake ($F_{1,18}$= 557.3, p < 0.001; Figure 6.6D). Consequently absolute sucrose intake was approximately four and a half times lower than that of the control treatment, this illustrates that the use of lower sucrose concentrations can be used as a robust way of mimicking starvation conditions.

Given the role of QMP in repressing adult worker reproduction in honey bees (Duncan *et al.*, 2016) — and the fact that honey bee QMP is known to repress reproduction in a variety of other species (prawns — species unspecified: Carlisle and Butler, 1956; *Kalotermes flavicollis*: Hrdý *et al.*, 1960; *Musca domestica*: Nayar, 1963; *D. melanogaster*: Sannasi, 1969; *B. terrestris*: Princen *et al.*, 2019b) — I next applied QMP to *O. bicornis* through: feeding, topical application and injection. This to try and repress oogenesis, once more via Notch signalling (Duncan *et al.*, 2016). Feeding differed significantly with treatment over time in QMP trial 1 (interaction term: $F_{1,6}$= 7.043, p = 0.038; Figure 6.6E; likely due to the short trial duration variance did not even out). In QMP trial 2 no such difference , nor any other difference could be found

(interaction term: $F_{1,20}$= 0.718, p = 0.407; time: $F_{1,20}$= 2.047, p = 0.168; treatment $F_{1,20}$= 0.200, p = 0.660; Figure 6.6F).

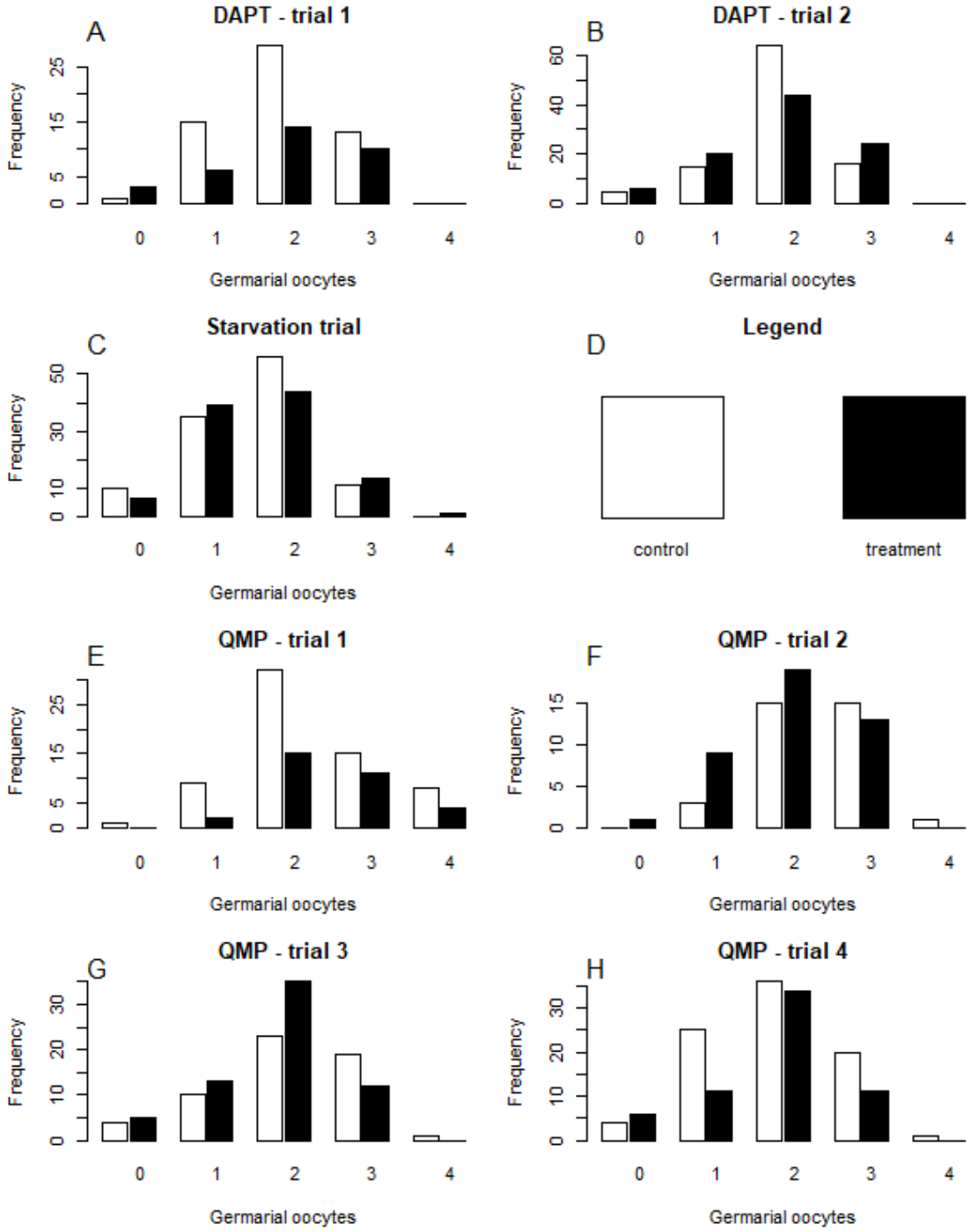

Figure 6.7: Germarial oocyte counts for respective trials. A-B) Distribution of germarial oocyte counts for DAPT trials 1 and 2 respectively (black = 1mM DAPT, white = solvent control). C) Distribution of germarial oocyte counts for the starvation trial (black = 10% sucrose - pollen, white = 50% sucrose + pollen). D) Legend showing treatment colour coding. E-H) Distribution of germarial oocyte counts for QMP trials 1 to 4 (trial 3 = injection and topical application; with black = QMP and white = solvent control).

Finally it should be noted that, upon dissection, all bees consistently had sucrose solution present in their crop, for any and all trials performed. Figure 6.7 shows the results for all *O. bicornis* trials performed, with the results of statistical testing summarised in Table 6.2. In short: no significant differences were found for any treatment or method in any of the trials; and the disorganisational phenotypes illustrated in Figure 6.2 were either rare pathophysiological phenomena or artefacts from dissection and fixation.

Other methods of analysis were briefly trialled (primarily using data of QMP trial 2), which included looking at germarial length, and oocyte maturation rate (as Chapter 4, but restricted to the germarium), to try and find even subtle effects in the germarial region (data not shown) — where as outlined previously one would mainly expect to find a phenotype. I also explored for subtle effects in the late stage vitellarium, looking at the ultimate and penultimate oocytes and their nurse cells. I modelled nurse cell chamber nuclei surface area (averaged by number of visible nurse cells) to gauge for early stages of the phenotype shown in Figure 6.2B (data not shown). I further modelled estimated nurse cell chamber volume to oocyte volume — since oocyte stages are not yet described and classified as they are in *D. melanogaster* (King, 1970) and *A. mellifera* (Wilson *et al.*, 2011) — to explore the possibility of terminal oocyte abortion. None of these additional metrics yielded any results, nor were any other distinct phenotypes observed.

Table 6.2: Overview of results for the *O. bicornis* trials. Df = degrees of freedom (numerator and denominator respectively) for the log likelihood ratio tests for model comparisons. N = sample size (i.e. females used — not number of ovarioles), T = treatment, c = control, value = value of the test statistic (χ^2). P = p-value of test; and corrected p = Holm corrected p-value (Holm, 1979).

Trial	N		Germarial counts (Log likelihood ratio)					Disorganisation (Fisher's exact)			
	T	C	Variable	Df	value	p	corrected p	Region	Odds ratio	p	corrected p
DAPT 1	8	11	treatment	1,3	<0.001	0.994	1.000	Germarium	0.566	0.699	1.000
			weight	1,3	0.317	0.574	1.000	Vitellarium	0.000	1.000	1.000
DAPT 2	24	24	treatment	1,3	0.099	0.753	1.000	Germarium	0.343	0.054	0.485
			weight	1,3	0.623	0.430	1.000	Vitellarium	7.590	0.033	0.334
Starvation	24	24	treatment	1,3	0.174	0.677	1.000	Germarium	1.182	0.720	1.000
			weight	1,3	0.388	0.533	1.000	Vitellarium	0.732	0.741	1.000
QMP 1	3	4	treatment	1,30[a]	0.265	0.393	1.000	Germarium	0.964	1.000	1.000
QMP 2	11	10	treatment	1,3	1.115	0.291	1.000				
			weight	1,3	0.291	0.984	1.000				
QMP 3	15	14	treatment	1,5	1.239	0.266	1.000	Germarium	0.600	0.545	1.000
			method	1,5	1.577	0.209	1.000				
			weight	1,5	0.001	0.992	1.000				
			method:weight	1,5	0.005	0.944	1.000				
QMP 4	24	24	treatment	1,3	0.020	0.888	1.000	Germarium	1.599	0.243	1.000
			weight	1,3	0.698	0.403	1.000	Vitellarium	1.380	1.000	1.000

[a] inflated sample size during QMP-trial 1 was due to ovarioles of individuals being pooled.

6.4.3 Repressing oogenesis post ovary activation

The inability to repress oogenesis in *O. bicornis*, particularly the lack of response to QMP, might suggest an inability to inhibit the ovary once it has been activated. Adult queenright honey bee workers with inactive ovaries are exposed to QMP since emergence (Winston, 1991), if not prior. QMP may therefore have a preventative mode of action, rather than a direct one. Studies that have shown a negative effect of QMP on reproduction in other species, have typically done so prior to adulthood and full reproductive capacity (Carlisle and Butler, 1956; Hrdý *et al.*, 1960; Nayar, 1963; Sannasi, 1969; Galang *et al.*, 2019; Princen *et al.*, 2019b). As illustrated in Chapter 5, *O. bicornis* emerges from hibernation with primed ovarioles. In order to ascertain whether QMP can elicit an effect post ovary maturation, I subjected queenless *A. mellifera* workers to QMP.

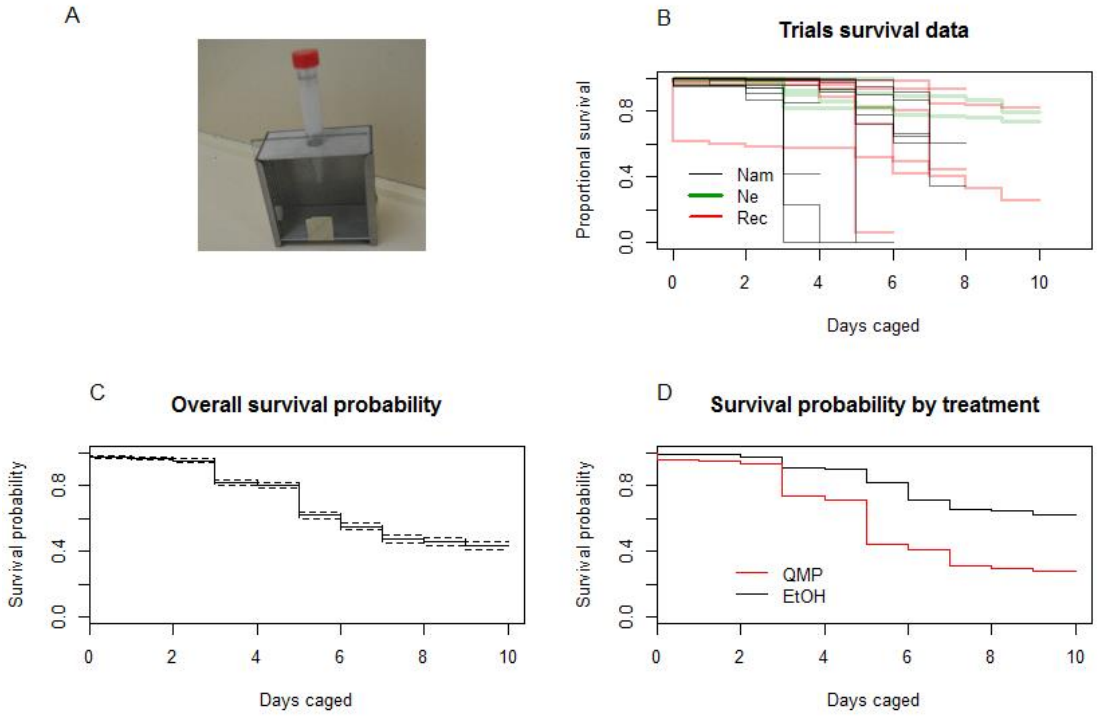

Figure 6.8: Survival of *A. mellifera* under laboratory condition. A) Cage design used to house *A. mellifera* workers. 15 ml polypropylene tube contained distilled water for thermoregulation[43], white caps on side contained complete bee food, and a slide in the middle of the cage was provided with either QMP or EtOH (solvent control). Holes for ventilation are present at the sides of the cage (not visible). B) Raw survival data, presented as proportional survival per cage over time. Nam = non age marked captured workers, Ne = newly emerged workers, and Rec = age marked recaptured workers (18-21 days old). Transparency and width of lines differs along categories, but these settings are not informative and only serve to make the figure legible. C) Kaplan-Meier survival probability curve denoting overall survival probability, with dotted lines representing confidence intervals. Data assumes individuals are independent observations and hence is used here for descriptive purposes only. Additionally, data includes newly emerged worker survival. D) Kaplan-Meier survival probability by treatment. P-value has been omitted (pseudoreplication) and figure is illustrative only.

Figure 6.8A illustrates the cages used, in addition to the survival rates and probabilities of the trials (Figure 6.8B-D). Twenty cages were trialled in total. First, ten cages of workers caught at random from a queenless hive were tried (Figure 6.8B). While these were not age-matched, these heterogeneously aged workers were assumed to be divided homogenously among replicates (Williams *et al.*, 2013). Secondly, newly emerged bees were age marked and

[43] One of the ways in which honey bee workers reduce temperature inside the hive (and these cages), is through the evaporation of water (Winston, 1991).

released into a queenless hive to be recaptured once ovary activation was sufficient (all cohorts were between 18 and 21 days old; six cages total). Finally, newly emerged workers were trialled (four cages), to investigate the possibility of initiating QMP exposure halfway through a trial. The social nature of *A. mellifera* requires a minimum amount of workers to be present in a cage (Rinderer and Baxter, 1978; Bosua *et al.*, 2018; Abou-Shaara and Elbanoby, 2018) for it to survive. This is density dependent (i.e. group and cage size dependent; Abou-Shaara and Elbanoby, 2018), which in the present study equated to a minimum of 50 bees for the cages used (80-100 bees per cage were used at the start of every experiment on average). Additionally, the inability of caged workers to remove dead bees causes stress and may spread disease. These factors may trigger mass deaths as illustrated for some cages in Figure 6.8B. Mass and sudden deaths occurred exclusively for non-age marked captured and age marked recaptured bees, presumably due to an increased likelihood of death by constipation. Figure 6.8D and Figure 6.9 further illustrate the increased mortality for caged workers exposed to QMP, and for captured and recaptured bees. There was no significant interaction between age matching categories and QMP treatments ($\chi_{2,9} = 0.004$, $p = 0.998$), nor did the increased mortality in captured bees (non-age marked and age marked) retain significance ($\chi_{2,7} = 5.937$, $p = 0.051$). Yet, QMP treated workers did show a significant increase in mortality ($\chi_{1,7} = 7.633$, $p = 0.006$; Figure 6.8D and Figure 6.9) under the binomial model; regardless of whether newly emerged workers were excluded from analysis.

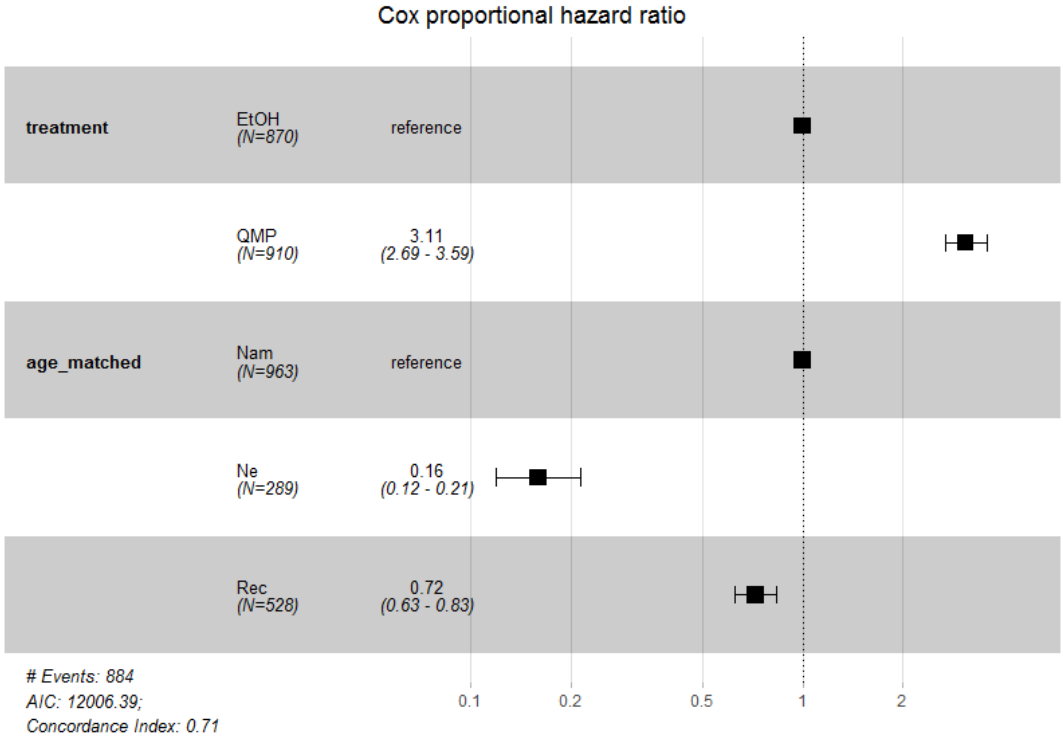

Figure 6.9: Cox proportional hazard model of *A. mellifera* in trials. Cox proportional hazard ratios (odds ratio of likelihood of instantaneous death) per treatment and age matching category are displayed. P- values are omitted throughout and figure is illustrative only. QMP treated adult workers with active ovaries are over thrice as likely to die than workers in controls (EtOH). Newly emerged (Ne) workers were also far less likely to die (0.16 times) than non age matched (Nam) workers.

With regard to food consumption, I did not find any difference in the uptake of CBF by treatment and age matching category (interaction term: $F_{2,13}$ = 0.123, p = 0.885), nor by treatment alone ($F_{1,14}$ = 1.257, p = 0.281; Figure 6.10B). This indicates that the increased mortality observed in QMP treated bees was likely not due to an increased intake of CBF. There was a significant effect of age matching category ($F_{1,13}$ = 8.554, p = 0.004), with random slopes of newly emerged bees differing significantly (Wald's t_{16} = -2.981, p = 0.009; Figure 6.10A; i.e. CBF uptake rose consistently over time for newly emerged workers).

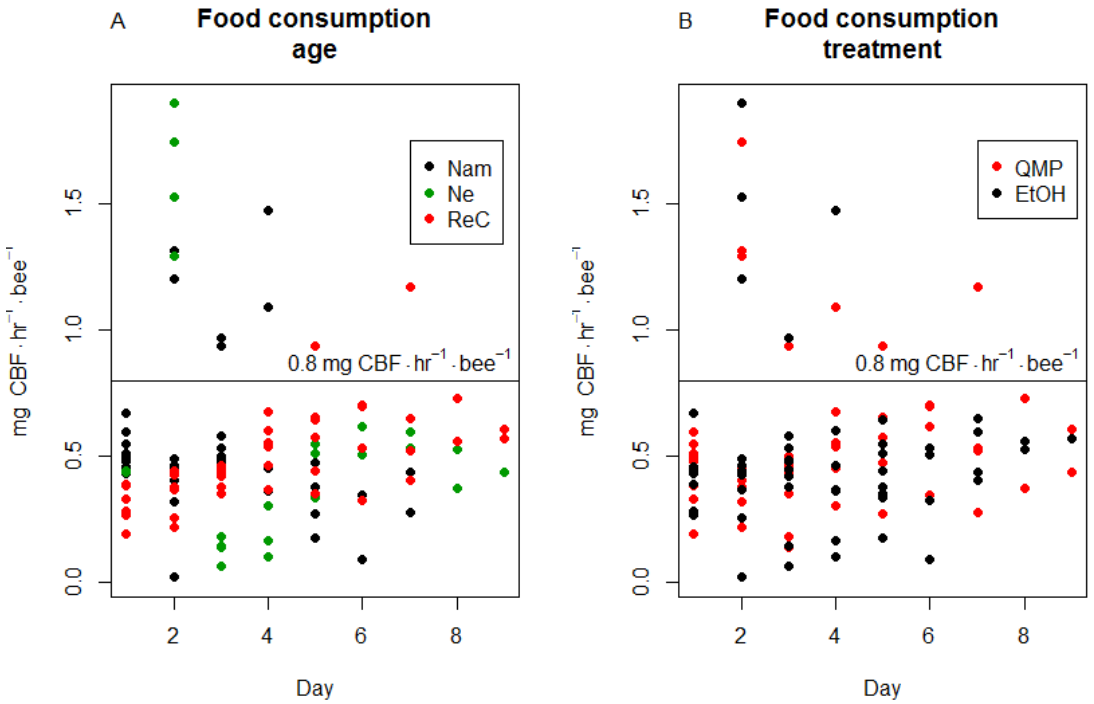

Figure 6.10: Caged *A. mellifera* worker food intake. A) Estimated CBF intake per bee per hour by age matching group (Nam = non age matched, Ne = newly emerged, and Rec = age marked and recaptured). B) Estimated CBF intake per bee per hour by treatment. Points above the horizontal line indicate CBF with too much honey which dripped into the cages. When these outliers were dropped from the model, it led to a substantially better fit (AIC scores and distribution of residuals).

Finally, there was no significant interaction effect among treatment groups and age matching categories on ovary scores ($\chi_{1,7} = 0.127$, $p = 0.278$), nor a significant effect of age matching ($\chi_{1,5} = 1.178$, $p = 0.722$), nor an effect of treatment ($\chi_{1,5} = 0.185$, $p = 0.333$; Figure 6.11A and B). Note that newly emerged workers were omitted from this analysis, since they do not show significant ovary activation after five days (Fisher's exact test: $p = 0.2361$, Figure 6.11C). Newly emerged workers showed significant ovary activation only after ten days (Fisher's exact test: $p < 0.001$, Figure 6.11D), and hence did not lend themselves to be exposed to QMP thereafter due to the drop off in survival when fed a protein source (Pirk *et al.*, 2010).

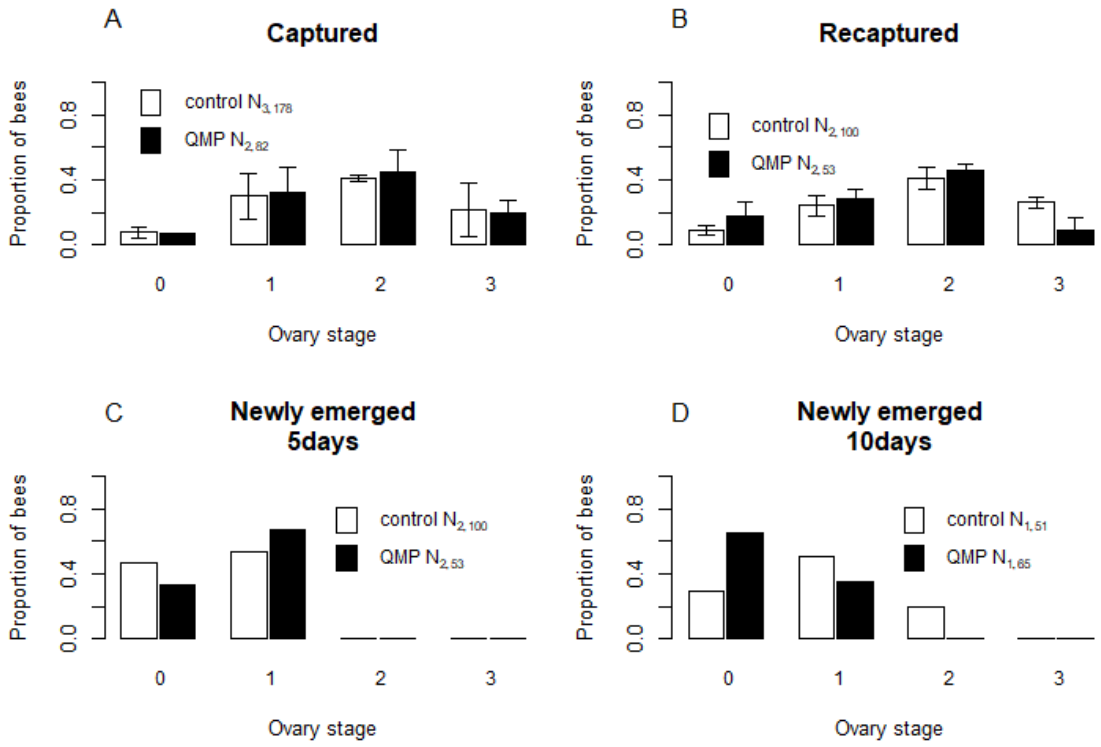

Figure 6.11: Proportions of ovary scores of *A. mellifera* trials. A) Proportions of ovary scores attributed to non-age matched captured workers upon dissection. B) Proportions of ovary scores attributed to age marked recaptured workers upon dissection. $N_{\#,\#} = N_{\text{number of cages, total number of bees}}$; with low sample sizes caused by low survival probability of captured and recaptured workers in cages (Figure 6.8C and Figure 6.9). Error bars denote standard deviations.

6.5 Discussion

Using *in situ* hybridisation, I have shown that *O. bicornis* maintains active Notch signalling in the germarium of ovaries with active oogenesis. This state is more in line with the situation in *D. melanogaster* (Song *et al.*, 2007), than with the state of Notch signalling in *A. mellifera* (Duncan *et al.*, 2016). This, even though *D. melanogaster* is some 330 million years diverged from *O. bicornis* in evolutionary time (Misof *et al.*, 2014), whereas *A. mellifera* is only a 95 million years diverged from *O. bicornis* (Branstetter *et al.*, 2017; Peters *et al.*, 2017). It follows that the function of Notch signalling has switched in the recent evolutionary history of *A. mellifera*, and it seems probable that this switch in signalling function occurred with respect to its co-option into the reproductive division of labour (Duncan *et al.*, 2016). Since Notch signalling is in essence a way for two cells to communicate (Bray, 2006), an alternate formulation is that the context of the communicating cells in question has changed in (relatively) recent evolutionary history (Schwanbeck *et al.*, 2011). That is, where in *O.*

bicornis and *D. melanogaster* an active germarial Notch signal facilitates oocyte specification, in *A. mellifera* such a signal may prevent specification and oocyte progression.

Consequently, as in Duncan *et al.* (2016), I used the Notch inhibitor DAPT to try and provide a functional test of Notch signalling in oocyte specification in this species. DAPT did not inhibit oocyte specification in *O. bicornis* within my set-up. This may be due to a number of reasons. The dose used (1mM DAPT as in Duncan *et al.*, 2016) may not have been sufficient. Not only do *O. bicornis* females and *A. mellifera* workers differ in body size, but the active ovaries of *O. bicornis* are inherently substantially larger than the inactive ovaries of newly emerged and queenright workers. Interspecific differences in the uptake (gut) and distribution (haemolymph) of DAPT could further complicate matters. To rule out such complications, the uptake, distribution and stability of DAPT in the *O. bicornis* haemolymph could be assayed directly through high performance liquid chromatography (HPLC).

In Duncan *et al.* (2016), DAPT was presented in solid food. Workers were also kept in complete darkness — which was not the case for *O. bicornis* here — and DAPT is known to be sensitive to light. A lack of uptake could further also be attributable to the concerns raised surrounding solubility (Stumm and Morgan, 2012; Williams *et al.*, 2012). While effective uptake can be assayed through HPLC; effective action of the inhibitor would need to be verified through the use of RT-qPCR of Notch responsive genes (E(spl)) in various tissues (e.g.: ovary, brain and fat body). Future trials could apply different methods of administration for this chemical (e.g. injection and topical application), as was performed for QMP here. Finally, it may be that DAPT did not act on the ovaries directly in (Duncan *et al.*, 2016). DAPT may have acted on for instance specific neuronal networks in *A. mellifera*, after which signals might be sent to the ovary. Hence, attempts could be made to cut out all intervening and compromising steps (uptake and brain), and assay ovaries directly. Tissue culture media exist, for at least *A. mellifera* ovaries (Rachinsky and Hartfelder, 1998), and have been used for BrdU immunocytochemistry previously (Tanaka and Hartfelder, 2004). If such a medium could be adapted for *O. bicornis* (and DAPT is soluble and effective in such media) then ovaries could be exposed and assayed directly. DAPT is generally considered a ‘highly specific γ-secretase inhibitor’ (Geling *et al.*, 2002), which made it the preferred candidate to investigate Notch signalling in the ovary here. Yet, following its lack of response, I subsequently opted for less specific ways to try and repress oocyte specification and oogenesis in *O. bicornis*.

Even with the denial of a protein source (pollen), I could not arrest oogenesis in the germarium of *O. bicornis*. Cane (2016) similarly denied pollen to *O. californica* females, and found that terminal oocytes did not grow[44]. He did not investigate the germarial region, nor did he explicitly report any other phenotypes. This implies, that while an adult pollen diet may be required to further vitellogenesis post emergence, protein levels may not regulate germarial Notch. However, this finding is still in line with the findings of Bonfini *et al.* (2015) in *D. melanogaster*, where they found no increase in germline stem cell niche size with yeast supplemented medium in comparison to standard medium. Starvation treatments in Bonfini *et al.* (2015) on the other hand, consisted of agar only (complete starvation), or glucose and protein depleted media, which did show a decrease in germline stem cell niche size. Since protein and glucose depletion were never regarded separately in their experiments, it may be that germarial Notch is responsive to glucose only. Not only is germarial Notch signalling known to be responsive to insulin signalling (Hsu and Drummond-Barbosa, 2011), but insulin signalling is known to be important to insect reproduction in general (Badisco *et al.*, 2013). Furthermore, insulin signalling has roles implicated for it in both the division of labour (Ament *et al.*, 2008) and caste differentiation of *A. mellifera* (Wheeler *et al.*, 2006; de Azevedo and Hartfelder, 2008; Mutti *et al.*, 2011; Wolschin *et al.*, 2011; Wheeler *et al.*, 2014).

During the starvation trial carried out here, I managed to decrease the estimated sucrose uptake over fourfold (in conjunction to protein starvation). Yet the disaccharide sucrose still breaks down into both glucose and fructose. Hence, while my treatment will have decreased the amount of glucose available (as in Bonfini *et al.*, 2015 where glucose could only be derived from maize starch and subsequently maltose), it may not have excluded glucose sufficiently. A future approach may be to try and completely exclude glucose (i.e. using fructose only, but also note that fructose impairs insulin signalling in rats: Baena *et al.*, 2016), to try and gauge the role of insulin signalling. Additional trials or experiments, may involve: a further decrease in sugar concentrations used (e.g. 2.5-5% treatment), temporary complete starvation (as

[44] I did not investigate terminal oocyte volumes here, since this was not the primary region of interest. Additionally, in order to clearly stain and image the germarium, ovarioles had to be individualised and peeled (removing the ovariole sheath and intima), and ovarioles had to be mounted on slides. Mature and terminal oocytes were often too large to mount, or were damaged and broken off during the peeling of the ovaries.

in Bonfini *et al.*, 2015)[45], or extending trial duration to try and burn through the females' reserves.

Ultimately, I tried to repress early oogenesis in *O. bicornis* through QMP, since it is known to do so in other species (Carlisle and Butler, 1956; Hrdý *et al.*, 1960; Nayar, 1963; Sannasi, 1969; Princen *et al.*, 2019b). Additionally, it should be directly responsive and relatable to the pathway under investigation (Duncan *et al.*, 2016). While QMP feeding assays were once more inconclusive (solubility issues); both injection and (repeated) topical exposure did not yield an effect either. Such results are inconclusive, since the existence of a dose dependent response needs to be considered; as has been done for Drosophila (Lovegrove *et al.*, 2019). Additionally, QMP is disseminated from the queen throughout the colony by worker licking (trophallaxis), antennation, and deposition into wax (Naumann *et al.*, 1991). Most QMP on workers, is found in the gut as well as the rest of the abdomen (Naumann *et al.*, 1991), hence it may be that topical application on the abdomen (instead of the thorax) could provide an effect and might be trialled in future. Yet it should be pointed out, that the exact mode of action of QMP is yet unknown (Jarriault and Mercer, 2012). QMP is known to affect dopamine levels in the brain (Jarriault and Mercer, 2012), where it enacts behavioural changes. But whether the brain further signals to the ovaries, or whether abdominal QMP acts directly on the ovaries, or whether the fatbody or haemolymph provide an intermediary signalling function is not clear. Given these uncertainties, another reasonable suggestion may be to apply QMP topically, but on the abdominal sterna where *O. bicornis* females might then lick it clean. Topical application here, was performed on the top of the thorax precisely to penetrate the cuticle without loss of substance to any form of cleaning behaviour.

A final possible avenue, to try and repress early oogenesis in *O. bicornis* may be to manipulate juvenile hormone (JH). Wasielewski *et al.* (2011b) found that methoprene (JH analogue), in conjunction with temperature, accelerated ovary activation. While their assessment of ovarian development consisted of measuring terminal oocytes (similar to Cane, 2016), effects on earlier stages of oogenesis cannot be excluded. Providing a block on JH and/or its synthesis (as used in pesticides: Quistad *et al.*, 1981; Staal, 1982; Kuwano *et al.*, 1983; Prestwich, 1986). Treating with an inhibitor of JH biosynthesis may arrest oogenesis and allow functional testing of whether Notch signalling is genuinely functionally associated with active oogenesis in *O. bicornis*. Alternatively, RNAi

[45] But note that complete starvation in *O. bicornis* already leads to high mortality after three days (Schenk *et al.*, 2018a).

of the JH hormone receptor Methoprene-tolerant (Met) might be tried (as has been performed in *Schistocerca gregaria* for instance; Gijbels *et al.*, 2019).

The most convincing negative result for *O. bicornis* here, was the absence of an effect of QMP injection in *O. bicornis.* Since this method has been demonstrated in other species (single injection: Carlisle and Butler, 1956; repeated injection: Sannasi, 1969). Yet, in all studies concerning QMP (old and new), QMP is administered either before or during ovary development/activation (Carlisle and Butler, 1956; Hrdý *et al.*, 1960; Nayar, 1963; Sannasi, 1969; Lovegrove *et al.*, 2019; Galang *et al.*, 2019; Princen *et al.*, 2019b). Indeed, Carlisle and Butler (1956) in particular, in their study using a single injection on prawns, are very explicit with this in their methodology. They initiate ovary activation by removing the eyestalks containing an 'ovary inhibiting hormone', and subsequently injected QMP. This then raised the question of whether ovary activation can indeed be repressed once oogenesis is underway. Since *O. bicornis* emerges with primed and active ovaries (see Chapter 4), this might then elegantly explain why ovary repression is seemingly so difficult in this species.

QMP is assured to work in *A. mellifera* workers, hence the ideal way to test the effectiveness of QMP post ovary activation, was to subject reproductively active workers to QMP. Such a design possesses its own hurdles, considering worker survival (see 6.4.3). Yet, no effect of QMP post-ovary activation could be detected. Once more, a dose-response argument could be made for QMP (Lovegrove *et al.*, 2019), albeit a nuanced one. It is already known that workers possessing more ovarioles, are more resistant to the repressive effects of QMP, and that they will activate their ovaries disproportionately upon release from QMP (Amdam *et al.*, 2006; Makert *et al.*, 2006; Traynor *et al.*, 2014; Ronai *et al.*, 2017)[46]. A straightforward explanation for this phenomenon might be that the 'excess' or 'surplus' ovarian tissue in these workers makes it harder for QMP to enact its role (assuming that every worker, on average, takes up as much QMP as any other worker). Such a hypothesis would elegantly explain why post ovary activation (and increased ovarian mass) QMP becomes ineffective. The ovarian mass of *O. bicornis* females upon emergence is likewise high, and might require a higher dose of QMP to elicit an effect. Other considerations are: the limited duration of the *A. mellifera* trials here (restricted by caged survival; 6.4.3), and a loss of QMP

[46] Ronai *et al.* (2017) suggests that five ovarioles per ovary may be ideal for worker ovary activation, but did not correct for the underlying distribution of ovariole numbers, which is not Gaussian (with a mode of 3-4 ovarioles; Velthuis, 1970) and the distribution is also known to vary among subspecies (Velthuis, 1970).

uptake by workers post-ovary activation (e.g. reduced worker trophallaxis in queenless workers: Mayer *et al.*, 1998). Finally, QMP might simply provide an non-reversible plastic phenotype, suppressing early oogenesis exclusively prior to tissue development.

The marked decrease in survival of *A. mellifera* workers exposed to QMP was striking, and retained significance even under appropriate tests. This result could not be explained by an increase in food uptake (Naumann and Laflamme, 1993). In fact, QMP is known to make workers more resistant to starvation (Fischer and Grozinger, 2008). Paoli *et al.* (2014) even reported an increased survival for caged workers subjected to QMP, compared to controls (regardless of whether essential amino acids were added to the diet). The differential survival presented here, might therefore make more sense in light of ovary activation. A decrease in queen acceptance in queenless hives over time is well known within the community[47], even though no explicit studies are available. Indeed, queen cells are known to be constructed within 48 hrs of queen loss (Fell and Morse, 1984; Hatch *et al.*, 1999), likely cued by the loss of QMP (Melathopoulos *et al.*, 1996). Subjecting the aged queenless workers to QMP here, could be considered a simulation of presenting a new queen to a hive that has been queenless for several weeks. Since workers could not remove the QMP on the slide (i.e. equivalent to rejecting and killing the queen), it may have caused stress. QMP might even be toxic to workers with activated ovaries, or might direct aggression (from workers with stage 0 or 1 ovaries) towards workers with fully activated ovaries (stage 2 or 3). It should be noted that deceased bees were not dissected to check for possible biases in ovary activation[48].

With a view to bypass the issues surrounding *A. mellifera* worker survival encountered here, it might be worthwhile to try feeding newly emerged caged workers royal jelly. While royal jelly does not necessarily lead to increased survival, Pirk *et al.* (2010) did find that it leads to higher levels of ovary activation. Perhaps ovaries may also activate faster, which could facilitate a design using newly emerged workers.[49] If the further use of caged workers to investigate the effects of QMP post ovary activation proves untenable, it may prove fruitful to make use of the fruit fly instead. This since the survival of *D. melanogaster* should not be limited in the same way as that of *A. mellifera*

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[47] E.g.: F. Ratnieks; http://www.lasiqueenbees.com/how-to/how-to-introduce-your-queen-into-a-hive; last updated: July 2016; access date: 23/02/2020

[48] This because incubating conditions facilitate rapid tissue degradation, and bees were only checked on once per day to reduce handling stress.

[49] Then again, the composition of RJ is rather complicated (Sabatini *et al.*, 2006) and may further confound results.

workers, while *D. melanogaster* is known to enjoy the priming effect of QMP (Lovegrove *et al.*, 2019). Survival, aggression, and secondary ovary regression could all be investigated in *D. melanogaster* in a full factorial design, to disentangle the priming and releasing effects of QMP, and perform a direct test of the reversibility of the ovarian phenotype (Figure 6.12).

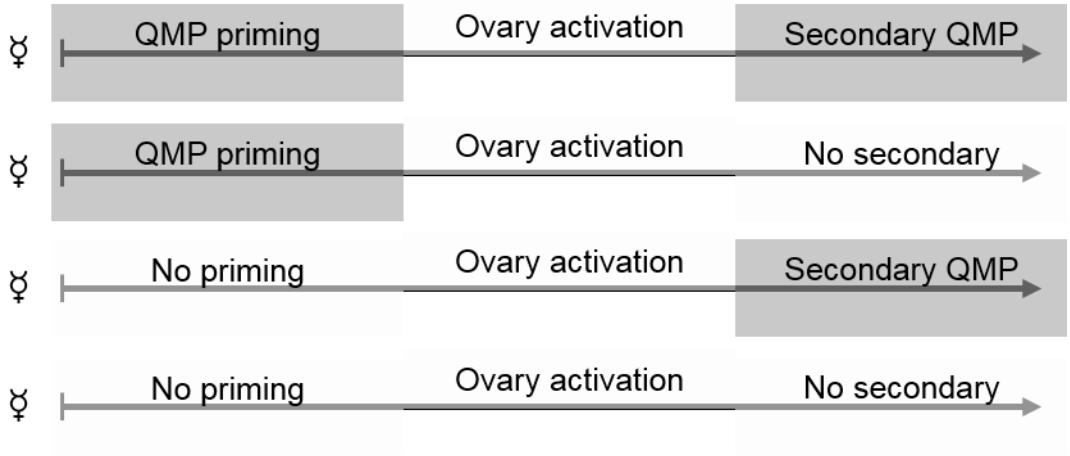

Figure 6.12: Suggested experimental design. Using virgin *D. melanogaster* females (☿), the reversibility of QMP could be studied more easily. The use of virgins is standard in *D. melanogaster* QMP experiments (Lovegrove *et al.*, 2019). The axes represent three (undefined) time periods.

Chapter 7 General discussion

Darwin's (1859) special difficulty — the evolution of neuter castes — continues to intrigue researchers. In the recent past, the RGPH has come to prominence in the field, whose foundations were laid out by West-Eberhard (1987; 1996; 2003; 2005). The RGPH broadly states that '*reproductive regulatory networks of solitary ancestors'* underlie the reproductive division of labour in eusocial species (Page *et al.*, 2009) — i.e. that there has been a decoupling of reproductive and parental care traits. This general framework can be further subdivided into more specific hypotheses and cases (e.g. within forager specialisation; Oldroyd and Beekman, 2008). These different hypotheses are not mutually exclusive and illustrate that multiple evolutionary routes can lead to eusociality (Linksvayer and Johnson, 2019). For instance, in *Polistes* wasps, it is thought that the regulatory network underlying diapause (e.g. JH) — which is typified by prolonged development, prolonged lifespan, cold-resistance, stress-resistance, nutrient storage, and delayed reproduction — underlies the gyne (i.e. 'queen') phenotype (Hunt *et al.*, 2007). Workers, in turn, are thought to be derived from the non-diapausing phenotype, possessing the reverse traits (Hunt *et al.*, 2007). This regulatory network is then thought to have been co-opted into nutritional pathways over evolutionary time, as parents *manipulated* (Alexander, 1974; Craig, 1979) this regulatory network and the resulting phenotypes through selective feeding of offspring (Hunt and Amdam, 2005; Hunt *et al.*, 2007). In a striking example of convergent evolution, the RGPH introduces how '*a set of conserved genes or molecular pathways*' have repeatedly been recruited into the underlying social structure of eusocial societies (Bloch and Grozinger, 2011; Berens *et al.*, 2015; Kapheim, 2016).

> *To validate this premise, cross-species comparisons across the eusocial spectrum are required:'Comparative studies that include solitary insects will help fill gaps in our understanding of how developmental plasticity evolves in solitary ancestors, as well as how genes involved in other aspects of developmental plasticity may have been co-opted for social evolution. Additional research on solitary insects will also enable more complete tests of the hypothesis that social function emerged from standing genetic variation, and will help to identify the mechanisms that produce this variation. Comparative studies of selection patterns in closely related solitary and social species will also be necessary to evaluate the degree to which plasticity precedes social evolution.'*
> *~ Kapheim (2019)*

7.1 The use of *O. bicornis*

Roubik (1992) pointed out that many advanced eusocial species are tropical and subtropical, and further outlined several reasons why this might be. It stands to reason that the most ideal candidates to become reference solitary model species for cross-species comparisons would be found in tropical regions — as they would be closely related to eusocial species, and possess many if not all of the required pre-adaptations to eusociality (Chapter 1). Unfortunately, tropical species are not accessible to all researchers, and for this reason I exerted considerable effort to further *O. bicornis* as such a model species (Chapter 3 and 4). Regardless of the shortcomings of a temperate species, *O. bicornis* is still expected to be relevant when investigating aspects of the RGPH. When *conserved genetic toolkits* (Kapheim, 2016) are of interest, the respective importance of phylogenetic distance (95 my diverged from *A. mellifera*; Peters *et al.*, 2017) should be somewhat lessened. Indeed, *D. melanogaster*, some 330 my diverged from *A. mellifera* (Misof *et al.*, 2014), has even been put forward as a model of study in this regard (Camiletti and Thompson, 2016).

Another solitary bee was initially considered for as a model species here, namely the alfalfa leafcutter bee (*Megachile rotundata*). Its most recent common ancestor with *A. mellifera* likewise existed some 95 my ago (Peters *et al.*, 2017) as it is a member of the Megachilidae just as *O. bicornis* is. *M. rotundata* possesses similar life-history traits to *O. bicornis*, with the notable exceptions of using leaves to line cells and the possession of a partial bivoltine lifecycle (Tepedino and Parker, 1988). Nevertheless, *O. bicornis* was elected over *M. rotundata*, chiefly based on the 2014 study that managed to elicit a full life cycle in a laboratory environment (Sandrock *et al.*, 2014).

Sandrock *et al.* (2014) definitively proved the feasibility of fully maintaining a solitary bee in an exclusively laboratory environment. I likewise managed to elicit the full range of reproductive behaviours, but could only do so with a small number of females at any one time. Based on personal observation, recommendations (van der Steen; Raw), and an overview of the literature; in Chapter 3 I laid out recommendations for future work. Specifically, I noted the prevailing successes reported using greenhouses (Holm, 1974; Raw, 1972), in conjunction with the simulated light conditions in Sandrock *et al.* (2014). Based on these I primarily emphasised the importance of natural light, and secondarily the presence natural floral resources[50]. Even though I put these

[50] Even though Sandrock *et al.*'s (2014) study indicated natural floral resources are not strictly necessary, Raw emphasised their use, and I achieved additional gains in nesting and egg laying behaviours when preferred floral resources (*Quercus* and *Ranunculus*) were mimicked.

forward as key factors in establishing *O. bicornis* females in a laboratory environment, I caution against '*threshold model*'–thinking, and argue that the natural environment should be mimicked as much as possible.

Such and further recommendations in Chapter 3, will hopefully aid future endeavours in establishing solitary bees in a laboratory environment. While methods for observing the provisioning and egg laying behaviour of *O. bicornis* are already present (e.g. Strohm *et al.*, 2002), lab rearing allows for further experimental control (e.g. behavioural manipulations, repeated injections, RNAi feeding, and the potential for genetic modification using CRISPR/Cas9, etc.). Combining these observation methods with laboratory maintenance would allow for studying, for instance, the conditions under which the eating of eggs occurs in this species (Strohm *et al.*, 2002) with reference to behavioural co-option into worker policing behaviour. Considering the difficulties in abating early oogenesis in Chapters 5 and 6, reproductive control in *O. bicornis* might predominantly be regulated during late stage oogenesis (e.g. Cane, 2016). Fully maintaining *O. bicornis* in the laboratory would then also allow directly measuring reproductive success (as in e.g.: Shukla *et al.*, 2013; Kant *et al.*, 2013; Sandrock *et al.*, 2014; Cane, 2016).

Another step undertaken to appropriate *O. bicornis* as a model species with regard to eusocial research, was the development and validation of microsatellite markers (Chapter 4). In future, these markers will be used to test whether females are indeed monogamous in this species (wrt: Hughes *et al.*, 2008), by determining whether extra-pair paternity occurs and with what frequency. Furthermore, Bretman and Tregenza (2005) point out that the possibility of post-copulatory female choice[51] can be an important factor underlying the effective mating system. Hence, in future, multiple mating could also be investigated by assaying spermatheca directly using the microsatellites developed in this study (Chapter 4) as was done in crickets (Bretman and Tregenza, 2005).

The microsatellite markers developed here may also advance the quality of genomic resources of *O. bicornis* in future. As microsatellite markers can further be used to create linkage maps for instance (Solignac *et al.*, 2004). While many more markers would be needed, these could likewise be mined (Beier *et al.*, 2017) from the *O. bicornis* genome (Beadle *et al.*, 2019), avoiding the labour intensive construction of genomic libraries (Ostrander *et al.*, 1992;

[51] Mechanisms of post-copulatory female choice consist of: preferential storage of sperm or dumping of sperm from disfavoured males, or even egg-sperm interactions whereby certain sperm phenotypes are more probable to fertilise (Parker, 1970; Eberhard, 1996; Bretman *et al.*, 2004).

Neumann and Seidelmann, 2006). Since inbreeding is known in Hymenoptera (Packer and Owen, 2001), and possibly exists within *O. bicornis* (Conrad *et al.*, 2010; Conrad and Ayasse, 2015; Conrad *et al.*, 2018), these markers can be used to test for it. Under laboratory conditions, as examined in Chapter 3, inbreeding avoidance and consequences to inbreeding could be ascertained directly through mating assays (e.g. Conrad and Ayasse, 2015) and reproductive success (e.g. Sandrock *et al.*, 2014) respectively. Finally, microsatellite markers such as these, can be used to asses gene-flow at population scales (e.g.: Conrad and Ayasse, 2015; Conrad *et al.*, 2018), and subsequently inform conservation efforts (Packer and Owen, 2001; Zayed, 2009).

While the work in Chapters 3 and 4 purposefully addressed establishing *O. bicornis* as a model species, Chapter 5 and 6 likewise contributed to this. The adaptation of immunohistochemistry (IHC; Chapter 5) and *in situ* hybridisation (ISH; Chapter 6) for ovarian tissue in this species, combined with the microstructural study and description of the ovary, should also aid in the set-up of *O. bicornis* as a model species. Overall, the work presented in this thesis has established new tools and approaches towards advancing *O. bicornis* as a model species for studying eusocial evolution

7.2 Reproductive constraint from reproductive control

The reproductive constraint suffered by adult honey bee workers, is both incomplete and reversible. The physiological self-restraint practiced by workers (Hoover *et al.*, 2003) is reversed as soon as a reproductive queen has vacated her throne. What is more, a single molecular switch can be overturned (germarial Notch signalling), and honey bee workers can activate their ovaries, even in the presence of QMP (Duncan *et al.*, 2016). The molecular switch in question is highly conserved (Duncan and Dearden, 2010) and is already known to regulate reproduction through environmental signals in other species (Xu *et al.*, 1992; Drummond-Barbosa and Spradling, 2001; Hsu and Drummond-Barbosa, 2011; Bonfini *et al.*, 2015). It stands to reason, that it is a conserved regulatory network that was recruited into the social context during the evolution of eusociality in *A. mellifera* (Kapheim, 2016). Investigating how proximate mechanisms work, can inform us on the causes of their ultimate evolution (Mayr, 1961; Tinbergen, 1963). For example, environmental signals controlling germarial Notch signalling in a hypothetical solitary ancestor of a eusocial species, would instruct us on how those environmental signals were commandeered by for instance, a dominant reproductive, to repress the physiological reproductive machinery in early eusocial workers.

The early block on queenright oogenesis in honey bee workers is morphologically similar to that of virgin queens (Tanaka and Hartfelder, 2004); which prompted me to investigate whether mating status could be ancestral to adult reproductive constraint. In Chapter 5 I established that mating status does not exert any kind of effect on the ovaries of *O. bicornis*, contrary to the situation in many other insects (Gillott and Friedel, 1977; Gillott, 2003; Colonello and Hartfelder, 2005; Avila *et al.*, 2011). This makes it extremely unlikely, for instance, that this constraint in early honey bee workers evolved by dominant reproductives monopolising access to mates. It is therefore probable, that both loss of honey bee worker spermatheca (Winston, 1991) as well as the block on oogenesis in queens (Patricio and Cruz-Landim, 2002; Tanaka and Hartfelder, 2004) evolved secondarily. That is to say, as colony size increases over evolutionary time, the queen-worker conflict shifts. Due to the increased number of workers, the probability succeeding the reproductive queen becomes ever smaller, which makes conflict over reproduction switch towards brood composition instead (e.g. sex ratio; Bourke, 1999). Consequently, no selective pressure is maintained on honey bee workers to lay fertilised eggs at that point (as workers are more related to their sons than they are to their daughters; Bourke, 1999), and extensive selective pressure is predicted to be exerted on queens by workers to be mated.

Negative results such as those found in Chapter 5, are typically difficult to resolve. To date, little is known of the physiological response of female *O. bicornis* to mating. The mating plug is thought to serve as an extra insurance to paternity, but is not incorporated by the female nor influence her decision to remate, and only serves as a physical block to future matings (Seidelmann, 2015). The post-copulatory display of the male is thought to prevent the female from remating (Seidelmann, 2014a), but the application of a pheromone during the display remains disputed (Ayasse *et al.*, 2000; Seidelmann and Rolke, 2019). Whether the post-copulatory display has any effect on female physiology in conjunction to its effect on behaviour is unknown. Consequently, current evidence would indicate that mating status can roughly be considered a Boolean trait[52] in this species. Therefore, the absence of any effect of mating status on the reproductive physiology of *O. bicornis* I demonstrated here can be considered robust.

[52] I.e. Boolean (true/false) in the sense that a female is either retained virginity or was mated. This is only true however, when we assume that the full suite of male and female mating behaviours (Seidelmann, 1995) were always observed by mated females.

In Chapter 6, I showed that germarial Notch signalling in the *O. bicornis* ovary is active when oogenesis is actively occurring, which stands in direct contrast to the situation in queenless *A. mellifera* workers. This result was more in line with the situation in *D. melanogaster* (Song *et al.*, 2007), whose most recent common ancestor with *A. mellifera* is some 235 my further removed than the most recent common ancestor shared between *A. mellifera* and *O. bicornis* (Misof *et al.*, 2014; Peters *et al.*, 2017). Given the role of germarial Notch signalling in regulating oogenesis in both *A. mellifera* and *D. melanogaster* (Song *et al.*, 2007; Duncan *et al.*, 2016), I subsequently set out to arrest oogenesis at the germarial checkpoint (Pritchett *et al.*, 2009) in *O. bicornis*. Initially, given the functional switch of germarial Notch signalling, I used the same Notch inhibitor used by Duncan *et al.* (2016) to try and achieve this. When no phenotype could be discerned, I next attempted to deprive females of nutrients (as in Bonfini *et al.*, 2015; and Cane, 2016). Finally, when that failed, I tried using QMP to arrest early oogenesis, as it is effective over a broad phylogenetic range (see Lovegrove *et al.*, 2019 and references therein).

None of these treatments had any success arresting early oogenesis in *O. bicornis*. Particularly, QMP exposure through injection is known to work (Nayar, 1963; Carlisle and Butler, 1956), yet did not yield a clear phenotype in *O. bicornis*. This while QMP exposure seemingly disrupts oogenesis by activating two ovarian checkpoints during *D. melanogaster* oogenesis (Lovegrove *et al.*, unpublished data). Such phenotypes (e.g. condensed nurse cell nuclei during early vitellogenesis) were rarely observed in *O. bicornis*. There may be numerous reasons for a lack of effect of QMP in *O. bicornis*. Experiments in *D. melanogaster* have typically been performed on females that are not yet reproductively mature (Camiletti *et al.*, 2013; Lovegrove *et al.*, 2019)[53], which could not be said of *O. bicornis* here[54]. Furthermore, the exact mode of action of QMP remains largely unknown (Jarriault and Mercer, 2012). Hence, unless it is known whether QMP affects the ovary directly, the lack of an effect by QMP injection does not allow me to draw stringent conclusions. As mentioned previously, demonstrating a negative claim is arduous, and shortcomings in experimental design (dose-response, uptake and administration, and time of dissection) further impede inference. Since, these factors were discussed at length in Chapter 6, they will not be renewed here.

[53] Ovaries of *D. melanogaster* are activated only within the first 60 hours after eclosion (Galang *et al.*, 2019).

[54] An equivalent experiment in *O. bicornis* would not only require exposing larvae to QMP, but also keeping overwintering pharates exposed to QMP throughout their diapause. This is arguably an unfeasible experiment, especially should oral uptake of QMP be required for overwintering pharates.

Instead, I will broaden the discussion here; as to why it may have been difficult to shut down early oogenesis in *O. bicornis* females generally.

7.2.1 Irreversible reversibility

Phenotypic plasticity in physiological and behavioural traits, are generally considered to be reversible (Whitman and Agrawal, 2009). One such reversible and physiologically plastic phenotype is supposedly the reproductive constraint exhibited by adult honey bee workers. The dietary response of germarial Notch signalling in the *D. melanogaster* ovary is likewise considered reversible (Bonfini *et al.*, 2015). It should be noted though, that this reversibility is generally only tested in a single direction; e.g. female *Drosophila* (personal communication Dr M.R. Lovegrove) or honey bee workers recover oogenesis after being released from QMP (Duncan *et al.*, 2016). A *'double reversal'* — for lack of a better phrasing — was tested in Chapter 6 on honey bee workers. While honey bee workers should provide the ideal subjects to study this, given that a response to QMP should be assured, the use of queenless *A. mellifera* workers was impractical due to low survival.Barring the possibility that queenless workers required more time to show an ovarian response to QMP, I tentatively indicated that the phenotype may not be *fully* (or *'doubly'*) reversible. This outlines a wider problem within the phenotypic plasticity literature. Reprising and generalising from Figure 6.12; research involving reversible phenotypic plasticity should consistently make use full factorial designs (Figure 7.1).

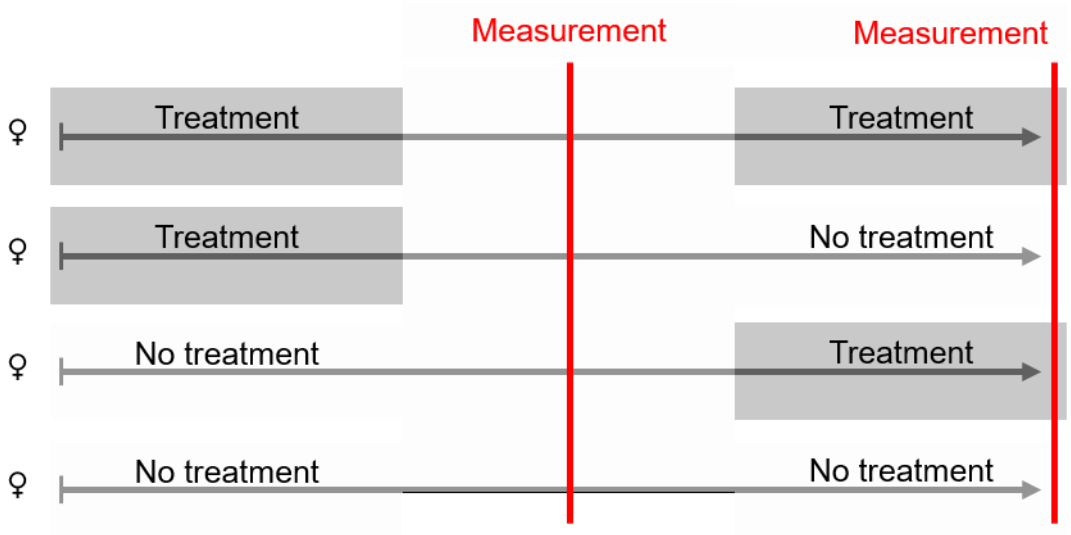

Figure 7.1: Full factorial design. This type of design should be emphasised and become standard for research into phenotypic plasticity, in order to demonstrate full reversibility of a plastic response.

Throughout this work, the insect ovary was regarded as a *'conveyer belt'* (5.4.1). This implies that any individual oocyte together with its associated nurse and follicle cells, does not provide any kind of positive or negative feedback to neighbouring oocytes and associated cells. This assumption of independence is ingrained in the literature surrounding insect oogenesis, implicit in language as *oocyte stages* (Wilson *et al.*, 2011), *checkpoints* (Pritchett *et al.*, 2009), and *rates of oogenesis and resorption* (Richard and Casas, 2012). But as Richard and Casas (2012) pointed out, different physiological processes are at work, which likely influence one another[55]. For instance, while ecdysone (from the prothoracic gland) is generally known to be a trigger for vitellogenin synthesis in the fat body, and patency of the oocyte — the local ecdysone produced by the follicle cells is directly incorporated into the oocyte (Lenaerts *et al.*, 2019a). Consequently, Deady *et al.* (2015) provided direct evidence of within ovary dependence, by showing that corpora lutea[56] produce ecdysone which aids maintaining a high rate of oogenesis in the *D. melanogaster* ovary. If we assume, that QMP operates on the ovary directly, then the putative evidence presented in Chapter 6, indicating that the ovarian response to QMP may not be fully reversible in honey bee workers, would similarly imply that processes within the different regions of the ovary[57] are not wholly independent of one another.

If this is the case, QMP mediated adult reproductive constraint would have to be considered a developmental constraint instead. As mentioned prior, in Chapter 6, studies that demonstrate the broad phylogenetic range of QMP without exception administer it either before or during ovary development (Carlisle and Butler, 1956; Hrdý *et al.*, 1960; Nayar, 1963; Sannasi, 1969; Princen *et al.*, 2019b; Galang *et al.*, 2019). QMP might maintain a block on the development of the ovarian tissue (i.e. organogenesis), instead of blocking early oogenesis directly. This subtle distinction would place adult reproductive constraint under the umbrella of developmental plasticity. The distinction is an important and meaningful one, as it informs us towards its evolution. Co-option of QMP mediated adult reproductive constraint might for instance be more firmly grounded under parental manipulation instead (Alexander, 1974; Michener and Brothers, 1974). Parental manipulation is known to be important in caste biasing in facultatively eusocial species for instance (e.g. Kapheim *et al.*, 2015a;

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[55] This is the case even within their model, where the speed at which the different physiological ovarian processes occur, are treated as rates — i.e. constants or parameters, rather than variables which change over time.

[56] Corpora lutea are former epithelial follicle cells, involuted from an ovulated mature egg.

[57] Terminal filament, germarium, and vitellarium.

Lawson *et al.*, 2017), and many studies have indicated the importance of larval nutrition in caste biasing/determination overall (e.g. review in Vespida: O'Donnell, 1998; the role of nutrition on honey bee caste is discussed in detail in 1.2.2). This overview then tends to paint a picture where development is exceedingly important, and where larval nutrient limitation through parental manipulation[58] may have presented the initial mechanism of achieving subfertility in female offspring.

The above conjecture, is contingent on both the mode of action of QMP (whether QMP acts on the ovary directly, without processing and signalling from the brain), as well as the possibility of secondarily arresting early oogenesis using QMP. The former remains enigmatic, and more work concerning the physiological mechanism of the anti-ovarian response of QMP is needed. This could be investigated by applying QMP directly to ovaries in culture media for example, as was likewise suggested for DAPT in section 6.5. The latter might be investigated through the use of *D. melanogaster* following the experimental design suggested in Figure 6.12.

7.2.2 Origin or elaboration?

In section 1.2.1, I discussed two phylogenetically informed sociogenomic studies that drew distinctions between *'primitively'* and *'highly'* eusocial species (Woodard *et al.*, 2011), or between *'origins'* and *'elaborations'* of eusociality (Kapheim *et al.*, 2015b)[59]. While these terms are subjective and may even differ slightly between studies, their general overtone points towards an identical and important consideration. That the selective pressures in small and incipient social organisations may be very different than those suffered by increasingly larger colonies (Bourke, 1999).

Primitive eusocial organisation is often regulated through dominance hierarchies, where the primary reproductive eats eggs laid by helpers (Fletcher and Ross, 1985). In small primitive social organisations headed by one or a few dominant reproductives, it is more difficult to fully police and repress the egg laying of helpers, in addition to any individual worker retaining a relatively high chance of replacing/succeeding a reproductive (Bourke, 1999). In other words, early on in eusocial evolution, reproductive conflict is dynamic. Under such

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[58] In larger colonies, manipulation of larvae would gradually be taken over by siblings, as is the case for worker nursing bees in *A. mellifera* for instance.

[59] Some authors even distinguish between three transitions (from subsocial to incipiently social, to primitively eusocial, to advanced eusocial: Rehan and Toth, 2015). Terminology within sociobiology is notoriously confounded (e.g.: Costa *et al.*, 1996; Costa and Fitzgerald, 2005) and further falls outside the remit of this thesis.

labile conditions, blocks on late oogenesis make more sense, as they allow for a quicker turn around when social context changes. This would make oocyte resorption, and the final checkpoint in oogenesis more susceptible to selective pressures. Other studies on solitary bees (Richards, 1994; Cane, 2016; Kapheim and Johnson, 2017a), tend to measure terminal oocyte length or volume. Even studies making use of *D. melanogaster* to investigate the effects of QMP, typically quantify the number of ovarioles, ovariole area or the number of mature oocytes (e.g.: Camiletti *et al.*, 2013; Lovegrove *et al.*, 2019) — i.e. measures which implicate either ovary development (organogenesis), late stage oogenesis or both.

In contrast, I specifically investigated early oogenesis throughout this work. This followed simply from the mechanism I studied (germarial Notch signalling; Duncan *et al.*, 2016), as well as from the inability to consistently enable *O. bicornis* females to forage and lay in a laboratory setting (Chapter 3). The lack of egg laying in the laboratory setup, disallowed determining how large mature eggs are, as egg sizes could already be different for sons and daughters for instance. More importantly however, no eggs were expected to reach full maturity, as late stage oocyte abortion and resorption are inevitably expected when females are reluctant to lay eggs. In Chapter 5, I largely circumvented this issue by approximating oocyte maturation rate within individual ovarioles. Yet, differences in actual egg laying and oocyte resorption cannot be excluded (as in Kant *et al.*, 2013; see section 5.5.2). This once more underlines the prudence of fully establishing a solitary bee species in a laboratory environment.

In more advanced eusocial organisation, policing behaviour by dominant reproductives is replaced by mutual worker policing (Ratnieks, 1988; Bourke, 1999). And morphological caste polyphenism is typically exacerbated, with workers being even more subfertile than they are in more primitive and smaller colonies (Bourke, 1999). The contrast between primitive and advanced eusocial organisation (*A. mellifera* falling in the latter category: Woodard *et al.*, 2011) indicates that mechanisms blocking early oogenesis (e.g. germarial Notch; Duncan *et al.*, 2016) may have become more important during stages of social *'elaboration'*, with increasing colony size and decreasing worker fertility (Bourke, 1999). Referring to Figure 1.4 (Gadagkar, 1996), this would mean that with increasing social complexity (and stability), the stabilising selection on early oogenesis would be eroded (Figure 1.4C) by the complete and stable lack of worker reproduction. Consequently, the phenotypic range of early oogenesis is widened (i.e. increased phenotypic plasticity), which is further expanded by diversifying selection on the individual castes (Figure 1.4D), but only when eusociality has already been established. Such a hypothesis might elegantly

explain why early oogenesis appears so canalised and hard to shut down in *O. bicornis* here.

To test the above hypothesis, it may be conducive to investigate how mechanisms blocking oogenesis operate at varying degrees of social complexity. This would preferably be carried out within a monophyletic clade spanning all transitions (e.g. Xylocopinae or Vespidae), as suggested by Rehan and Toth (2015)[60]. Another important addition to future research would include more detailed measures, possibly considering multiple physiological processes at play in the ovary (e.g. rate of oocyte determination, rate of vitellogenesis, oocyte resorption, etc.). It may not always be feasible to measure all processes and factors on the same tissue, since multiple techniques might be required (e.g. IHC and confocal microscopy, vital stains, etc.). In consideration of this, Richard and Casas (2012) provide a quantitative framework for ovarian dynamics. Since, as Whitman and Agrawal (2009) eloquently stated, different aspects of ovarian physiology will represent different underlying trade-offs, which will vary along their respective capacity for phenotypic plasticity.

> *'For example, some insects exhibit canalized egg size, and when confronted with poor nutrition or end of season, such insects maintain egg size, but express plasticity in clutch size or oocyte development rates (Chapter 11). In other species, clutch size or oocyte development may be canalized (Stearns 1992, Nylin and Gotthard 1998, Fox and Czesak 2000). Given trade-offs, and that particular traits can evolve to be plastic or canalized, the evolutionary outcome is presumably based on the relative advantages of different strategies in different habitats. Furthermore, what at first may appear to be a non-adaptive passive response (for example, lowered clutch size under poor nutrition), may in fact be an evolved plastic response to maintain egg size, oocyte development rate, or female survival.'*
> *~ Whitman and Agrawal (2009)*

In conclusion: that which is measured, even within a single ovary, should be chosen with care and deliberation.

7.2.3 The multifactorial nature of reproductive constraint

Mechanisms regulating reproductive constraint might be polygenic[61]. As suggested in section 1.2.3, the various functions of Notch signalling, alongside its numerous interactions with other signalling pathways (Schwanbeck *et al.*, 2011; Guruharsha *et al.*, 2012), potentiate it to unify multiple mechanisms. For example, not only has germarial Notch signalling been shown to regulate

[60] However, as pointed out in section 7.1; this may not always be feasible to carry out outside of tropical and subtropical climates (cf. Roubik, 1992, p. 380-389).

[61] I.e. control of a single trait, or phenotype, by multiple genes. Or in what follows; control of a phenotype by multiple pathways.

reproductive constraint in the honey bee directly (Duncan *et al.*, 2016), it is also responsive to other signalling pathways such as insulin signalling in *D. melanogaster* (Drummond-Barbosa and Spradling, 2001) — leaving it sensitive to dietary changes as well (Bonfini *et al.*, 2015). The conserved pathway's multiple levels of signalling control, such as gene dosage sensitivity and *cis* and *trans* regulation further indicate its polygenic potential (Guruharsha *et al.*, 2012). Moreover, Notch signalling is pleiotropic in nature. The cellular context under which Notch is active determines its outcome: cell-fate specification, self-renewal, differentiation, proliferation or apoptosis (Schwanbeck *et al.*, 2011). It is mainly the latter two functions that are of interest here. The role of Notch signalling in mediating apoptosis (Miele and Osborne, 1999) would lend itself towards explaining the germarial disorganisation and apoptosis seen in queenright honey bee workers (Tanaka and Hartfelder, 2004). Specifically, Numb/Notch is known to regulate apoptosis directly (Lundell *et al.*, 2003). Numb being the self-same Notch inhibitor that was posited to regulate QMP-mediate adult reproductive constraint (Duncan *et al.*, 2016).

Figure 7.2 displays a model for germarial Notch signalling that is both polygenic (two regulators) and pleiotropic (two outcomes). The model is informed by the traits of Notch signalling outlined in the previous paragraph, and incorporates with it, the refinements of *origin* and *elaboration* delineated in the previous section (7.2.2). In short, I make use of the reversal of the relationship between juvenile hormone (JH) and vitellogenin (Vg) that occurred during the evolutionary history of *A. mellifera*. JH production is negatively associated with Vg in advanced eusocial species like the honey bee (Amdam *et al.*, 2003), contrary to the positive relation found in solitary and primitively eusocial species (Robinson and Vargo, 1997; as well as other insects).

While we are currently unaware of the state of germarial Notch signalling in primitively eusocial species, a functional reversal of germarial Notch from its ancestral solitary state in *O. bicornis* to its state in the highly eusocial *A. mellifera* (Duncan *et al.*, 2016) has at least been demonstrated here (section 6.4.1). If we assume that the cellular context (Schwanbeck *et al.*, 2011) that informs Notch signalling is dependent upon JH[62], then switching from blocking oogenesis at a late stage towards blocking it at an early stage could be explained by a single role reversal. This combined reversal in signalling (germarial Notch and JH-Vg) can be argued to have occurred during the *elaboration* of honey bee eusociality. This because workers in primitively eusocial societies retain developed ovaries, and settle labile reproductive

[62] Or in other words: if we assume the reversal of germarial Notch signalling was enabled by the reversal of the JH-Vg axis.

conflict through dominance hierarchies (Fletcher and Ross, 1985). As colony size increases, workers are less likely to become reproductives (Bourke, 1999), and consequently: workers retaining active and vitellogenic ovaries are costly to produce and selected against. Hence, during an *elaboration* of sociality (i.e. increase in colony size), a putative pre-vitellogenic block on oogenesis is less costly and therefore highly adaptive. Given the roles of JH and ecdysone in triggering vitellogenin synthesis, patency and uptake in the oocyte across insect taxa (Roy *et al.*, 2018), leave both JH and ecdysone as likely suspects in facilitating the shift in the block on oogenesis.

The early block on oogenesis would swiftly become fixed. Ultimately, the early block on oogenesis is maintained by QMP, which has been found to be highly derived (Van Oystaeyen *et al.*, 2014), and its broad phylogenetic range makes it likely that it targets a highly conserved yet presently unknown pathway (Lovegrove *et al.*, 2019). In honey bees, the ovarian QMP-response is regulated by the Notch pathway via its inhibitor Numb (Duncan *et al.*, 2016). Yet, QMP mediation of Notch signalling may still have arisen gradually, as JH may have already been responsive to the social environment in a hypothetical primitively eusocial honey bee (as it is the case in the primitively eusocial *B. terrestris* for instance; Amsalem *et al.*, 2014; Shpigler *et al.*, 2014). Even if JH is not responsive to social environment in solitary bees (Kapheim and Johnson, 2017a).

The central assumption for this model would be that JH mediates at least the cellular context with respect to germarial Notch signalling. This may be possible, considering the prominent roles of both JH and Notch in oogenesis (Xu *et al.*, 1992; Robinson and Vargo, 1997). Galang *et al.* (2019) were unable to rescue newly eclosed *D. melanogaster* females subjected to QMP with pyriporixifen (a JH analogue). This indicates that QMP is likely not acting directly upon JH (the same was true for ecdysone).

Note that this model mainly serves as an example. The situation is likely far more complex. JH and the insulin signalling pathway are also known to interact in mosquito vitellogenesis (Hansen *et al.*, 2014; Perez-Hedo *et al.*, 2014), body size in *D. melanogaster* (Mirth and Shingleton, 2014; Mirth *et al.*, 2014)[63], *A. mellifera* caste determination (Mutti *et al.*, 2011) and queen longevity (Corona *et al.*, 2007), *Pogonomyrmex rugosus* caste determination (Libbrecht *et al.*, 2013) and vitellogenesis in *Triboleum castaneum* (Sheng *et al.*, 2011). Given the association between JH and insulin signalling (canonically insulin

[63] Insulin signalling also has a role in vitellogenesis in *D. melanogaster*, but this role is independent of JH (Richard *et al.*, 2005).

signalling upregulates downstream JH production; Tatar *et al.*, 2001), their respective roles might even be opposite to those depicted in Figure 7.2. Insulin signalling might provide cellular *context*, whilst JH titres provide a sustained *environmental signal*. Furthermore, ecdysone, another important insect hormone, was completely disregarded in Figure 7.2; and only a single tissue (ovary) was considered.

The specifics of the presented model (Figure 7.2) are not the main point here, nor are their associated semantics (*context* vs *signal*). What is important, is the overarching message of interacting signalling networks, and how they might come together to regulate reproductive constraint in adult honey bee workers. Given the underlying complexity of the problem — with its multiple independent origins and elaborations — it is unlikely that any single mechanism will underlie reproductive constraint in its entirety, even within one species.

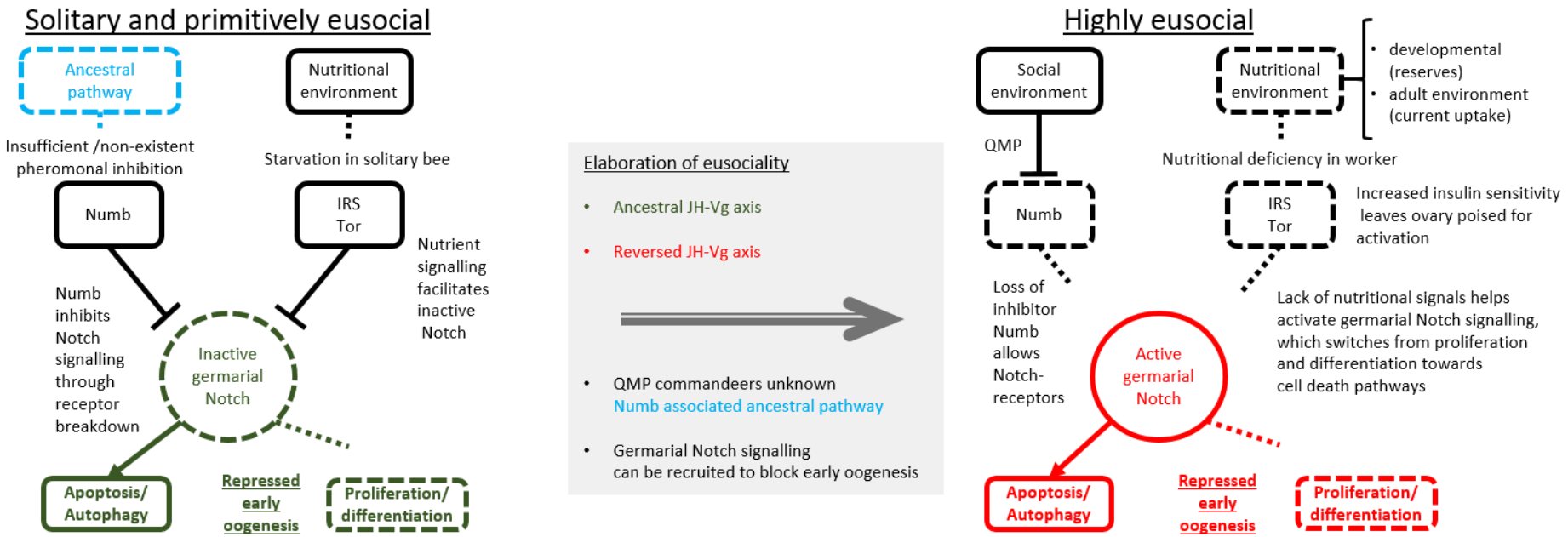

Figure 7.2: A hypothetical multifactorial origin of QMP-mediated adult reproductive constraint. An example of a conceptual model factoring in how different signalling pathways might interact in mediating reproductive constraint. In the hypothetical ancestral state (solitary or primitively eusocial), JH positively regulates Vg (Robinson and Vargo, 1997). During the elaboration of honey bee eusociality however, the probability of workers reproducing becomes perpetually smaller and more stable with increasing colony size (Bourke, 1999). Workers retaining active and vitellogenic ovaries are costly and selected against, hence a putative block earlier on in oogenesis is highly adaptive and quickly driven to fixation once it occurs. For the sake of simplicity in the model, we assume that the JH-vitellogenin axis provides the context for the germarial cells, upon which Notch signalling acts. Once the JH-Vg axis is reversed (Amdam *et al.*, 2003), the end result of germarial Notch signalling is likewise reversed, leading to an early block on oogenesis. After which it could be recruited into reproductive constraint through the exploitation of an unknown conserved pathway by QMP (Duncan *et al.*, 2016; Lovegrove *et al.*, 2019). IRS = insulin receptor substrate, Tor = target of rapamycin, and QMP = queen mandibular pheromone.

The benefits of a model such as the one presented in Figure 7.2, is that it generates testable hypotheses and clear experimental design. As the main premise and auxilliary hypotheses can all be tested. For instance, a phylogenetic analysis into the occurrence of the loss of JH's gonadotropic role (Robinson and Vargo, 1997), in association with the occurrence of early or late repression of oogenesis. Functional tests could be performed using combinations of starvation assays, QMP treatments, and fluoromevalonate or precocene-I treatments (JH biosynthesis inhibitors; Quistad *et al.*, 1981; Amsalem *et al.*, 2014). As stated earlier, under the model in Figure 7.2, QMP treatment in conjunction with JH inhibition would be expected to repress early oogenesis. Additionally, gene expression of insulin receptor substrate (*IRS*; or *insulin like peptide, ILP*) and Notch associated genes (*E(spl)*-genes) could be quantified. Further functional tests could be carried out in *D. melanogaster* as a tractable genetic model (Camiletti and Thompson, 2016). This, to for example overexpress germarial insulin receptors in combination with QMP treatment in adults.

Multifactorial models will help refine hypotheses and define overarching frameworks. They may help explain other outstanding questions, such as whether and why honey bee queens themselves, are unaffected by QMP (Jarriault and Mercer, 2012). For instance, are queens unaffected by their own QMP due to their being mated? Is this why virgin honey bee queen ovaries regress functionally? Or are queens unaffected by QMP due to their nutritional reserves? As the field continues to grow, more interacting signalling pathways may also be identified. Studies identifying gene clusters that are equipped to respond plastically to environmental stimuli, like the one performed by Duncan *et al.* (2020), will help identify even more networks and consequently mechanisms to study in future.

7.3 Concluding remarks

Gadagkar (1996) made the specific case for developmental morphological plasticity, yet the same principles should hold for reversible physiological plasticity. As Gadagkar (1996) denoted, and as was depicted in Figure 1.4; less phenotypic variation and consequently plasticity are expected initially for reproductive traits in solitary ancestors (Figure 1.4A). In other words: it is to be expected that levels of plasticity should be more rigid in a univoltine solitary species such as *O. bicornis*. More work is of course needed to establish a tractable solitary model species for use in the temperate regions of Europe. As are more comprehensive and functional tests into reproductive constraint needed. Such functional tests should acknowledge and incorporate multiple signalling pathways. Finally, the broad phylogenetic range of QMP (Lovegrove *et al.*, 2019), should facilitate these studies in a broad range of species.

Appendices

Appendix A. Personal communications

Personal communication Dr Christoph Sandrock

From: Christoph Sandrock <ch.sandrock@gmail.com>
Sent: 27 April 2016 21:22
To: Jens Van Eeckhoven
Subject: Re: Osmia rufa; husbandry
Attachments: mudbox.png; mudcollection.png

Dear Jens,

absolutely no problem, my desk in the office was just burried with lots of things. Though bees would still be my favourite topic I am actually involved in other entomology projects (i.e. insect proteins for feed stuff), so I can basically only get back to my former work at home.

Anyway, to be honest the preparation for the trial with Osmia was a nightmare and the way how it finally worked still seems to be a mystery in the community since many obviously failed to repeat it, but indeed in worked! Especially the pollen collection was surprising, and planned totally different originally. I intended to provide the Osmia with potted poppies (only pollen no nectar!), sown consecutively in a huge green house. Some people were obviously already wondering what my real intention would be with planting all these poppies... But finally the bees were simply destroying the flowers and I realized that pollen provisions would be far to less even with 18 pots per cage per day. So I had to react spontaneously with a second set up not to miss the season.
In any case, as opposed to any other of my papers, I feel that almost bad with this paper simply because it is the sole study where I didn't manage to run real replicates (it is rather than something where I could finally be glad to get it published somehow...). This was because many things went wrong and I was running out of time and also cocoons to set up fresh colonies. So if I can help you not to run into similar problems I will try to do so.

But maybe step by step. Your specific question addressed the nesting material. Unfortunately I could not find the receipt for the exact mixture, which puzzles me a bit. But the raw material was normal (natural) pottery clay I bought in a handicraft store (for creating any mess kids would be able to...) and the silica sand was from the commercial provider ‘Knauf’ (used for concrete and the like) and had a grain size 0.1.-0.5 mm. If I am not totally mistaken the ratio was 50-50.

I attached two pics, one showing the tilted mud box with the mixture and especially the water reservoire with the wick. Depending on how much water was in the jar, the gradient with the preferred water shifted during the day. The jar was completely filled in each cage each morning. The net is to prevent the bees from drowning. They are very busy, but not always very clever... In the second picture you can see some bees in action digging in the mud. Throughout the study they were really digging holes, like little pigs :-).

I would just like to ask you not to use the pictures for tallks or so, or at least not without referring to me as a source.

I will prepare some more pictures, but to get a better feeling of what you may need for information, I would be interested in whether you also plan to run an indoor-colony in a climate chamber, or a kind of (semi-)field experiment?
I just ask to know better if the pictures of the climate chamber would also be useful for you or whether you may prefer pics of the artificial flowers etc. Please let me know what could help you most and I will work on some of the original (quite large) pictures another evening (maybe when Norton is not paralysing my laptop with whatever so important updates...).

By the way, who is your professor supervising your studies?

Hope this helps you already!
Cheers

Christoph

2016-04-26 17:32 GMT+02:00 Jens Van Eeckhoven <bsjve@leeds.ac.uk>:

Dear Christoph,

I was wondering if you have had a chance to look into the request I sent you earlier. It would really be a great help to our project if you could provide us with any information you have to spare on setting up an Osmia bicornis culture.

I hope you do not think me incessant or annoying, I was merely worried you had forgotten.

With kind regards,

Jens

From: Christoph Sandrock <ch.sandrock@gmail.com>
Sent: 18 April 2016 08:06
To: Jens Van Eeckhoven
Subject: Re: Osmia rufa; husbandry

Dear Jens,

Thank you very much for your mail. I am very sorry for my late reply. Your request reached me right during some business trips.

I do not have the relevant data with me on my current laptop. But I will look it up asap when I get back home on Wednesday evening.

Best regards,

Christoph

2016-04-11 18:29 GMT+02:00 Jens Van Eeckhoven <bsjve@leeds.ac.uk>:

Dear Dr. Sandrock,

I recently read your paper on sublethal neonicotinoid exposure in Osmia.

My professor and I are hoping to set up an Osmia culture of our own in the lab over the summer,

since we want to study reproduction in a solitary bee species (to compare with eusocial bees).

Hence I was wondering if you could maybe send some pictures of the set-up you used for Osmia. If it's not too much trouble of course.

I was also wondering about the clay and silica sand mixture you were using.

What was the ratio you used for that?

We would be very grateful for any tips or help you can provide.

With kind regards,

Jens Van Eeckhoven

PhD-candidate

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University of Leeds
Leeds LS2 9JT
UK
+447592296434

Personal communication Dr Karsten Seidelmann

From: Karsten Seidelmann <karsten.seidelmann@zoologie.uni-halle.de>
Sent: 07 September 2016 12:35
To: Jens Van Eeckhoven
Subject: AW: Laboratory culture of Osmia bicornis

Dear Mr. Eeckhoven,

it is a very ambitious attempt to set up a laboratory culture of a solitary bee. Many researchers tried this before, but to my knowledge, all failed.
Also our research group tried several years ago to set up a caged culture of Osmia bicornis. However, we were not successful. When using a greenhouse or a cage in the field with flowering plants as food resource, some females started to nest. But the reproduction was by no means comparable to that of free flying bees. There might be several reasons for this: light intensity, food resources, limited flight distances, temperatures and temperature profiles, and so on. Nevertheless I was able to use a cage in the lab to study mating behavior of O. bicornis. Males started to search for females after a couple of days and performed normally at matings.
In the case you are interested in quantitative reproduction parameters, I am afraid I have to suggest to move to the field. In case you are about to study physiological parameters of reproduction, you might be successful also in cages or green houses, but the results are probably not really comparable to measurements on free flying bees.
To sum up, I expect that you have to invest a lot of efforts for just a few results of limited value. Therefore I would suggest to establish a solid rearing in the field. You can release the bees in batches to have females nesting from April through July. And you can collect nests and delay the development of larvae by storing the nest at cold temperatures. So you might have bee larvae for your experiments over nearly 4/5 month. That is all I can suggest.

However, I am not really an expert in the field of laboratory culture of bees. You might wish to contact Dr. Sjef van der Steen (Netherlands). He tried to set up an artificial year-round lab culture of Osmia several years ago. He might give you exhaustive information on this topic.

I was not able to see the pictures. They have been removed from the drop-box folder.

Best wishes,
K. Seidelmann

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Dr. Karsten Seidelmann
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Email: karsten.seidelmann@zoologie.uni-halle.de
Tel: +49-345-5526442 Fax: +49-345-5527152

Von: Jens Van Eeckhoven [mailto:bsjve@leeds.ac.uk]
Gesendet: Donnerstag, 1. September 2016 16:04
An: karsten.seidelmann@zoologie.uni-halle.de
Betreff: Laboratory culture of Osmia bicornis

Dear Dr. Seidelmann,

I am a recent PhD-student at the University of Leeds working with Elizabeth Duncan. We are hoping to set up an *Osmia bicornis* culture in the laboratory, since we want to study reproduction in a solitary bee species (to compare with eusocial bees). Hence, I was hoping that considering your expertise with the species, you might be able to provide some advice?
Our set-up follows a recent paper (Sandrock *et al*., 2014), the author of which we also contacted. He confessed to some difficulties and could not really provide a straightforward answer on how he got them to mate and nest in the laboratory.

Pictures of the set-up are in the links below (seeing as it is a pilot project, we only have one cage for now). Following Sandrock *et al.* (2004) we made artificial flowers using both 10 mL plastic tubes as well as Eppendorf tubes in racks (+ photoprint paper for attraction). We found that Eppendorf tubes worked best. We have also tried various types of mud (loam soil from commercial stores, various mixtures of Fuller's earth with silica sand). We have had some fleeting success when at least one of the females (possibly up to 3) started to lay; this happened after pollen was introduced to them on pieces of string which were knotted (to resemble catkins; see video) instead of in a petridish. The consistency of the pollen loaf made by the female(s) was oddly coloured and the embryo looked weird under the microscope, so we suspect there were problems with microbial activity.

When that trial was ended and new bees were used for a new trial, the bees stopped doing much of anything altogether. Do you see anything wrong with the set-up? Do you have any ideas we could try out? Or is there something crucial we may have overlooked in our attempt?

We would be very grateful for any tips or help you can provide.

With kind regards,
Jens Van Eeckhoven

Video: https://www.dropbox.com/s/wd2mldxzabydk7h/Foraging%20pollen.3gp?dl=0
Photos: https://www.dropbox.com/s/9b4ngyv3wvgazlq/Osmia.7z?dl=0

Jens Van Eeckhoven
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Personal communication Dr Sjef Van Der Steen

From: Steen, Sjef van der <sjef.vandersteen@wur.nl>
Sent: 28 September 2016 16:45
To: Jens Van Eeckhoven
Subject: RE: Laboratory culture of Osmia bicornis

Dear Jens,
As far as I remember I worked in badges of about 40 males and 20 females and added new badges every week. Don't make it too crowded in the flight cage and provide the bees with sufficient nesting holes. Hope this helps.

Best regards

Sjef

From: Jens Van Eeckhoven [mailto:bsjve@leeds.ac.uk]
Sent: maandag 26 september 2016 18:50
To: Steen, Sjef van der
Subject: Re: Laboratory culture of Osmia bicornis

Dear Sjef,

Perhaps one more question, and then I will stop badgering you for at least a while. In the paper it said you used a 40x40x40cm flight cage and tested a total of 263 females. Do you by any chance remember how many Osmia were in the cage at any given time, i.e. do they get overcrowded and how many females would be best to place in one cage simultaneously?

With kind regards,
Jens

From: Steen, Sjef van der <sjef.vandersteen@wur.nl>
Sent: 22 September 2016 10:54:14
To: Jens Van Eeckhoven
Subject: RE: Laboratory culture of Osmia bicornis

Dear Jens,
So did I. The main issue is that the bee collected pollen are very very dry otherwise you get a dough. I checked the grinded (ground?) pollen by waving over the ground pollen with a piece of paper to see the dust whirled up. If so it was okay.
How long are the bees kept at 4 degree? Please keep me informed about the progress, I am very anxious about the results and if I can be of any help. Let me know.
Best regards

Sjef

From: Jens Van Eeckhoven [mailto:bsjve@leeds.ac.uk]
Sent: donderdag 22 september 2016 11:43
To: Steen, Sjef van der
Subject: Re: Laboratory culture of Osmia bicornis

Dear Sjef,

This has been a great help. I am very glad it has been done before and you have given me many new things to contemplate. We indeed have cocoons now, kept at 4°C in the fridge.

How did you get the pollen ground fine enough? We are currently using an electric coffee grinder.

Many thanks and kind regards,

Jens

From: Steen, Sjef van der <sjef.vandersteen@wur.nl>
Sent: 21 September 2016 14:10:38
To: Jens Van Eeckhoven
Subject: RE: Laboratory culture of Osmia bicornis

Dear Jens,
Attached you will find the procedure i developed for the indoor rearing of Osmia. The most important issue is to have the bee collected, dried pollen grinded as small as possible as it appeared Osmia can (at least indoors) only collect the pollen that whirled when the bees flew over it. Next daylight or artificial daylight is important and of course vital males and females in the cocoons that merge in the flight cage. Do you have male and female cocoons now?
Please check the protocol out and if you have any questions don't hesitate to ask me.

Good luck and all the best

Sjef

Dr Jozef J.M. (Sjef) van der Steen
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Website www.wageningenur.nl/bijen
nieuwsbrief http://www.pri.wur.nl/NL/publicaties/nieuwsbrief-bijen/

From: Jens Van Eeckhoven [mailto:bsjve@leeds.ac.uk]
Sent: maandag 19 september 2016 13:57
To: Steen, Sjef van der
Subject: Re: Laboratory culture of Osmia bicornis

Dear Dr. van der Steen,

I wrote you earlier about the indoor rearing of Osmia. I was wondering if you have given this any further thought? Any tips or help you could provide would be much appreciated.

With kind regards,
Jens Van Eeckhoven

From: Steen, Sjef van der <sjef.vandersteen@wur.nl>
Sent: 09 September 2016 11:09:02
To: Jens Van Eeckhoven
Subject: RE: Laboratory culture of Osmia bicornis

Dear jens, I will be back in the office next wednesday and answer your. I do think there are good opportunities to establish a small indoor rearing of osmia
All the best
Sjef

Verzonden met mijn Windows Phone

Van: Jens Van Eeckhoven<mailto:bsjve@leeds.ac.uk>
Verzonden: 7-9-2016 16:09
Aan: Steen, Sjef van der<mailto:sjef.vandersteen@wur.nl>
Onderwerp: Laboratory culture of Osmia bicornis

Dear Dr. van der Steen,

I am a recent PhD-student at the University of Leeds working with Elizabeth Duncan. We are hoping to set up an Osmia bicornis culture in the laboratory, since we want to study reproduction in a solitary bee species (to compare with eusocial bees). Prof. Dr. Karsten Seidelmann pointed me in your direction, saying that you have attempted something similar in the past. I was hoping that you could perhaps share some of your experience on your attempt? Our set-up follows a recent paper (Sandrock et al., 2014), the author of which we also contacted. He confessed to some difficulties and could not really provide a straightforward answer on how he got them to mate and nest in the laboratory.

Pictures of the set-up are in the link below (seeing as it is a pilot project, we only have one cage for now). Following Sandrock et al. (2004) we made artificial flowers using both 10 mL plastic tubes as well as Eppendorf tubes in racks (+ photoprint paper for attraction). We found that Eppendorf tubes worked best (for a while). We have also tried various types of mud (loam soil from commercial stores, various mixtures of Fuller's earth with silica sand). We have had some fleeting success when at least one of the females (possibly up to 2-3) started to lay; this happened after pollen was introduced to them on pieces of string which were knotted (to resemble catkins; see video) instead of in a petridish. The consistency of the pollen loaf made by the female(s) was oddly coloured and the embryo looked weird under the microscope, so we suspect there were problems with microbial activity.

When that trial was ended and new bees were used for a new trial, the bees stopped doing much of anything altogether. Do you see anything wrong with the set-up? Do you have any ideas we could try out? Or is there something crucial we may have overlooked in our attempt?

We would be very grateful for any tips or help you can provide.

With kind regards,
Jens Van Eeckhoven

Video and photos: https://www.dropbox.com/sh/8315mjitxlc9jb7/AACyPba6eEenKcfffkIaVNPza?dl=0

Jens Van Eeckhoven
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+447592296434
bsjve@leeds.ac.uk

Personal communication Prof Dr Anthony Raw[64]

Jens Van Eeckhoven Sep 1, 2016

Dear Prof. Dr. Raw,

I am a recent PhD-student at the University of Leeds working with Elizabeth Duncan. We are hoping to set up an Osmia bicornis culture in the laboratory, since we want to study reproduction in a solitary bee species (to compare with eusocial bees). Hence, I was hoping that considering your expertise with the species, you might be able to provide some advice?

Our set-up follows a recent paper (Sandrock et al., 2014), the author of which we also contacted. He confessed to some difficulties and could not really provide a straightforward answer on how he got them to mate and nest in the laboratory.

Pictures of the set-up are in the links below (seeing as it is a pilot project, we only have one cage for now). Following Sandrock et al. (2004) we made artificial flowers using both 10 mL plastic tubes as well as Eppendorf tubes in racks (+ photoprint paper for attraction). We found that Eppendorf tubes worked best. We have also tried various types of mud (loam soil from commercial stores, various mixtures of Fuller's earth with silica sand). We have had some fleeting success when at least one of the females (possibly up to 3) started to lay; this happened after pollen was introduced to them on pieces of string which were knotted (to resemble catkins; see video) instead of in a petridish. The consistency of the pollen loaf made by the female(s) was oddly coloured and the embryo looked weird under the microscope, so we suspect there were problems with microbial activity.

When that trial was ended and new bees were used for a new trial, the bees stopped doing much of anything altogether. Do you see anything wrong with the set-up? Do you have any ideas we could try out? Or is there something crucial we may have overlooked in our attempt?

We would be very grateful for any tips or help you can provide.

With kind regards,
Jens Van Eeckhoven

Video: https://www.dropbox.com/s/wd2mldxzabydk7h/Foraging%20pollen.3gp?dl=0
Photos: https://www.dropbox.com/s/9b4ngyv3wvgazlq/Osmia.7z?dl=0

64 No contact information could be found. This conversation was transcribed from ResearchGate.

Anthony Raw Sep 2, 2016

Dear Jens
I'm not sure just what aspect of the bee's reproduction you want to study. I opened the video you, but couldn't open the photographs.
I had mated females nesting in cages (50 cm x 50 cm and 60 cm high). I provided cut flowers of wallflowers and Quercus.
I can see why you want to control the mating, but why not give them fresh flowers. I think you are trying to change and control too many factors.

Jens Van Eeckhoven Sep 5, 2016

Dear Dr. Raw,

Thank you for your quick reply. I had archived the photos using 7zip on our old university computers, which is probably why the file could not be opened. I attached the photos here should you still wish to see them.

And indeed, we are attempting to control a lot of factors, and perhaps too many. We are looking to compare orthologue genes of Apis using RNAi. This to find some of these genes' possible ancestral functions in reproduction in the related solitary species Osmia bicornis.

Since the pupa are easily stored in the fridge, we were hoping this would allow us to perform experiments some time outside of spring as well. This is why the set-up we are trying is so artificial. In any case, thank you for your consideration. If our endeavours remain unsuccessful we will surely try using actual flowers and catkins, unfortunately that would have to wait until next spring.

With kind regards,
Jens

Anthony Raw Sep 5, 2016

The bees like Siberian wallflower (Cheiranthus allioni). The flowers supply pollen and nectar. If you keep removing the dead flowers the plants continue to bloom in a cold greenhouse until October.

Personal communication Chris Whittles[65]

No detailed transcripts are available, as the majority of information was provided over the phone.

From: Jens Van Eeckhoven
Sent: 07 August 2017 21:10
To: MasonBees UK
Cc: Elizabeth Duncan
Subject: Re: Related individuals of Osmia bicornis

Dear Chris,

Thank you again for all the information you passed on to me last Friday, to be fair it was quite illuminating. I also added my supervisor, Dr. Liz Duncan, in CC since we are both very excited to be working with you.

I knocked up a quick experimental design and did a power calculation. Based on these we were thinking of ordering about 10 tubes per site, and this for three to four sites. This since you mentioned there were about that many sites for which you have consistently sent back cocoons (making them less artificial and genetically more uniform in a sense). These sites would be best suited for the experiments considering this. We are hoping to get about 2-4 females from each of the tubes, because you mentioned a tube might yield 9 individuals on average (and with a tendency towards male bias in the red mason bee, I'm hoping that that is not too optimistic a guesstimate).

Ultimately the number of tubes will depend entirely upon your stock/yield this year (including parasite load, etc). We could also make do with less tubes originating from more/other sites, or simply do with less tubes altogether if that is not possible. We could then make it a preliminary analysis, but we would need at least 2 tubes from each site for the experiment we have in mind.

We were also interested in acquiring some of the longer and better cardboard tubes and holders you told me about, if that is at all possible. Both for trying them out in the field, as well as in our laboratory set-up. Maybe about 4 to start with (depending on price as well)?

Looking forward to hearing from you,
with kind regards,

Jens

[65] Director of MasonBees Ltd; contact@masonbees.co.uk

From: MasonBees UK <contact@masonbees.co.uk>
Sent: 03 August 2017 16:45:10
To: Jens Van Eeckhoven
Subject: RE: Related individuals of Osmia bicornis

Dear Jens,

Yes , any time after 1100hrs

Kind Regards

Chris

From: Jens Van Eeckhoven [mailto:bsjve@leeds.ac.uk]
Sent: 03 August 2017 14:37
To: MasonBees UK <contact@masonbees.co.uk>
Subject: Re: Related individuals of Osmia bicornis

Dear Chris,

Thank you for your quick reply. And that sounds great! Unfortunately I am fairly busy today, but would you have time to talk on the phone some time tomorrow?

Kind regards,
Jens

From: MasonBees UK <contact@masonbees.co.uk>
Sent: 03 August 2017 12:18:37
To: Jens Van Eeckhoven
Subject: RE: Related individuals of Osmia bicornis

Dear Jens,

Your PhD sounds interesting. I think the way forward is to talk on the phone. I do not yet know how many cocoons I am going to have this year, as it has been another difficult year. If I can help I will but I need to understand your requirements.

Kind Regards

Chris

Chris Whittles
Director
MasonBees Ltd
07774 733585

From: Jens Van Eeckhoven [mailto:bsjve@leeds.ac.uk]
Sent: 03 August 2017 10:19
To: contact@masonbees.co.uk
Subject: Related individuals of Osmia bicornis

Dear Sir/Madam,

I am a PhD student at the University of Leeds working in the Duncan lab. We are researching bees, amongst which the elegant *Osmia bicornis* (formerly *Osmia rufa*). I was hoping to set up an experiment on kin recognition in this species, as well as investigating population structure to an extent (commercial ones included).

For this purpose, I was wondering if it would be possible to order whole nests of red mason bees instead of just loose cocoons? Since individuals within nests are presumably related, except when nests are superseded of course (which will be accounted for).

Additionally, I was wondering whether the red mason bees you keep are bred at multiple locations, or whether they are plots close to one another? In the former situation, I would be interested in acquiring several nests from each of these multiple locations.

Nests, in all cases, would have to be chosen at random, but would preferably have a sufficient diameter since we would want there to be females in each nest. There is however no distinct need for you to part way with the nesting material, which you presumably reuse. As long as cocoons are kept separated as distinct ‘nest’ or ‘family’ units when shipped (and identified by location), that would suffice.

If this is at all possible, would you mind sending some more information? I have yet to work out sample sizes for the experiments, but in fairness this will in part depend on your price (and the number of locations). Could you include a price estimate per nest? Additionally, are the nests opened and mixed at the end of summer, or in other words, what would be the deadline for ordering them as nests/’family units’?

With kind regards,

Jens Van Eeckhoven

Jens Van Eeckhoven
Faculty of Biological Sciences
University of Leeds
Leeds LS2 9JT, UK
Phone: +44 (0)7592296434

Appendix B. Trap-nesting field sites

Table B.1: Specific trap-nesting sites in the greater Leeds area. Location = codes corresponding to sites (see Figure 4.1), Lat = latitude, Lon = longitude, Elev = elevation from sea level in metres, and Ecol = the general ecology of the individual trap-nesting site (primary ecology occurring first, secondary or surrounding ecology placed second and third). Location codes are: CS = canal side, KVR = Kirkstall Valley Reserve, MVT = Meanwood Valley Trail, SHP = Sugarwell Hill Park, LUF = Leeds University farm, and UoL = University of Leeds. Localities in red were successful trap nests of leafcutter bees or potter wasps, the locality in green contained one nest tube with *O. bicornis*.

Location	Lat	Lon	Elev	Ecol
CS01	53.79336	-1.5587	23	Urban
KVR01	53.81116	-1.60263	20	Grassland, wetland and woodland
KVR02	53.81072	-1.60198	22	Grassland, wetland and woodland
KVR03	53.80907	-1.59916	31	Grassland, wetland and woodland
KVR04	53.80876	-1.60022	22	Grassland, wetland and woodland
KVR05	53.80821	-1.59724	23	Grassland, wetland and woodland
MVT01	53.82266	-1.56727	51	Open woodland
MVT02	53.82986	-1.57249	40	Grassland and woodland
MVT03	53.84019	-1.57759	66	Grassland and wetland
MVT04	53.83946	-1.57565	76	Grassland and woodland
MVT05	53.84014	-1.57559	67	Grassland and woodland
MVT06	53.84007	-1.57414	85	Grassland and woodland
MVT07	53.8539	-1.56661	121	Acid heath and woodland
MVT08	53.85389	-1.56825	125	Acid heath and woodland
MVT09	53.85353	-1.56751	123	Acid heath and woodland
MVT10	53.85348	-1.56651	124	Acid heath and woodland
MVT11	53.85756	-1.57629	109	Grassland and woodland
SHP01	53.81924	-1.5492	36	Woodland and grassland
SHP02	53.81784	-1.54561	32	Woodland and grassland
TN01	53.78738	-1.45124	51	Woodland
TN02[a]	53.78637	-1.4516	52	Mown grassland and woodland
TN03[a]	53.78612	-1.45155	51	Mown grassland and woodland
TN04[a]	53.78609	-1.45153	51	Mown grassland and woodland
LUF01	53.86875	-1.32966	51	Orchard and farmland
LUF02	53.87071	-1.32307	43	Farmland (Canola)
LUF03	53.87074	-1.32013	45	Farmland (Canola)
LUF04	53.87079	-1.31803	46	Farmland (Canola)
LUF05	53.86211	-1.32702	48	Farmland (Canola)
LUF06	53.86239	-1.32509	40	Farmland (Canola)
LUF07	53.86273	-1.32264	46	Farmland (Canola)
LUF08	53.8635	-1.32987	49	Orchard and farmland
UoL01	53.80524	-1.55509	71	Allotment and urban

[a] These were located within or near the Temple Newsam apiary (Leeds Beekeepers Association; LBKA) — hence while plenty of forage was present, heavy competition was as well.

Appendix C. Microsatellite marker validation

Table C.1: Relatedness matrix (Kalinowski *et al.*, 2006) for the North Shropshire subpopulation. NS = North Shropshire, C-J = nest tube, f1-2 = position of female within the nest. U = unrelated, HS = half-sib. For each of the suspected related pairs (red), one individual was removed.

NS	NSCf1	NSDf1	NSEf1	NSFf2	NSGf1	NSHf1	NSIf2	NSJf1
NSCf1	-							
NSDf1	U	-						
NSEf1	U	U	-					
NSFf2	U	U	U	-				
NSGf1	U	U	U	U	-			
NSHf1	U	U	U	U	U	-		
NSIf2	U	U	U	U	U	HS	-	
NSJf1	U	U	U	U	U	U	U	-

Table C.2: Relatedness matrix (Kalinowski *et al.*, 2006) for the Surrey subpopulation. Su = Surrey, A-J = nest tube, f1-2 = position of female within the nest. U = unrelated, HS = half-sib. For each of the suspected related pairs (red), one individual was removed.

Su	SuAf1	SuBf1	SuDf1	SuEf1	SuFf2	SuGf2	SuIf1	SuJf2
SuAf1	-							
SuBf1	U	-						
SuDf1	U	U	-					
SuEf1	U	U	U	-				
SuFf2	U	U	U	U	-			
SuGf2	U	HS	U	U	U	-		
SuIf1	U	U	U	U	U	U	-	
SuJf2	U	U	U	U	U	U	U	-

Table C.3: Relatedness matrix (Kalinowski *et al.*, 2006) for the first German subpopulation. G1 = German location 1, A-N = nest tube, f1 = first female in the nest. U = unrelated, HS = half-sib. For each of the suspected related pairs (red), one individual was removed.

G1	G1Af1	G1Bf1	G1Cf1	G1Df1	G1Ef1	G1Ff1	G1Gf1	G1Hf1	G1If1	G1Jf1	G1Kf1	G1Lf1	G1Mf1	G1Nf1
G1Af1	-													
G1Bf1	U	-												
G1Cf1	U	U	-											
G1Df1	U	U	U	-										
G1Ef1	U	U	U	U	-									
G1Ff1	U	HS	U	U	U	-								
G1Gf1	U	U	U	U	U	U	-							
G1Hf1	U	U	U	U	U	U	U	-						
G1If1	U	U	U	U	U	U	U	U	-					
G1Jf1	U	U	U	U	U	U	HS	U	U	-				
G1Kf1	U	U	U	HS	U	U	U	U	U	U	-			
G1Lf1	U	U	U	U	U	U	U	U	U	U	U	-		
G1Mf1	U	U	U	U	U	U	U	U	U	U	U	U	-	
G1Nf1	U	U	HS	HS	U	U	U	U	U	U	U	U	U	-

Table C.4: Relatedness matrix (Kalinowski *et al.*, 2006) for the second German subpopulation. G2 = German location 2, A-J = nest tube, f1 = position of female in nest. U = unrelated, HS = half-sib. For each of the suspected related pairs (red), one individual was removed.

G2	G2Af1	G2Bf1	G2Cf1	G2Df1	G2Ef1	G2E2f	G2Ff1	G2Gf1	G2Hf1	G2If1	G2Jf1
G2Af1	-										
G2Bf1	HS	-									
G2Cf1	U	U	-								
G2Df1	U	U	U	-							
G2Ef1	U	U	U	U	-						
G2E2f	U	U	U	U	U	-					
G2Ff1	U	U	U	U	U	U	-				
G2Gf1	U	U	U	U	HS	U	U	-			
G2Hf1	U	U	U	U	U	U	U	U	-		
G2If1	U	U	U	U	U	U	U	U	U	-	
G2Jf1	U	U	U	U	U	U	U	U	U	U	-

Table C.5: Absence of linkage disequilibrium between markers in the overall population. Genepop log likelihood ratio statistic for genetic disequilibrium of all unrelated females. χ^2 = Chisquared statistic, Df = degrees or freedom, and p = uncorrected p-value.

Locus1	Locus2	χ^2	Df	p
Obic95Fam	Obic77Hex	1.467	6	0.962
Obic95Fam	Obic1Hex	1.545	6	0.956
Obic77Hex	Obic1Hex	3.799	6	0.704
Obic95Fam	Obic1206Hex	2.311	6	0.889
Obic77Hex	Obic1206Hex	7.097	8	0.526
Obic1Hex	Obic1206Hex	3.213	6	0.782
Obic95Fam	Obic450Fam	6.291	6	0.391
Obic77Hex	Obic450Fam	2.911	8	0.940
Obic1Hex	Obic450Fam	2.513	6	0.867
Obic1206Hex	Obic450Fam	1.684	8	0.989
Obic95Fam	Obic73Hex	4.699	6	0.583
Obic77Hex	Obic73Hex	3.574	8	0.893
Obic1Hex	Obic73Hex	4.505	6	0.609
Obic1206Hex	Obic73Hex	1.622	8	0.99
Obic450Fam	Obic73Hex	0.000	8	1.000
Obic95Fam	Obic220Hex	1.342	6	0.969
Obic77Hex	Obic220Hex	6.112	8	0.635
Obic1Hex	Obic220Hex	6.989	6	0.322
Obic1206Hex	Obic220Hex	3.201	8	0.921
Obic450Fam	Obic220Hex	3.555	8	0.895
Obic73Hex	Obic220Hex	10.28	8	0.246
Obic95Fam	Obic1374Fam	3.503	6	0.744
Obic77Hex	Obic1374Fam	2.218	8	0.974
Obic1Hex	Obic1374Fam	1.367	6	0.968
Obic1206Hex	Obic1374Fam	2.750	8	0.949
Obic450Fam	Obic1374Fam	5.043	8	0.753
Obic73Hex	Obic1374Fam	5.820	8	0.667
Obic220Hex	Obic1374Fam	2.972	8	0.936
Obic95Fam	Obic1238Fam	3.435	6	0.753
Obic77Hex	Obic1238Fam	6.718	8	0.567
Obic1Hex	Obic1238Fam	1.663	6	0.948
Obic1206Hex	Obic1238Fam	12.40	8	0.134
Obic450Fam	Obic1238Fam	5.922	8	0.656
Obic73Hex	Obic1238Fam	2.376	8	0.967
Obic220Hex	Obic1238Fam	1.928	8	0.983
Obic1374Fam	Obic1238Fam	5.154	8	0.741
Obic95Fam	Obic415Fam	0.505	6	0.998
Obic77Hex	Obic415Fam	6.343	8	0.609
Obic1Hex	Obic415Fam	7.391	6	0.286
Obic1206Hex	Obic415Fam	1.074	8	0.998
Obic450Fam	Obic415Fam	0.420	8	1.000
Obic73Hex	Obic415Fam	8.618	8	0.376

Obic220Hex	Obic415Fam	8.780	8	0.361
Obic1374Fam	Obic415Fam	5.170	8	0.739
Obic1238Fam	Obic415Fam	2.436	8	0.965
Obic95Fam	Obic629Hex	1.253	6	0.974
Obic77Hex	Obic629Hex	0.676	8	1.000
Obic1Hex	Obic629Hex	0.861	6	0.990
Obic1206Hex	Obic629Hex	0.428	8	1.000
Obic450Fam	Obic629Hex	1.299	8	0.996
Obic73Hex	Obic629Hex	3.699	8	0.883
Obic220Hex	Obic629Hex	3.394	8	0.907
Obic1374Fam	Obic629Hex	0.744	8	0.999
Obic1238Fam	Obic629Hex	3.581	8	0.893
Obic415Fam	Obic629Hex	7.815	8	0.452
Obic95Fam	Obic740Fam	0.000	2	1.000
Obic77Hex	Obic740Fam	1.354	4	0.852
Obic1Hex	Obic740Fam	0.664	2	0.718
Obic1206Hex	Obic740Fam	0.000	4	1.000
Obic450Fam	Obic740Fam	3.744	4	0.442
Obic73Hex	Obic740Fam	3.342	4	0.502
Obic220Hex	Obic740Fam	2.276	4	0.685
Obic1374Fam	Obic740Fam	3.997	4	0.406
Obic1238Fam	Obic740Fam	0.000	4	1.000
Obic415Fam	Obic740Fam	2.446	4	0.654
Obic629Hex	Obic740Fam	0.000	4	1.000
Obic95Fam	Obic168Hex	4.753	6	0.576
Obic77Hex	Obic168Hex	5.284	8	0.727
Obic1Hex	Obic168Hex	4.326	6	0.633
Obic1206Hex	Obic168Hex	0.950	8	0.999
Obic450Fam	Obic168Hex	8.078	8	0.426
Obic73Hex	Obic168Hex	5.523	8	0.700
Obic220Hex	Obic168Hex	9.049	8	0.338
Obic1374Fam	Obic168Hex	1.896	8	0.984
Obic1238Fam	Obic168Hex	1.053	8	0.998
Obic415Fam	Obic168Hex	2.982	8	0.935
Obic629Hex	Obic168Hex	7.790	8	0.454
Obic740Fam	Obic168Hex	3.189	4	0.527
Obic95Fam	Obic1252Fam	0.000	4	1.000
Obic77Hex	Obic1252Fam	0.538	6	0.997
Obic1Hex	Obic1252Fam	1.633	4	0.803
Obic1206Hex	Obic1252Fam	0.000	6	1.000
Obic450Fam	Obic1252Fam	3.254	6	0.776
Obic73Hex	Obic1252Fam	2.552	6	0.863
Obic220Hex	Obic1252Fam	1.071	6	0.983
Obic1374Fam	Obic1252Fam	0.000	6	1.000
Obic1238Fam	Obic1252Fam	4.151	6	0.656
Obic415Fam	Obic1252Fam	2.24	6	0.896
Obic629Hex	Obic1252Fam	0.000	6	1.000
Obic740Fam	Obic1252Fam	0.000	4	1.000

Obic168Hex	Obic1252Fam	1.547	6	0.956
Obic95Fam	Obic113Fam	1.693	6	0.946
Obic77Hex	Obic113Fam	9.650	8	0.290
Obic1Hex	Obic113Fam	0.858	6	0.990
Obic1206Hex	Obic113Fam	8.189	8	0.415
Obic450Fam	Obic113Fam	1.983	8	0.982
Obic73Hex	Obic113Fam	2.934	8	0.938
Obic220Hex	Obic113Fam	4.482	8	0.811
Obic1374Fam	Obic113Fam	1.667	8	0.990
Obic1238Fam	Obic113Fam	7.792	8	0.454
Obic415Fam	Obic113Fam	2.392	8	0.967
Obic629Hex	Obic113Fam	4.210	8	0.838
Obic740Fam	Obic113Fam	0.000	4	1.000
Obic168Hex	Obic113Fam	10.43	8	0.236
Obic1252Fam	Obic113Fam	4.096	6	0.664
Obic95Fam	Obic1181Fam	2.181	6	0.902
Obic77Hex	Obic1181Fam	2.469	8	0.963
Obic1Hex	Obic1181Fam	2.060	6	0.914
Obic1206Hex	Obic1181Fam	1.247	8	0.996
Obic450Fam	Obic1181Fam	0.433	8	1.000
Obic73Hex	Obic1181Fam	1.274	8	0.996
Obic220Hex	Obic1181Fam	2.840	8	0.944
Obic1374Fam	Obic1181Fam	2.743	8	0.949
Obic1238Fam	Obic1181Fam	0.000	8	1.000
Obic415Fam	Obic1181Fam	2.854	8	0.943
Obic629Hex	Obic1181Fam	0.000	8	1.000
Obic740Fam	Obic1181Fam	0.000	4	1.000
Obic168Hex	Obic1181Fam	6.722	8	0.567
Obic1252Fam	Obic1181Fam	0.000	6	1.000
Obic113Fam	Obic1181Fam	4.524	8	0.807
Obic95Fam	Obic1176Hex	0.000	4	1.000
Obic77Hex	Obic1176Hex	3.090	6	0.798
Obic1Hex	Obic1176Hex	0.550	4	0.968
Obic1206Hex	Obic1176Hex	1.264	6	0.974
Obic450Fam	Obic1176Hex	2.431	6	0.876
Obic73Hex	Obic1176Hex	1.317	6	0.971
Obic220Hex	Obic1176Hex	0.953	6	0.987
Obic1374Fam	Obic1176Hex	1.005	6	0.985
Obic1238Fam	Obic1176Hex	5.510	6	0.48
Obic415Fam	Obic1176Hex	1.403	6	0.966
Obic629Hex	Obic1176Hex	0.000	6	1.000
Obic740Fam	Obic1176Hex	0.000	2	1.000
Obic168Hex	Obic1176Hex	5.147	6	0.525
Obic1252Fam	Obic1176Hex	1.690	2	0.430
Obic113Fam	Obic1176Hex	1.166	6	0.979
Obic1181Fam	Obic1176Hex	0.679	6	0.995

Table C.6: Linkage disequilibrium by marker & subpopulation. Genepop log likelihood ratio statistic for genetic disequilibrium of unrelated females. Sub = subpopulation (with: NS = North Shropshire, Su = Surrey, G1-2 = Germany 1 and 2), and p = uncorrected p-value. Significant p- values are in red, NAs are those instances where no contingency table could be drawn up (e.g. marker Obic740 only had one allele).

Sub	Locus1	Locus2	p
G1	Obic95	Obic77	0.968
G1	Obic95	Obic1	0.834
G1	Obic77	Obic1	0.349
G1	Obic95	Obic1206	0.315
G1	Obic77	Obic1206	1.000
G1	Obic1	Obic1206	0.842
G1	Obic95	Obic450	0.525
G1	Obic77	Obic450	0.449
G1	Obic1	Obic450	1.000
G1	Obic1206	Obic450	1.000
G1	Obic95	Obic73	1.000
G1	Obic77	Obic73	0.682
G1	Obic1	Obic73	0.791
G1	Obic1206	Obic73	1.000
G1	Obic450	Obic73	1.000
G1	Obic95	Obic220	0.902
G1	Obic77	Obic220	0.109
G1	Obic1	Obic220	0.218
G1	Obic1206	Obic220	0.369
G1	Obic450	Obic220	0.533
G1	Obic73	Obic220	0.346
G1	Obic95	Obic1374	1.000
G1	Obic77	Obic1374	0.933
G1	Obic1	Obic1374	0.787
G1	Obic1206	Obic1374	1.000
G1	Obic450	Obic1374	0.817
G1	Obic73	Obic1374	1.000
G1	Obic220	Obic1374	0.291
G1	Obic95	Obic1238	1.000
G1	Obic77	Obic1238	0.467
G1	Obic1	Obic1238	0.791
G1	Obic1206	Obic1238	0.240
G1	Obic450	Obic1238	1.000
G1	Obic73	Obic1238	0.403
G1	Obic220	Obic1238	0.737
G1	Obic1374	Obic1238	1.000
G1	Obic95	Obic415	1.000
G1	Obic77	Obic415	0.079
G1	Obic1	Obic415	0.056
G1	Obic1206	Obic415	1.000
G1	Obic450	Obic415	0.810

G1	Obic73	Obic415	0.316
G1	Obic220	Obic415	0.262
G1	Obic1374	Obic415	1.000
G1	Obic1238	Obic415	0.456
G1	Obic95	Obic629	1.000
G1	Obic77	Obic629	0.878
G1	Obic1	Obic629	0.650
G1	Obic1206	Obic629	1.000
G1	Obic450	Obic629	1.000
G1	Obic73	Obic629	0.429
G1	Obic220	Obic629	0.841
G1	Obic1374	Obic629	1.000
G1	Obic1238	Obic629	1.000
G1	Obic415	Obic629	0.470
G1	Obic95	Obic740	NA
G1	Obic77	Obic740	NA
G1	Obic1	Obic740	NA
G1	Obic1206	Obic740	NA
G1	Obic450	Obic740	NA
G1	Obic73	Obic740	NA
G1	Obic220	Obic740	NA
G1	Obic1374	Obic740	NA
G1	Obic1238	Obic740	NA
G1	Obic415	Obic740	NA
G1	Obic629	Obic740	NA
G1	Obic95	Obic168	1.000
G1	Obic77	Obic168	0.880
G1	Obic1	Obic168	1.000
G1	Obic1206	Obic168	1.000
G1	Obic450	Obic168	1.000
G1	Obic73	Obic168	1.000
G1	Obic220	Obic168	0.822
G1	Obic1374	Obic168	1.000
G1	Obic1238	Obic168	1.000
G1	Obic415	Obic168	1.000
G1	Obic629	Obic168	1.000
G1	Obic740	Obic168	NA
G1	Obic95	Obic1252	1.000
G1	Obic77	Obic1252	0.764
G1	Obic1	Obic1252	0.621
G1	Obic1206	Obic1252	1.000
G1	Obic450	Obic1252	1.000
G1	Obic73	Obic1252	0.279
G1	Obic220	Obic1252	0.585
G1	Obic1374	Obic1252	1.000
G1	Obic1238	Obic1252	0.125
G1	Obic415	Obic1252	0.326
G1	Obic629	Obic1252	1.000

G1	Obic740	Obic1252	NA
G1	Obic168	Obic1252	1.000
G1	Obic95	Obic113	1.000
G1	Obic77	Obic113	0.543
G1	Obic1	Obic113	1.000
G1	Obic1206	Obic113	0.492
G1	Obic450	Obic113	1.000
G1	Obic73	Obic113	0.403
G1	Obic220	Obic113	0.335
G1	Obic1374	Obic113	1.000
G1	Obic1238	Obic113	0.748
G1	Obic415	Obic113	0.880
G1	Obic629	Obic113	0.461
G1	Obic740	Obic113	NA
G1	Obic168	Obic113	0.470
G1	Obic1252	Obic113	0.323
G1	Obic95	Obic1181	0.336
G1	Obic77	Obic1181	0.972
G1	Obic1	Obic1181	0.837
G1	Obic1206	Obic1181	1.000
G1	Obic450	Obic1181	1.000
G1	Obic73	Obic1181	1.000
G1	Obic220	Obic1181	0.893
G1	Obic1374	Obic1181	1.000
G1	Obic1238	Obic1181	1.000
G1	Obic415	Obic1181	1.000
G1	Obic629	Obic1181	1.000
G1	Obic740	Obic1181	NA
G1	Obic168	Obic1181	0.227
G1	Obic1252	Obic1181	1.000
G1	Obic113	Obic1181	0.448
G1	Obic95	Obic1176	1.000
G1	Obic77	Obic1176	0.213
G1	Obic1	Obic1176	0.759
G1	Obic1206	Obic1176	1.000
G1	Obic450	Obic1176	0.445
G1	Obic73	Obic1176	1.000
G1	Obic220	Obic1176	0.621
G1	Obic1374	Obic1176	0.605
G1	Obic1238	Obic1176	0.614
G1	Obic415	Obic1176	0.955
G1	Obic629	Obic1176	1.000
G1	Obic740	Obic1176	NA
G1	Obic168	Obic1176	0.076
G1	Obic1252	Obic1176	0.430
G1	Obic113	Obic1176	0.558
G1	Obic1181	Obic1176	0.712
G2	Obic95	Obic77	0.643

G2	Obic95	Obic1	0.554
G2	Obic77	Obic1	1.000
G2	Obic95	Obic1206	1.000
G2	Obic77	Obic1206	0.489
G2	Obic1	Obic1206	0.418
G2	Obic95	Obic450	0.082
G2	Obic77	Obic450	0.520
G2	Obic1	Obic450	0.285
G2	Obic1206	Obic450	0.431
G2	Obic95	Obic73	0.095
G2	Obic77	Obic73	1.000
G2	Obic1	Obic73	0.462
G2	Obic1206	Obic73	0.706
G2	Obic450	Obic73	1.000
G2	Obic95	Obic220	0.735
G2	Obic77	Obic220	1.000
G2	Obic1	Obic220	1.000
G2	Obic1206	Obic220	1.000
G2	Obic450	Obic220	0.571
G2	Obic73	Obic220	0.502
G2	Obic95	Obic1374	0.485
G2	Obic77	Obic1374	0.86
G2	Obic1	Obic1374	0.642
G2	Obic1206	Obic1374	1.000
G2	Obic450	Obic1374	0.302
G2	Obic73	Obic1374	0.114
G2	Obic220	Obic1374	1.000
G2	Obic95	Obic1238	0.368
G2	Obic77	Obic1238	0.247
G2	Obic1	Obic1238	0.550
G2	Obic1206	Obic1238	0.324
G2	Obic450	Obic1238	0.087
G2	Obic73	Obic1238	0.757
G2	Obic220	Obic1238	1.000
G2	Obic1374	Obic1238	0.076
G2	Obic95	Obic415	0.777
G2	Obic77	Obic415	1.000
G2	Obic1	Obic415	0.443
G2	Obic1206	Obic415	0.718
G2	Obic450	Obic415	1.000
G2	Obic73	Obic415	0.184
G2	Obic220	Obic415	0.751
G2	Obic1374	Obic415	0.127
G2	Obic1238	Obic415	0.784
G2	Obic95	Obic629	0.535
G2	Obic77	Obic629	1.000
G2	Obic1	Obic629	1.000
G2	Obic1206	Obic629	1.000

G2	Obic450	Obic629	0.522
G2	Obic73	Obic629	0.367
G2	Obic220	Obic629	0.218
G2	Obic1374	Obic629	0.689
G2	Obic1238	Obic629	1.000
G2	Obic415	Obic629	0.840
G2	Obic95	Obic740	NA
G2	Obic77	Obic740	NA
G2	Obic1	Obic740	NA
G2	Obic1206	Obic740	NA
G2	Obic450	Obic740	NA
G2	Obic73	Obic740	NA
G2	Obic220	Obic740	NA
G2	Obic1374	Obic740	NA
G2	Obic1238	Obic740	NA
G2	Obic415	Obic740	NA
G2	Obic629	Obic740	NA
G2	Obic95	Obic168	0.191
G2	Obic77	Obic168	0.786
G2	Obic1	Obic168	0.400
G2	Obic1206	Obic168	1.000
G2	Obic450	Obic168	0.143
G2	Obic73	Obic168	0.778
G2	Obic220	Obic168	0.158
G2	Obic1374	Obic168	0.822
G2	Obic1238	Obic168	1.000
G2	Obic415	Obic168	0.467
G2	Obic629	Obic168	0.233
G2	Obic740	Obic168	NA
G2	Obic95	Obic1252	NA
G2	Obic77	Obic1252	NA
G2	Obic1	Obic1252	NA
G2	Obic1206	Obic1252	NA
G2	Obic450	Obic1252	NA
G2	Obic73	Obic1252	NA
G2	Obic220	Obic1252	NA
G2	Obic1374	Obic1252	NA
G2	Obic1238	Obic1252	NA
G2	Obic415	Obic1252	NA
G2	Obic629	Obic1252	NA
G2	Obic740	Obic1252	NA
G2	Obic168	Obic1252	NA
G2	Obic95	Obic113	1.000
G2	Obic77	Obic113	0.149
G2	Obic1	Obic113	0.651
G2	Obic1206	Obic113	1.000
G2	Obic450	Obic113	1.000
G2	Obic73	Obic113	0.901

G2	Obic220	Obic113	0.826
G2	Obic1374	Obic113	1.000
G2	Obic1238	Obic113	1.000
G2	Obic415	Obic113	0.344
G2	Obic629	Obic113	1.000
G2	Obic740	Obic113	NA
G2	Obic168	Obic113	0.823
G2	Obic1252	Obic113	NA
G2	Obic95	Obic1181	1.000
G2	Obic77	Obic1181	1.000
G2	Obic1	Obic1181	1.000
G2	Obic1206	Obic1181	1.000
G2	Obic450	Obic1181	1.000
G2	Obic73	Obic1181	0.529
G2	Obic220	Obic1181	0.455
G2	Obic1374	Obic1181	0.254
G2	Obic1238	Obic1181	1.000
G2	Obic415	Obic1181	0.494
G2	Obic629	Obic1181	1.000
G2	Obic740	Obic1181	NA
G2	Obic168	Obic1181	0.448
G2	Obic1252	Obic1181	NA
G2	Obic113	Obic1181	1.000
G2	Obic95	Obic1176	1.000
G2	Obic77	Obic1176	1.000
G2	Obic1	Obic1176	1.000
G2	Obic1206	Obic1176	1.000
G2	Obic450	Obic1176	0.667
G2	Obic73	Obic1176	0.518
G2	Obic220	Obic1176	1.000
G2	Obic1374	Obic1176	1.000
G2	Obic1238	Obic1176	1.000
G2	Obic415	Obic1176	0.519
G2	Obic629	Obic1176	1.000
G2	Obic740	Obic1176	NA
G2	Obic168	Obic1176	1.000
G2	Obic1252	Obic1176	NA
G2	Obic113	Obic1176	1.000
G2	Obic1181	Obic1176	1.000
NS	Obic95	Obic77	0.772
NS	Obic95	Obic1	1.000
NS	Obic77	Obic1	0.429
NS	Obic95	Obic1206	1.000
NS	Obic77	Obic1206	0.112
NS	Obic1	Obic1206	0.570
NS	Obic95	Obic450	1.000
NS	Obic77	Obic450	1.000
NS	Obic1	Obic450	1.000

NS	Obic1206	Obic450	1.000
NS	Obic95	Obic73	1.000
NS	Obic77	Obic73	0.894
NS	Obic1	Obic73	0.288
NS	Obic1206	Obic73	0.629
NS	Obic450	Obic73	1.000
NS	Obic95	Obic220	0.772
NS	Obic77	Obic220	0.433
NS	Obic1	Obic220	0.139
NS	Obic1206	Obic220	0.889
NS	Obic450	Obic220	0.717
NS	Obic73	Obic220	0.315
NS	Obic95	Obic1374	0.358
NS	Obic77	Obic1374	0.781
NS	Obic1	Obic1374	1.000
NS	Obic1206	Obic1374	0.484
NS	Obic450	Obic1374	1.000
NS	Obic73	Obic1374	0.479
NS	Obic220	Obic1374	0.777
NS	Obic95	Obic1238	0.488
NS	Obic77	Obic1238	0.302
NS	Obic1	Obic1238	1.000
NS	Obic1206	Obic1238	0.109
NS	Obic450	Obic1238	1.000
NS	Obic73	Obic1238	1.000
NS	Obic220	Obic1238	0.885
NS	Obic1374	Obic1238	1.000
NS	Obic95	Obic415	1.000
NS	Obic77	Obic415	0.660
NS	Obic1	Obic415	1.000
NS	Obic1206	Obic415	1.000
NS	Obic450	Obic415	1.000
NS	Obic73	Obic415	0.231
NS	Obic220	Obic415	0.110
NS	Obic1374	Obic415	0.592
NS	Obic1238	Obic415	0.828
NS	Obic95	Obic629	1.000
NS	Obic77	Obic629	1.000
NS	Obic1	Obic629	1.000
NS	Obic1206	Obic629	1.000
NS	Obic450	Obic629	1.000
NS	Obic73	Obic629	1.000
NS	Obic220	Obic629	1.000
NS	Obic1374	Obic629	1.000
NS	Obic1238	Obic629	0.167
NS	Obic415	Obic629	0.285
NS	Obic95	Obic740	1.000
NS	Obic77	Obic740	1.000

NS	Obic1	Obic740	0.718
NS	Obic1206	Obic740	1.000
NS	Obic450	Obic740	0.469
NS	Obic73	Obic740	0.188
NS	Obic220	Obic740	0.320
NS	Obic1374	Obic740	0.136
NS	Obic1238	Obic740	1.000
NS	Obic415	Obic740	0.294
NS	Obic629	Obic740	1.000
NS	Obic95	Obic168	0.485
NS	Obic77	Obic168	0.309
NS	Obic1	Obic168	0.288
NS	Obic1206	Obic168	0.622
NS	Obic450	Obic168	0.278
NS	Obic73	Obic168	0.106
NS	Obic220	Obic168	0.107
NS	Obic1374	Obic168	0.471
NS	Obic1238	Obic168	1.000
NS	Obic415	Obic168	0.827
NS	Obic629	Obic168	1.000
NS	Obic740	Obic168	0.203
NS	Obic95	Obic1252	1.000
NS	Obic77	Obic1252	1.000
NS	Obic1	Obic1252	0.711
NS	Obic1206	Obic1252	1.000
NS	Obic450	Obic1252	0.484
NS	Obic73	Obic1252	1.000
NS	Obic220	Obic1252	1.000
NS	Obic1374	Obic1252	1.000
NS	Obic1238	Obic1252	1.000
NS	Obic415	Obic1252	1.000
NS	Obic629	Obic1252	1.000
NS	Obic740	Obic1252	1.000
NS	Obic168	Obic1252	1.000
NS	Obic95	Obic113	0.429
NS	Obic77	Obic113	0.139
NS	Obic1	Obic113	1.000
NS	Obic1206	Obic113	0.047
NS	Obic450	Obic113	1.000
NS	Obic73	Obic113	1.000
NS	Obic220	Obic113	0.717
NS	Obic1374	Obic113	0.434
NS	Obic1238	Obic113	0.051
NS	Obic415	Obic113	1.000
NS	Obic629	Obic113	0.480
NS	Obic740	Obic113	1.000
NS	Obic168	Obic113	0.570
NS	Obic1252	Obic113	1.000

NS	Obic95	Obic1181	1.000
NS	Obic77	Obic1181	0.581
NS	Obic1	Obic1181	0.427
NS	Obic1206	Obic1181	1.000
NS	Obic450	Obic1181	0.805
NS	Obic73	Obic1181	1.000
NS	Obic220	Obic1181	0.595
NS	Obic1374	Obic1181	1.000
NS	Obic1238	Obic1181	1.000
NS	Obic415	Obic1181	0.485
NS	Obic629	Obic1181	1.000
NS	Obic740	Obic1181	1.000
NS	Obic168	Obic1181	1.000
NS	Obic1252	Obic1181	1.000
NS	Obic113	Obic1181	0.820
NS	Obic95	Obic1176	NA
NS	Obic77	Obic1176	NA
NS	Obic1	Obic1176	NA
NS	Obic1206	Obic1176	NA
NS	Obic450	Obic1176	NA
NS	Obic73	Obic1176	NA
NS	Obic220	Obic1176	NA
NS	Obic1374	Obic1176	NA
NS	Obic1238	Obic1176	NA
NS	Obic415	Obic1176	NA
NS	Obic629	Obic1176	NA
NS	Obic740	Obic1176	NA
NS	Obic168	Obic1176	NA
NS	Obic1252	Obic1176	NA
NS	Obic113	Obic1176	NA
NS	Obic1181	Obic1176	NA
Su	Obic95	Obic77	NA
Su	Obic95	Obic1	NA
Su	Obic77	Obic1	NA
Su	Obic95	Obic1206	NA
Su	Obic77	Obic1206	0.523
Su	Obic1	Obic1206	NA
Su	Obic95	Obic450	NA
Su	Obic77	Obic450	1.000
Su	Obic1	Obic450	NA
Su	Obic1206	Obic450	1.000
Su	Obic95	Obic73	NA
Su	Obic77	Obic73	0.275
Su	Obic1	Obic73	NA
Su	Obic1206	Obic73	1.000
Su	Obic450	Obic73	1.000
Su	Obic95	Obic220	NA
Su	Obic77	Obic220	1.000

Su	Obic1	Obic220	NA
Su	Obic1206	Obic220	0.616
Su	Obic450	Obic220	0.775
Su	Obic73	Obic220	0.107
Su	Obic95	Obic1374	NA
Su	Obic77	Obic1374	0.527
Su	Obic1	Obic1374	NA
Su	Obic1206	Obic1374	0.522
Su	Obic450	Obic1374	0.326
Su	Obic73	Obic1374	1.000
Su	Obic220	Obic1374	1.000
Su	Obic95	Obic1238	NA
Su	Obic77	Obic1238	1.000
Su	Obic1	Obic1238	NA
Su	Obic1206	Obic1238	0.238
Su	Obic450	Obic1238	0.594
Su	Obic73	Obic1238	1.000
Su	Obic220	Obic1238	0.585
Su	Obic1374	Obic1238	1.000
Su	Obic95	Obic415	NA
Su	Obic77	Obic415	0.807
Su	Obic1	Obic415	NA
Su	Obic1206	Obic415	0.814
Su	Obic450	Obic415	1.000
Su	Obic73	Obic415	1.000
Su	Obic220	Obic415	0.573
Su	Obic1374	Obic415	1.000
Su	Obic1238	Obic415	1.000
Su	Obic95	Obic629	NA
Su	Obic77	Obic629	0.812
Su	Obic1	Obic629	NA
Su	Obic1206	Obic629	0.808
Su	Obic450	Obic629	1.000
Su	Obic73	Obic629	1.000
Su	Obic220	Obic629	1.000
Su	Obic1374	Obic629	1.000
Su	Obic1238	Obic629	1.000
Su	Obic415	Obic629	0.179
Su	Obic95	Obic740	NA
Su	Obic77	Obic740	0.508
Su	Obic1	Obic740	NA
Su	Obic1206	Obic740	1.000
Su	Obic450	Obic740	0.328
Su	Obic73	Obic740	1.000
Su	Obic220	Obic740	1.000
Su	Obic1374	Obic740	1.000
Su	Obic1238	Obic740	1.000
Su	Obic415	Obic740	1.000

Su	Obic629	Obic740	1.000
Su	Obic95	Obic168	NA
Su	Obic77	Obic168	0.333
Su	Obic1	Obic168	NA
Su	Obic1206	Obic168	1.000
Su	Obic450	Obic168	0.443
Su	Obic73	Obic168	0.763
Su	Obic220	Obic168	0.779
Su	Obic1374	Obic168	1.000
Su	Obic1238	Obic168	0.591
Su	Obic415	Obic168	0.584
Su	Obic629	Obic168	0.087
Su	Obic740	Obic168	1.000
Su	Obic95	Obic1252	NA
Su	Obic77	Obic1252	1.000
Su	Obic1	Obic1252	NA
Su	Obic1206	Obic1252	1.000
Su	Obic450	Obic1252	0.406
Su	Obic73	Obic1252	1.000
Su	Obic220	Obic1252	1.000
Su	Obic1374	Obic1252	1.000
Su	Obic1238	Obic1252	1.000
Su	Obic415	Obic1252	1.000
Su	Obic629	Obic1252	1.000
Su	Obic740	Obic1252	1.000
Su	Obic168	Obic1252	0.461
Su	Obic95	Obic113	NA
Su	Obic77	Obic113	0.715
Su	Obic1	Obic113	NA
Su	Obic1206	Obic113	0.715
Su	Obic450	Obic113	0.371
Su	Obic73	Obic113	0.636
Su	Obic220	Obic113	0.536
Su	Obic1374	Obic113	1.000
Su	Obic1238	Obic113	0.531
Su	Obic415	Obic113	1.000
Su	Obic629	Obic113	0.550
Su	Obic740	Obic113	1.000
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Su	Obic95	Obic1181	NA
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Su	Obic1206	Obic1181	0.536
Su	Obic450	Obic1181	1.000
Su	Obic73	Obic1181	1.000
Su	Obic220	Obic1181	1.000
Su	Obic1374	Obic1181	1.000

Su	Obic1238	Obic1181	1.000
Su	Obic415	Obic1181	1.000
Su	Obic629	Obic1181	1.000
Su	Obic740	Obic1181	1.000
Su	Obic168	Obic1181	0.342
Su	Obic1252	Obic1181	1.000
Su	Obic113	Obic1181	0.284
Su	Obic95	Obic1176	NA
Su	Obic77	Obic1176	1.000
Su	Obic1	Obic1176	NA
Su	Obic1206	Obic1176	0.532
Su	Obic450	Obic1176	1.000
Su	Obic73	Obic1176	1.000
Su	Obic220	Obic1176	1.000
Su	Obic1374	Obic1176	1.000
Su	Obic1238	Obic1176	0.104
Su	Obic415	Obic1176	1.000
Su	Obic629	Obic1176	1.000
Su	Obic740	Obic1176	1.000
Su	Obic168	Obic1176	1.000
Su	Obic1252	Obic1176	NA
Su	Obic113	Obic1176	1.000
Su	Obic1181	Obic1176	1.000

Table C.7: Hard-Weinberg equilibrium probability tests by subpopulation and marker. Genepop probability test using unrelated females. Sub = subpopulation (with: NS = North Shropshire, Su = Surrey, G1-2 = Germany 1 and 2), and p = uncorrected p-values. Significant p- values are in red, NAs are those instances lack data. Overall there seem to be no generally consistent deviations from Hardy-Weinberg equilibrium, given the low sample sizes per subpopulation in addition to multiple testing.

Locus	Sub	p	Locus	Sub	p
Obic113	G1	0.011	Obic415	G1	0.115
Obic113	G2	1.000	Obic415	G2	1.000
Obic113	NS	1.000	Obic415	NS	1.000
Obic113	Su	0.006	Obic415	Su	0.100
Obic1176	G1	0.082	Obic450	G1	1.000
Obic1176	G2	0.500	Obic450	G2	1.000
Obic1176	NS	0.422	Obic450	NS	0.228
Obic1176	Su	0.302	Obic450	Su	0.442
Obic1181	G1	0.012	Obic629	G1	0.0415
Obic1181	G2	1.000	Obic629	G2	0.149
Obic1181	NS	1.000	Obic629	NS	0.784
Obic1181	Su	0.743	Obic629	Su	1.000
Obic1206	G1	1.000	Obic73	G1	0.228
Obic1206	G2	0.340	Obic73	G2	0.315
Obic1206	NS	0.575	Obic73	NS	0.324
Obic1206	Su	1.000	Obic73	Su	0.808
Obic1238	G1	0.845	Obic740	G1	0.988
Obic1238	G2	0.571	Obic740	G2	0.370
Obic1238	NS	0.234	Obic740	NS	0.057
Obic1238	Su	1.000	Obic740	Su	0.101
Obic1252	G1	0.049	Obic77	G1	1.000
Obic1252	G2	1.000	Obic77	G2	1.000
Obic1252	NS	0.458	Obic77	NS	1.000
Obic1252	Su	0.732	Obic77	Su	1.000
Obic1374	G1	0.271	Obic95	G1	0.445
Obic1374	G2	0.024	Obic95	G2	0.626
Obic1374	NS	0.777	Obic95	NS	1.000
Obic1374	Su	0.860	Obic95	Su	1.000
Obic168	G1	0.966	Obic220	G1	1.000
Obic168	G2	0.003	Obic220	G2	1.000
Obic168	NS	0.105	Obic220	NS	1.000
Obic168	Su	0.441	Obic220	Su	1.000
Obic1	G1	0.178			
Obic1	G2	1.000			
Obic1	NS	NA			
Obic1	Su	NA			

Table C.8: Marker statistics by subpopulation. Cervus generated marker statistics. Sub = subpopulation (with: NS = North Shropshire, Su = Surrey, G1-2 = Germany 1 and 2), k =number of alleles, N = number of females tested, Ho = observed heterozygosity, He = expected heterozygosity, ΔH = difference between observed and expected heterozygosity, PIC = polymorphic information content (estimated power of marker), and F_{null} = estimated frequency of null alleles with ND = not determined (too little information; i.e. sample size). Significant values are denoted in red, i.e. difference between expected and observed hetrozygosity |ΔH| > 0.2; or where estimated null allele frequency > 0.1. Overall there seem to be no generally consistent abnormalities (no more than expected by chance), given the low sample sizes.

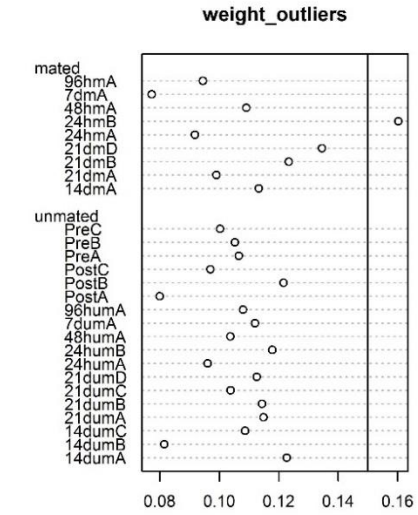
Sub	Locus	k	N	Ho	He	ΔH	PIC	F_{null}
G1	Obic113	5	10	0.200	0.368	-0.168	0.337	0.404
G2	Obic113	4	9	0.667	0.608	0.059	0.533	ND
NS	Obic113	3	7	0.286	0.275	0.011	0.240	ND
Su	Obic113	5	7	0.286	0.659	-0.373	0.571	ND
G1	Obic1176	4	10	0.800	0.726	0.074	0.628	-0.086
G2	Obic1176	5	9	0.667	0.771	-0.104	0.684	ND
NS	Obic1176	7	7	0.714	0.879	-0.165	0.792	ND
Su	Obic1176	5	7	0.714	0.846	-0.132	0.752	ND
G1	Obic1181	7	10	0.500	0.8	-0.300	0.726	0.232
G2	Obic1181	6	9	0.889	0.784	0.105	0.699	ND
NS	Obic1181	4	7	0.714	0.648	0.066	0.553	ND
Su	Obic1181	4	7	0.571	0.714	-0.143	0.600	ND
G1	Obic1206	5	10	0.600	0.653	-0.053	0.571	0.014
G2	Obic1206	5	9	0.667	0.752	-0.085	0.661	ND
NS	Obic1206	4	7	0.857	0.703	0.154	0.599	ND
Su	Obic1206	2	7	0.286	0.264	0.022	0.215	ND
G1	Obic1238	5	10	0.800	0.763	0.037	0.681	-0.041
G2	Obic1238	5	9	0.778	0.719	0.059	0.640	ND
NS	Obic1238	4	7	0.571	0.648	-0.077	0.553	ND
Su	Obic1238	4	7	0.714	0.659	0.055	0.570	ND
G1	Obic1252	8	10	0.700	0.868	-0.168	0.804	0.080
G2	Obic1252	5	9	0.667	0.791	-0.124	0.704	ND
NS	Obic1252	5	7	0.714	0.802	-0.088	0.704	ND
Su	Obic1252	5	6	0.833	0.818	0.015	0.708	ND
G1	Obic1374	5	10	0.500	0.726	-0.226	0.635	0.157
G2	Obic1374	5	9	0.556	0.752	-0.196	0.661	ND
NS	Obic1374	3	7	0.714	0.67	0.044	0.551	ND
Su	Obic1374	4	7	0.571	0.692	-0.121	0.585	ND
G1	Obic168	5	10	0.700	0.742	-0.042	0.653	-0.011
G2	Obic168	4	9	0.556	0.739	-0.183	0.637	ND
NS	Obic168	3	7	0.286	0.615	-0.329	0.501	ND
Su	Obic168	2	7	0.286	0.44	-0.154	0.325	ND
G1	Obic1	3	10	0.300	0.484	-0.184	0.41	0.248
G2	Obic1	5	9	0.667	0.549	0.118	0.485	ND

NS	Obic1	2	7	0.143	0.143	0.000	0.124	ND
Su	Obic1	1	7	0.000	0.000	0.000	0.000	ND
G1	Obic220	2	10	0.500	0.479	0.021	0.351	-0.047
G2	Obic220	3	9	0.556	0.542	0.014	0.426	ND
NS	Obic220	2	7	0.571	0.527	0.044	0.370	ND
Su	Obic220	2	7	0.571	0.527	0.044	0.370	ND
G1	Obic415	4	10	0.400	0.611	-0.211	0.535	0.157
G2	Obic415	5	9	0.444	0.405	0.039	0.368	ND
NS	Obic415	3	7	0.571	0.473	0.098	0.386	ND
Su	Obic415	4	7	0.429	0.714	-0.285	0.615	ND
G1	Obic450	2	10	0.500	0.395	0.105	0.305	-0.142
G2	Obic450	2	9	0.444	0.366	0.078	0.286	ND
NS	Obic450	2	7	0.143	0.363	-0.220	0.280	ND
Su	Obic450	2	7	0.286	0.440	-0.154	0.325	ND
G1	Obic629	6	10	0.500	0.800	-0.300	0.730	0.220
G2	Obic629	6	9	0.889	0.797	0.092	0.713	ND
NS	Obic629	6	7	0.857	0.835	0.022	0.741	ND
Su	Obic629	4	7	0.857	0.736	0.121	0.626	ND
G1	Obic73	5	10	0.700	0.695	0.005	0.611	-0.011
G2	Obic73	3	9	0.778	0.68	0.098	0.568	ND
NS	Obic73	3	7	0.429	0.560	-0.131	0.464	ND
Su	Obic73	3	7	0.714	0.703	0.011	0.580	ND
G1	Obic740	8	10	0.900	0.868	0.032	0.804	-0.041
G2	Obic740	8	9	0.667	0.856	-0.189	0.784	ND
NS	Obic740	8	7	0.714	0.901	-0.187	0.818	ND
Su	Obic740	7	7	0.714	0.879	-0.165	0.792	ND
G1	Obic77	3	10	0.300	0.279	0.021	0.247	-0.075
G2	Obic77	2	9	0.333	0.294	0.039	0.239	ND
NS	Obic77	3	7	0.429	0.385	0.044	0.325	ND
Su	Obic77	2	7	0.286	0.264	0.022	0.215	ND
G1	Obic95f	6	10	0.800	0.779	0.021	0.703	-0.031
G2	Obic95f	5	9	0.667	0.693	-0.026	0.603	ND
NS	Obic95f	3	7	0.857	0.703	0.154	0.580	ND
Su	Obic95f	5	7	0.714	0.758	-0.044	0.657	ND

Appendix D. Modelling oogenesis

Zuur *et al.* (2010) provide a protocol for data exploration, which was adopted here using R 3.5.1 (R Core Team, 2016). Outliers can have an overbearing effect on analysis and conclusions, hence they were investigated using Cleveland plots (Fig. D.1). Data-points were considered outliers when: extremely high and no intermediate values were present. Data was further explored graphically (Fig. D.2 and Fig. D.3). Collinearity was only found for vitellarium and total ovariole length, implying that total ovariole length was mostly dependent on the vitellarium. None of the covariates used in the models (e.g. weight and time) showed any clear signs of collinearity or confounding. Linear mixed effect models and generalised linear mixed effect models were built using lme4 (Bates *et al.*, 2015). For linear mixed effect models, assumptions for normality and homoscedasticity were checked graphically (Fig. D.4), since graphical tools are advocated (Zuur *et al.*, 2010). Additionally, AN(C)OVA is considered fairly robust against violations of normality (Zuur *et al.*, 2010). AN(C)OVA was performed using *lmerTest* (Kuznetsova *et al.*, 2016; Luke, 2017). For oocyte volumes, slopes were allowed to vary according to the position of the oocyte in the ovariole [i.e. random effect = (1 + oocyte position | individual); Fig. D.4]. These slopes approximate oocyte maturation rate, which are then grouped in the model across **individuals** [i.e. (1 + oocyte position | **individual**)], and subsequently compared across weight, time and treatment. Oocyte volume was $\log_{10}$-transformed, since growth processes tend to be exponential[66], which also provided the best model fit (AIC; data not shown) of all models tried (including polynomial terms). Generalised linear mixed models (i.e. oocytes and cell counts; Table D.1) were checked for overdispersion using a point estimate (Harrison, 2014). Overdispersed models were corrected for by adding an observation level random effect (OLRE; Harrison, 2014). Model factors were tested likelihood-ratio tests (Whittingham *et al.*, 2006; Mundry and Nunn, 2008; Forstmeier and Schielzeth, 2011; Bates *et al.*, 2015), only removing interaction effects where appropriate (Engqvist, 2005). Germarial and terminal filament cell counts suffered from low number of degrees of freedom (due to sample quality many observations were unmeasurable/uncountable). Hence complex models for cell counts failed to converge and simpler models containing only the relevant random effects and the main fixed effect of interest (i.e. time) were used (Table D.1). All results are displayed in Table D.2.

[66] Even though the oocyte itself does not duplicate, oocyte growth still approximates an exponential growth curve, since the follicle cells around the oocyte do continuously divide while depositing yolk. Additionally, nurse cells expand and dump RNA and proteins into the growing egg.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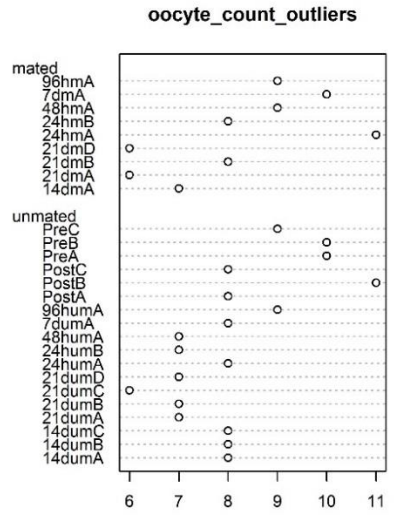
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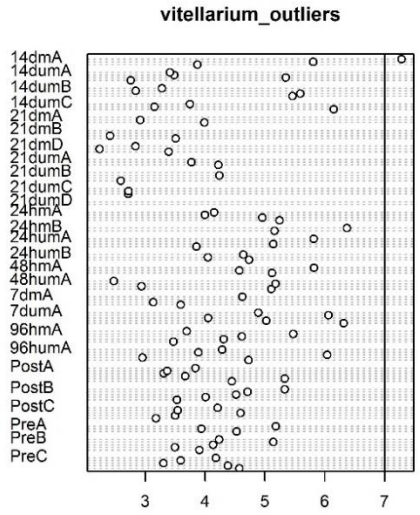
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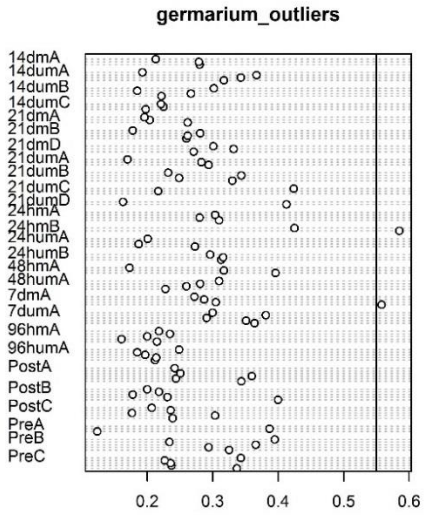
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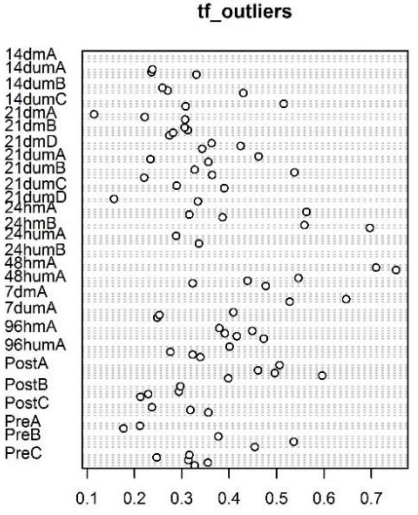
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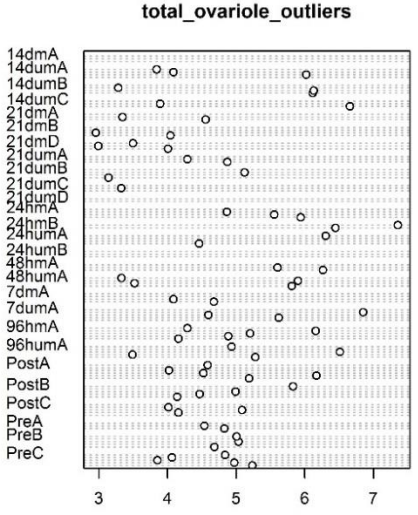
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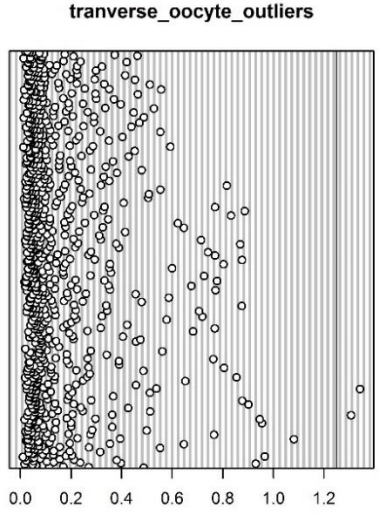
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0.4
0.6
0.8
1.0
1.2

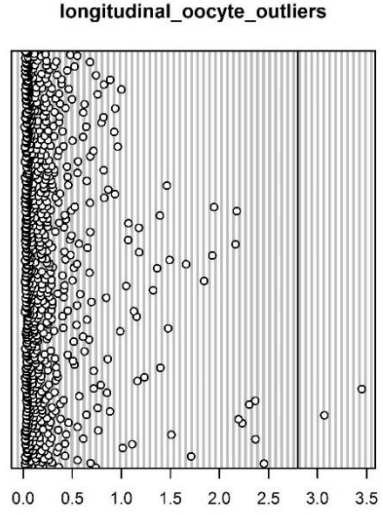
longitudinal_oocyte_outliers
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5

Fig. D.1 Cleveland plots for the detection of outliers. Data-points right of the vertical line were deemed outliers and removed prior to analysis.

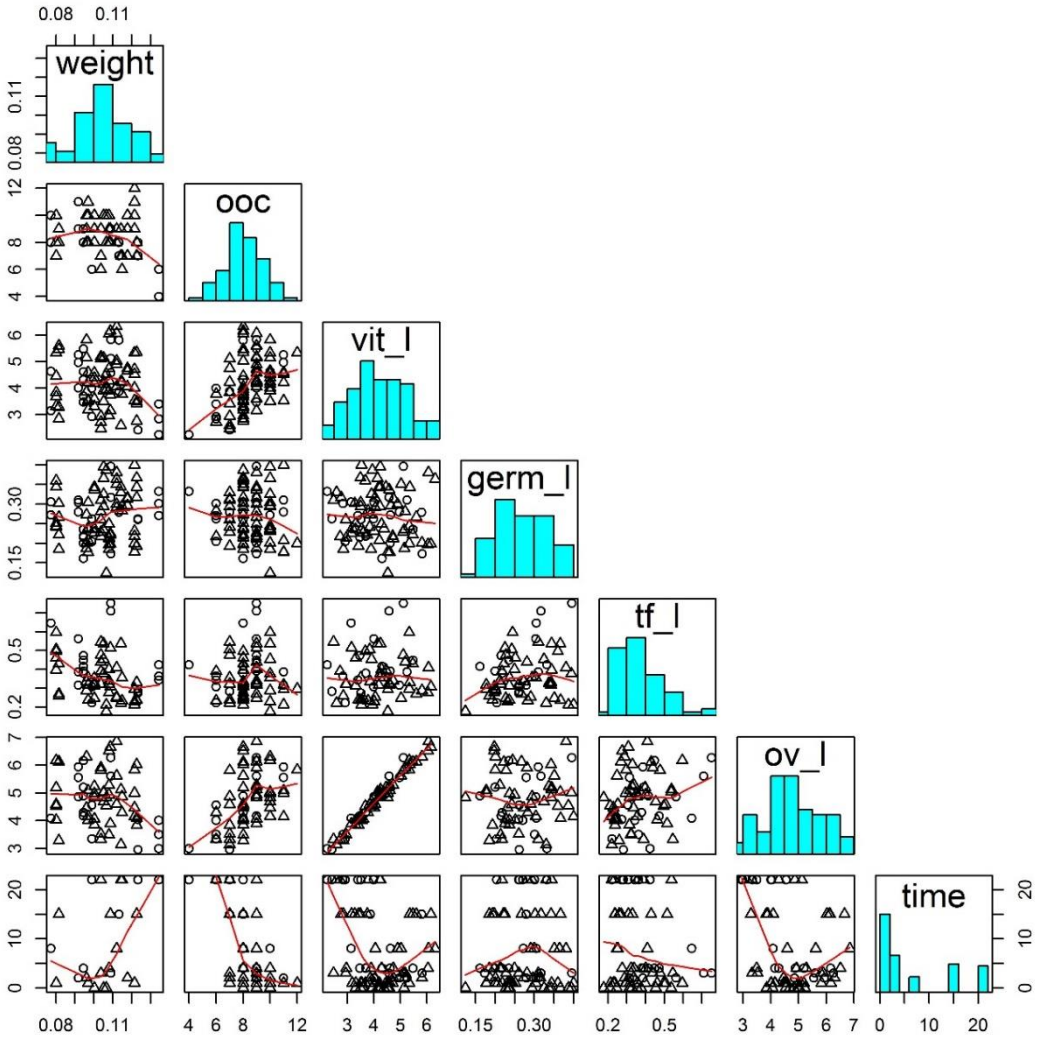

Fig. D.2 Graphical exploration of ovariole lengths data. Mated and unmated females were plotted using different symbols, red lines represent LOWESS smoothing and diagonal histograms show raw data distributions. Weight = female weight (g); ooc = total number of discernible oocytes (includes data of poor samples with damaged tissue which were removed prior to analysis); vit_l = vitellarium length (mm); germ_l = germarium length (mm); tf_l = terminal filament (mm); ov_l = total ovariole length (mm); and time = time-point of the experiment (days). Only vitellarium and total ovariole length showed a clear and strong correlation, implying total ovariole length was mostly dependent on the vitellarium.

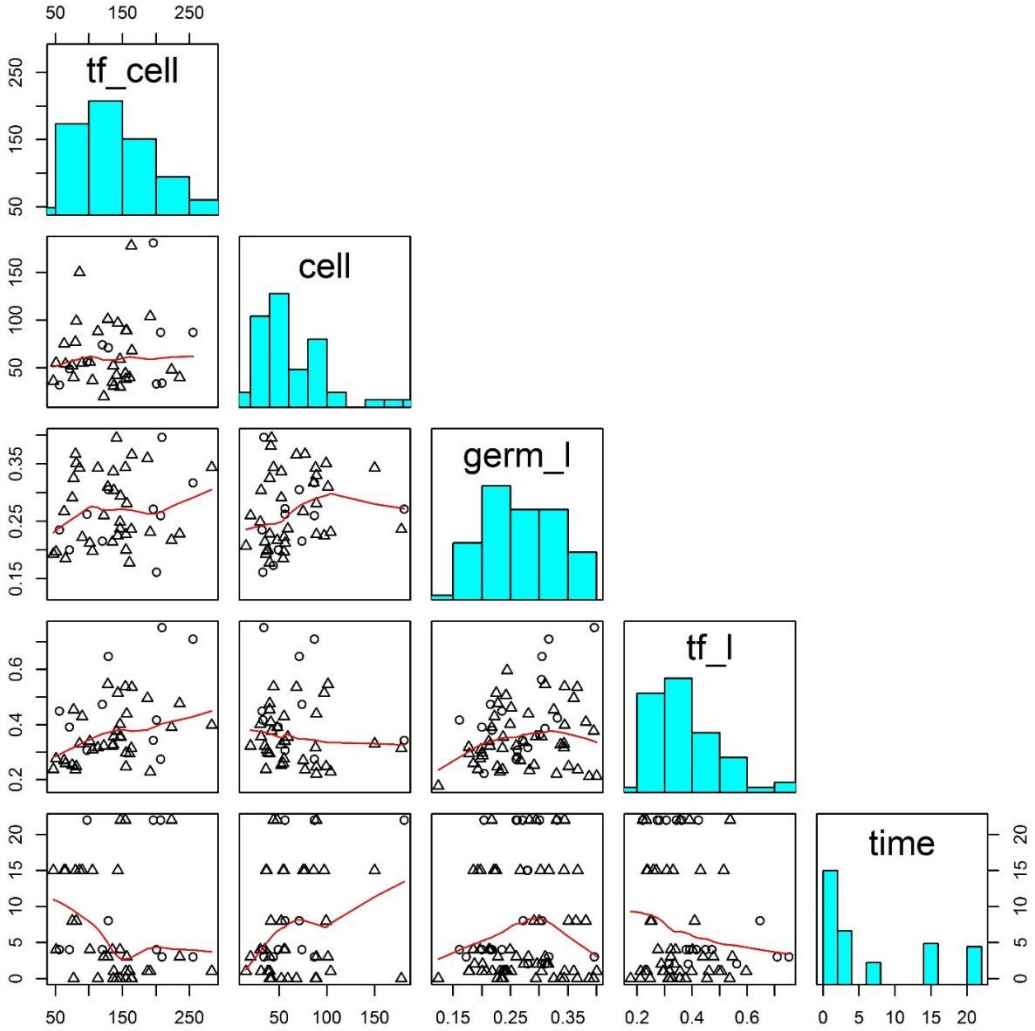

Fig. D.3 Graphical exploration of cell counts and their relative tissue lengths (germarium and terminal filament). Mated and unmated females were plotted using different symbols, red lines represent LOWESS smoothing and diagonal histograms show raw data distributions. tf_cell = terminal filament cell count; cell = cell count of germarial cells from the terminal filament until the first discernible oocyte; germ_l = germarium length (mm); tf_l = terminal filament (mm); and time = time-point of the experiment (days).

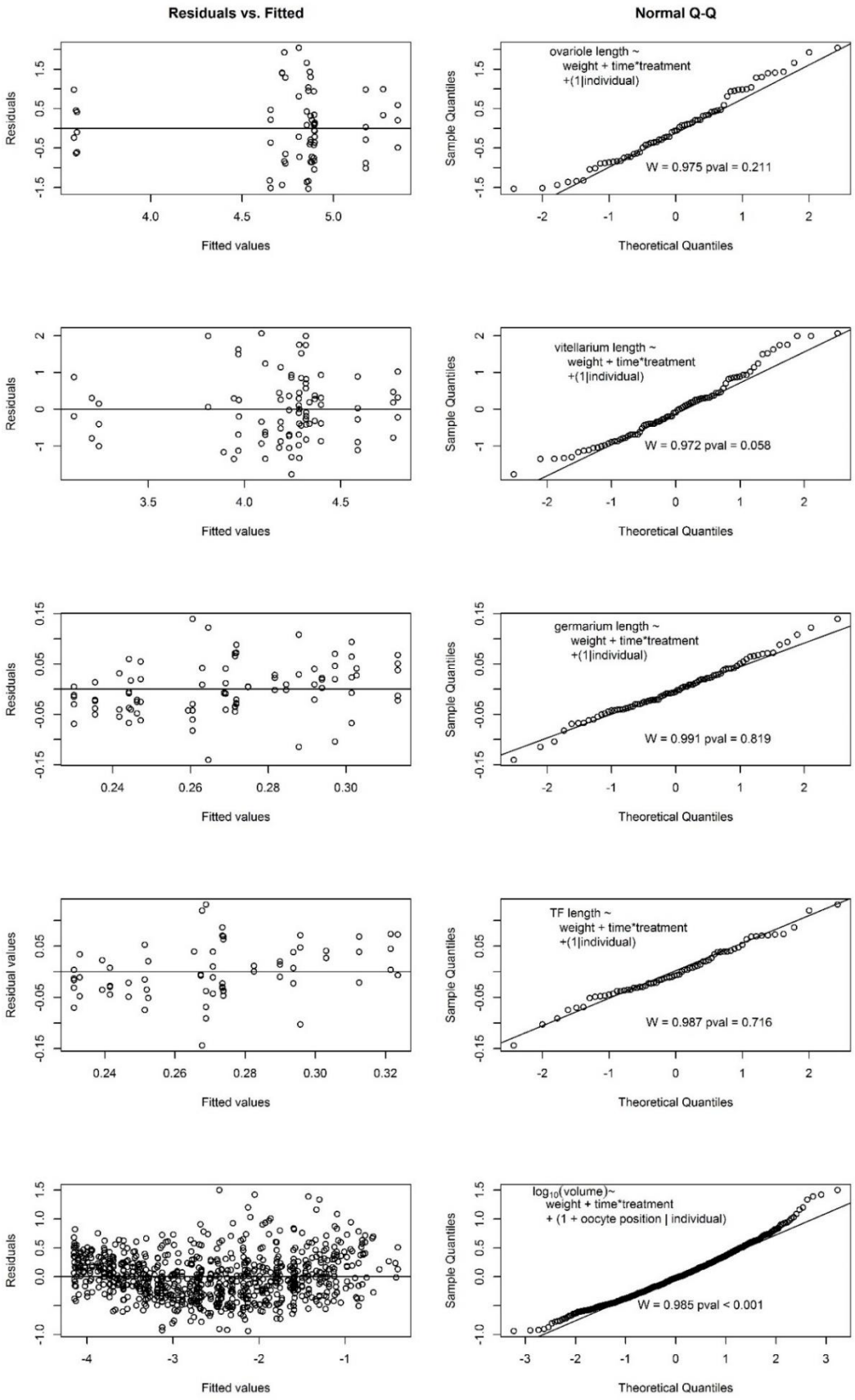

Fig. D.4 Model assumptions for linear mixed models. Homoscedasticity and qq-plots with model structures. Shapiro-Wilk's test statistic (W) and p-values are illustrative only.

Table D.1 Log - link models and their respective overdispersion parameter (OP) point estimate following Harrison (2014). Random effects are in brackets. Models with parameters > 1 were corrected by adding a observation level random effect (OLRE in red; Harrison, 2014).

Model	OP
oocyte count ~ weight + time-point * treatment + (1|individual)	0.142
early germarial cell count ~ time-point + (1|individual) + (1|OLRE)	10.83
terminal filament cell count ~ time-point + (1|individual) + (1|OLRE)	7.476

Table D.2 Modeling results for all ovariole measurements. Model = independent variable of the model; factor = explanatory variables of the model (with treatment = mating status); d.f. = numerator and denominator degrees of freedom for the test statistic, acquired through Satterthwaite approximation and rounded to its integer; value = value of test statistic; and sig = significance levels (taken to be: p < 0.001 = ***; p < 0.01 = **; and P < 0.05 = *).

Model	factor	d.f.	value	p	sign
Total ovariole length[a]	Weight	$F_{1,61}$	0.002	0.962	
	Time	$F_{1,61}$	8.698	0.004	**
	Treatment	$F_{1,61}$	2.711	0.105	
	Time * Treatment	$F_{1,61}$	6.349	0.024	*
Vitellarium length[a]	Weight	$F_{1,19}$	0.329	0.573	
	Time	$F_{1,28}$	10.49	0.003	**
	Treatment	$F_{1,20}$	3.242	0.087	
	Time * Treatment	$F_{1,29}$	4.882	0.035	*
Germarium length[a]	Weight	$F_{1,22}$	1.506	0.233	
	Time	$F_{1,26}$	0.104	0.750	
	Treatment	$F_{1,22}$	0.136	0.715	
	Time * Treatment	$F_{1,27}$	0.268	0.609	
Terminal filament length[a]	Weight	$F_{1,19}$	1.802	0.196	
	Time	$F_{1,21}$	0.762	0.392	
	Treatment	$F_{1,18}$	0.474	0.500	
	Time * Treatment	$F_{1,21}$	0.456	0.507	
Log_{10} (oocyte volume)[a,b]	Weight	$F_{1,20}$	0.072	0.791	
	Time	$F_{1,22}$	26.36	< 0.001	***
	Treatment	$F_{1,20}$	0.555	0.465	
	Time * Treatment	$F_{1,22}$	1.052	0.316	
Oocyte count[c]	Weight	$\chi^2_{3,6}$	0.361	0.948	
	Time	$\chi^2_{1,5}$	9.414	0.009	**
	Treatment	$\chi^2_{1,5}$	0.053	0.818	
	Time * Treatment	$\chi^2_{1,5}$	0.599	0.807	
Germarial cell count[c]	Time	$\chi^2_{1,4}$	1.423	0.233	
Terminal filament cell count[c]	Time	$\chi^2_{1,4}$	0.004	0.949	

[a] linear mixed effect models (Gaussian; identity link function)
[b] Oocyte maturation rate model: i.e. the slopes for log_{10}(oocyte volume) ~ oocyte position in the ovariole, are compared across time and treatment
[c] generalised linear mixed effect models (Poisson distributed; log link function)

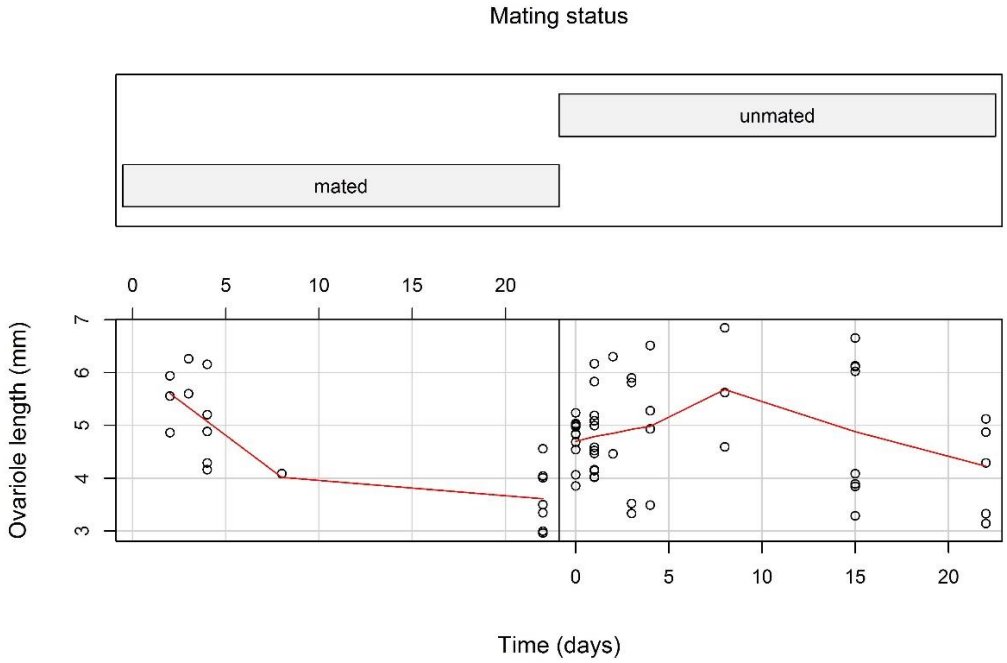

Fig. D.5 Conditional plot of ovariole lengths. Detail of the ovariole lengths data, with treatments separated (mating status; left to right), showing missing intermediate data points in the mated group. Points were not jittered, hence may overlap and mask one another. Red lines represent LOWESS smoothing.

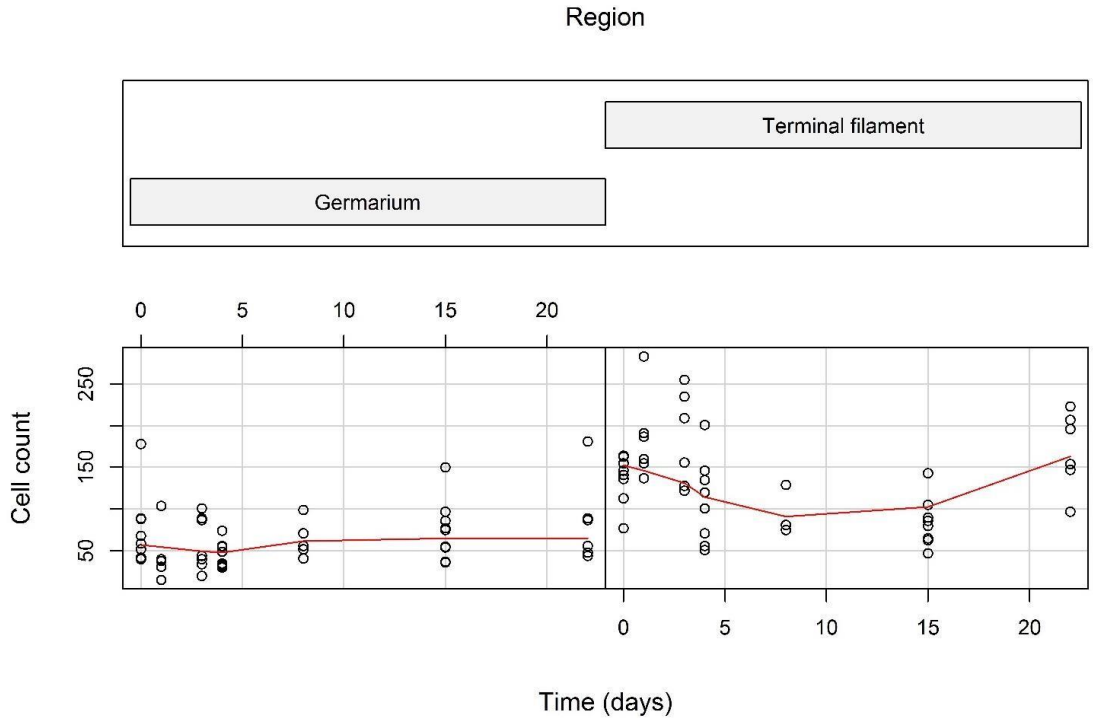

Fig. D.6 Cell counts showed no indication of *reproductive senescence*. Semi-automated cell counts (ImageJ) showed a highly variable number of presumptive germline stem cells, with no signs of decreasing cell populations. Points were not jittered, hence may overlap and mask one another. Red lines represent LOWESS smoothing.

Appendix E. NICD

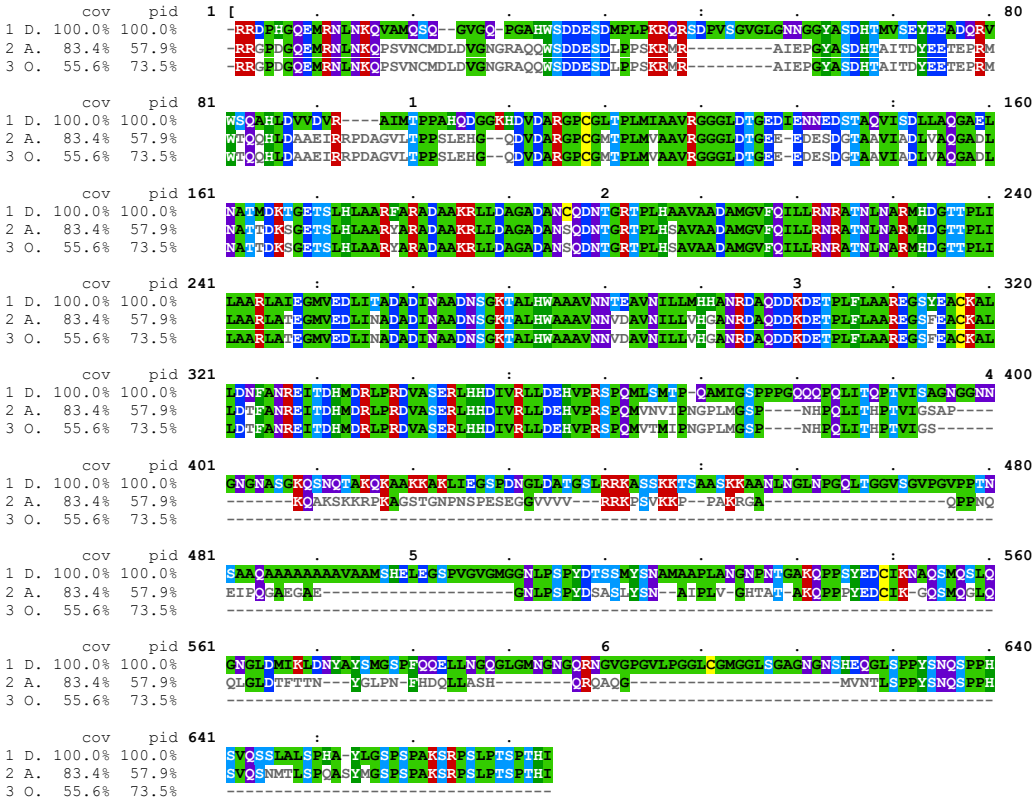

Fig. E.1 Alignment of the NICD antibody epitope (C17.9C6, Developmental Studies Hybridoma Bank) for the relevant species. D. = *D. melanogaster* NICD epitope (aa1791-2504; flybase ID: FBgn0004647). A. = *A. mellifera* NICD epitope (Duncan *et al.*, 2016). O. = the equivalent region in *O. bicornis* obtained through xBLAST (Altschul *et al.*, 1990) to *D. melanogaster* of the Notch sequence obtained from *de novo* transcriptome assembly (Dr E.J. Duncan, see text). Cov = covariance, p = percentage identity. Identities normalised by aligned length, and colored by identity. Alignment performed using BioEdit (ClustalW multiple alignment; Hall, 1999), and visualised using EMBL-EBI (Madeira *et al.*, 2019). The **putative** *O. bicornis* NICD seems to be missing a large portion of the NICD antibody epitope.

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