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Mating status and the evolution of eusociality: Oogenesis is independent of mating status in the solitary bee *Osmia bicornis*.

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Abstract

The fundamental trait underlying eusociality is the reproductive division of labour. In honeybees (*Apis mellifera*), queens lay eggs while workers forage, defend and care for brood. The division of labour is maintained by pheromones including queen mandibular pheromone (QMP) produced by the queen. QMP constrains reproduction in adult honeybee workers, but in the absence of their queen workers can activate their ovaries and, although they cannot mate, they lay haploid male eggs. The reproductive ground plan hypothesis suggests that reproductive constraint may have evolved by co-opting mechanisms of reproductive control in solitary ancestors. In many insects mating is required to activate or accelerate oogenesis. Here, we use the solitary bee *Osmia bicornis* (Megachilidae) to test whether reproductive constraint evolved from ancestral control of reproduction by mating status. We present a structural study of the *O. bicornis* ovary, and compare key stages of oogenesis with honeybee workers. Importantly, we show that mating did not affect any aspect of the reproductive physiology of *O. bicornis*. We therefore conclude that mechanisms governing reproductive constraint in honeybees were unlikely to have been co-opted from mechanisms pertaining to mating status.

Keywords

Apis mellifera, *Osmia bicornis*, reproduction, reproductive constraint, mating, ovary, ovariole, germarium, terminal filament

1 Introduction

The central tenet of eusociality is the reproductive division of labour, with specific reproductive and non-reproductive castes. The non-reproductive caste is kept functionally sterile in the presence of a dominant female or 'queen' by mechanisms collectively known as reproductive constraints. In Hymenopteran worker castes these reproductive constraints can be behavioural (Beekman and Oldroyd, 2008), through physical reduction of fecundity during development (Khila and Abouheif, 2010; Hartfelder and Steinbruck, 1997), or adulthood where queen presence affects ovarian physiology (Tanaka *et al.*., 2006; Duncan *et al.*., 2016; Ronai *et al.*., 2017). In honeybees, reproductive constraint is mediated through pheromones produced by the queen, queen mandibular pheromone, and her brood (Winston, 1991).

Understanding how the reproductive division of labour evolved is a key question in evolutionary biology (Smith and Szathmary, 1997). The reproductive ground plan hypothesis (RGPH; Amdam *et al.*., 2006) suggests that the reproductive division of labour originated from a decoupling of maternal behaviour (non-reproductive; worker) and reproductive status (reproductive; queen). Consequently, it has been hypothesised that ancestral mechanisms that controlled the reproduction of solitary individuals in response to environmental stimuli such as nutrition or temperature (Engelmann, 1970) (hereafter referred to as reproductive control) have been de-coupled from these environmental factors and co-opted into constraining reproduction in 'worker' castes in the evolution of eusociality (hereafter referred to as reproductive constraint). Understanding what these ancestral mechanisms were, how they mediate reproductive control in response to environmental stimuli, and finally; how they have been co-opted into reproductive constraint is integral to our understanding of the evolution of eusociality.

Reproductive constraint is often effected by the presence of a dominant female or queen, which is mainly signalled through queen pheromones (Winston, 1991; Van Oystaeyen *et al.*., 2014; Holman, 2018). Within the eusocial hymenoptera queen signals, other than those

produced by the honeybee (*Apis mellifera*), are thought to be derived from cuticular hydrocarbons (CHCs; Holman *et al.*., 2010; Van Oystaeyen *et al.*., 2014). Cuticular hydrocarbons serve pleiotropic roles in insect communication (reviewed in Oi *et al.*., 2015). CHCs signal mating status, species recognition, colonial and/or kin recognition (Oi *et al.*., 2015). The current paradigm suggests that queen pheromones act as conserved honest queen fertility signals (Van Oystaeyen *et al.*., 2014; Oi *et al.*., 2015). These honest signals of fertility are thought to be derived from the by-products of ovary development, from sex pheromones, and/or from oviposition deterring pheromones (Oi *et al.*., 2015). Fecundity and insemination are closely linked in Hymenopteran queens, where queen signals change significantly with mating status (A. mellifera: Slessor *et al.*., 1990; Leptothorax gredleri: Oppelt and Heinze, 2009), as well as queen ovary activation requiring mating in advanced social species (Melipona quadrifasciata anthidioides: Martins and Serrão, 2004; de Souza *et al.*., 2007; Tanaka *et al.*., 2009; A. mellifera: Tanaka and Hartfelder, 2004; Tanaka *et al.*., 2006). The effects of mating have also been shown to be important for queen pheromone production in *A. mellifera* (Richard *et al.*., 2007) and CHC profiles in *Bombus terrestris* (Jansen *et al.*., 2016). Taken together, this warrants investigating whether mating status had an ancestral role in reproductive control, and whether such mating-linked reproductive control may have been a precursor to adult reproductive constraint in social species.

By conducting a cross species comparison between *A. mellifera* and a related solitary species (Osmia bicornis, mrca 100 mya: Branstetter *et al.*., 2017) we investigated whether mating status may have had an ancestral role in reproductive control, and subsequently reproductive constraint. *O. bicornis* is an excellent species to address this question as it possesses many pre-adaptations relevant to the evolution of eusociality, among others: monogamy, limited dispersal /female philopatry, and high levels of maternal care (Seidelmann, 1995). Additionally, female mating status is thought to be signalled through CHC in *O. bicornis* via a male anti-aphrodisiac applied during post-copulatory display (Ayasse and Dutzler, 1998; Seidelmann, 2014), although this has recently been contested (Seidelmann and Rolke, 2019). We compare oogenesis in *O. bicornis* with the honeybee *A. mellifera.* Although eusociality in the honeybee is considered to be highly derived, it is the most well studied eusocial bee species in terms of oogenesis (Tanaka and Hartfelder, 2004;

97 Wilson *et al.*., 2011) and molecular mechanisms of reproductive constraint (Ronai *et al.*.,
98 2016; Duncan *et al.*., 2016). Here we use *O. bicornis* to test the hypothesis that
99 reproductive constraint evolved from ancestral control of reproduction in response to
100 mating status.

101 2 Materials and methods

102 2.1 Study species and husbandry

103 *O. bicornis* were obtained as cocoons from a commercial supplier (Dr Schubert plant
104 breeding; Landsberg, Germany) and hatched under controlled laboratory conditions
105 (Sandrock *et al.*., 2014); Briefly, bees were kept between 21-23˚C and in at 18:6 h light: dark
106 cycle. Bees were supplied with: makeshift flowers and catkins with ground pollen, 50%
107 sucrose solution (filter sterilised; 0.22 µm; Millipore), additional fondant paste (Candipolline
108 Gold), Fabre's hives (Oxford bee company), and mud for nest building (70% Fuller's earth by
109 Intra Laboratories and 30% white silica sand by Cristobalite). Bees were fed *ad libitum*
110 throughout the study.
111 Females were hatched in isolation in individual plastic containers in the dark, and
112 subsequently housed according to treatment for three days (mesh cage; 60 x 60 x 90 cm).
113 The mated group was kept in a in a 3:1 ratio (9 females:3 males; Fliszkiewicz *et al.*., 2013),
114 the unmated group contained 12 females and no males. Two one-hour observations were
115 performed on the same day to observe attempts at mating. Mating status was confirmed
116 upon dissection of the females by visual examination of the spermathecae. Mating plugs
117 were rarely found in our laboratory set-up (these regress within one day; Seidelmann,
118 1995). Three days after the introduction of males mated females were marked red (Uni
119 Posca marker) on the thorax, and all females (both mated and unmated) were placed
120 together in a larger cage (65 x 90 x 140 cm). Females were dissected for their ovaries at
121 eight different time points: pre-eclosion (dissected from cocoon), post-eclosion (within 24
122 hours of hatching), and 24 hours, 48 hours, 96 hours, 7 days, 14 days and 21 days after
123 being placed with males.

A. mellifera mellifera workers were kept according to standard practices in British National hives at the University of Leeds 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 (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).

2.2 Staining

Bees were sedated by chilling at 4°C, weighed, and dissected in PBS (phosphate buffered saline). Ovarioles were separated using fine forceps, and the intima and ovariole sheath (or peritoneal sheath) were removed to facilitate staining and increase image quality. Ovarioles from each individual bee were treated and kept together. Tissue was fixed for ten minutes in a 1:1 mixture of 4% formaldehyde in PBS and heptane on a nutating mixer at room temperature (RT), rinsed three times in 0.1% PTx (PBS + 0.1% Triton x). Tissue was permeabilised for 90 minutes in PTx at RT on a nutating mixer. Excessive PTx was removed and stained with 0.33 µM Phalloidin Dylight 488 (Thermo Fisher Scientific) added, and left to incubate at RT in darkness for three hours. 900 µl 0.1% PTx was added, along with 5 µg/ml of DAPI (4',6-diamidino-2-phenylindole; Molecular Probes), and left to incubate at RT in the dark for ten minutes. Excess DAPI was removed by washing 3 x 5 min in PTx, and tissue was cleared overnight through 80% ultrapure glycerol prior to mounting. 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 projection. Whether

confocal images presented are maximum intensity projections or single slices (i.e. “optical section”) is indicated in figure legends.

2.3 Morphological measurements

Measurements of 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, we 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 (known as corpus luteum that consist of degenerating postovulatory follicle cells; Büning, 1994) and number of mature oocytes (defined as stage 7 and 8 oocytes in Wilson *et al.*., 2011) were counted to investigate rates of oogenesis. Mature oocytes were generally not mounted on slides, and hence were not part of vitellarium length measurement. 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 similar to Cane, 2016):

$$\textit{prolate spheroid} = 4\pi/3 * (\textit{polar radius})^2 * \textit{equatorial radius} \qquad \textbf{Eq. 1}$$

By fitting these into a model (see 2.4), ‘*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 (see 2.2), and was semi-automated to limit observer bias.

2.4 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 *lmerTest* for linear mixed models (Kuznetsova *et al.*., 2016), or through model comparison for generalised linear mixed models (Bates *et al.*., 2015). Appendix A outlines the Zuur *et al.*. (2010) protocol implemented (Fig. A.1-4), and full models are

displayed (Fig. A.4 and Table A.1) along with all results (Table A.2). 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 A.2 are Satterthwaite approximations.

3 Results

3.1 Structure of the *O. bicornis ovary*

Nomenclature follows Büning (1994). Briefly, the vagina opens up into the median oviduct where a spermatheca is present and a male mating plug may be present shortly after mating (Fig. 1A; Seidelmann, 1995). The median oviduct progresses into two lateral oviducts with epithelial plugs, which separate the lateral oviduct from the three ovarioles (Fig. 1A). Ovariole number is stereotyped in this species and three ovarioles are consistently found per ovary (Fig. 1A,B; as in other *Osmia*; Maeta and Kurihara, 1971). Degrading follicle cells form a globular yellow body known as a corpus luteum (Fig. 1B inset; Büning, 1994) when they are shed from the mature egg (Fig. 1C), as it leaves the lateral oviduct. Corpora lutea accumulate in-between the ovariole and the intima, and in *Drosophila melanogaster* these produce ecdysone that help maintain high rates of oogenesis (Deady *et al.*., 2015).
Like *A. mellifera,* the *O. bicornis* ovary is of the polytrophic meroistic type where the developing oocyte is connected to sister cells known as nurse cells or trophocytes (Fig. 1D,E). The individual ovarioles can 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. Oocytes within the vitellarium are vitellogenic (yolk is being deposited into the oocytes; Fig. 1D,E).

3.2 Comparison of ovariole structure in *O. bicornis* and queen-less *A. mellifera* workers

The *O. bicornis* terminal filament (containing putative germline stem cells in *A. mellifera*; Tanaka and Hartfelder, 2004) lacks the coin-shaped cells present in *A. mellifera* and other

insects (O. bicornis: Fig. 2A,B; A. mellifera: Fig. 2C,D; Büning, 1994). It only possesses cell nuclei resembling those of the interspersed clusters of putative germline stem cells (see anterior of terminal filament in Fig. 2C; Tanaka and Hartfelder, 2004) which are separated by cortical actin in *O. bicornis* (e.g. arrowhead in Fig. 2B). In *O. bicornis* the terminal filament transitions abruptly into the germarium by a transverse septum (arrows Fig. 2A,B). This in contrast to the terminal filament of *A. mellifera* (Fig. 2C), where the coin-shaped cells of the terminal filament - which are generally arranged in a single layer stack of cells - gradually give rises to cells with more rounded morphology and the tissue thickens to several cell layers thick in the germarium (Fig 2C).

Within the germarium the cystocyte clusters are formed. These cystocyte clusters contain the presumptive oocyte and a set of sister cells which are destined to become the nurse cells (trophocytes). In *A. mellifera* the presumptive oocyte and nurse cells are connected by a polyfusome (white arrows Fig. 2C,D). Cells within the cystocyte cluster undergo successive rounds of cell-division followed by incomplete cytokinesis, and the cystocyte cluster migrates posteriorly down the germarium during this process. The fusome connects the cells of the cystocyte cluster acting as an intracellular bridge. Following specification of the oocyte from the cystocyte cluster, the fusome will break down giving rise to 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 Fig. 2D). In *D. melanogaster*, the fusome is asymmetrically divided during cell division, contributing to oocyte specification and microtubule polarisation (Greenbaum *et al.*., 2011). In *O. bicornis* the polyfusome is either lacking, or too transitive to be observed. The germarium contains a cystocyte cluster immediately following the transverse septum of the terminal filament (arrow in Fig. 2A, and circles in Fig. 2E). This cluster will often already possess ring canals (asterisks in Fig. 2B, circle in Fig. 2F). Overall, the germarium is much shorter than in *A. mellifera*, and there are generally only a few cystocyte clusters visible in the germarium before oocytes are specified and readily discernible (Fig. 1E inset, and Fig. 2E). Once the oocyte is formed, rod-like actin elements can be detected in the ooplasm around the nuclear envelope of the oocyte nucleus (arrows in Fig. 2F).

Following the germarium, the vitellarium starts at the first constriction of the ovariole as the oocyte begins to bud out from the nurse cells and becomes surrounded by a distinct layer of follicle cells (Fig. 1D, 1E), and resembles that of *A. mellifera*. The ovariole can thus be viewed

as a conveyor-belt with germline stem cells giving rise to cystocytes which move from the terminal filament, into the germarium where nurse cells and oocytes are specified, moving further into the vitellarium. The similarities and differences between oogenesis in *A. mellifera* and *O. bicornis* are summarised in Table 1.

3.3 Oogenesis in *O. bicornis*

Unlike honeybee queens which eclose with oocytes arrested in previtellogenic development (Tanaka and Hartfelder, 2004), *O. bicornis* eclose with both pre- and post-vitellogenic oocytes and the first fully mature oocytes (Fig. 1C) are detected 96 hours after eclosion (Fig. 3). Corpora lutea (yellow bodies), which consist of post-ovulatory follicle cells, start accumulating soon after (Fig. 3).

Examining the number of oocytes, mature oocytes and corpora lutea 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* ($\chi^2_{1,5} = 9.414$, $p = 0.009$; Fig. 3). This also translated into a decrease in both length of the vitellarium and total ovariole length over time (Fig. A.5). However, the vitellarium disproportionately determines total length (Fig. A.2) and drives the effects in total ovariole length.

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 Fig. A.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*, and the ambiguity surrounding the existence of a germline stem cell niche in the Hymenoptera in general (Büning, 1994); allow 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 cells over time (terminal filament: $\chi^2_{1,4} = 0.004$, $p = 0.949$; and early germarium: $\chi^2_{1,4} = 1.423$, $p = 0.233$; Fig. A.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.

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

To address our hypothesis that reproductive constraint evolved from ancestral control of reproduction in response to mating status we examined: whether mating affected the rate of oogenesis (Fig. 4), in the solitary bee *O. bicornis*. We found no effect of mating status on the rate of reproduction over time (interaction: $F_{1,22} = 1.052$, $p = 0.316$; Fig. 4A), nor a difference with regard to mating separately ($F_{1,20} = 0.555$, $p = 0.465$). However, a significant effect of time was found ($F_{1,22} = 26.36$, $p < 0.001$), with the rate of oogenesis increasing over time in both treatments (Fig. 4A). This suggests that oogenesis will initiate and accelerate regardless of mating status, once oocyte stores generated prior to eclosion start to deplete. No structural differences were found between ovarioles of hibernating, mated, and unmated females (Fig. 4B-D, for full overview see Fig. B.2). 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 with any of the measurements taken (Table A.2). Suggesting that mating status has no effect on oogenesis in this solitary bee and does not cause arrest of oogenesis as has been seen in some social species (Tanaka *et al.*., 2006; de Souza *et al.*., 2007).

4 Discussion

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 activate oogenesis and other parts 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 (Tanaka *et al.*., 2006). Virgin queens of the eusocial *M. 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

and 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. We therefore hypothesised that mating status may have had an ancestral role in reproductive control, and subsequently been co-opted into reproductive constraint in eusocial insects like the honeybee.

We could not detect any response in the ovary with regard to mating in solitary *O. bicornis* females. Even after enough time had transpired for the initial oocyte stores to be depleted, *O. bicornis* females showed no difference in oogenesis with regard to mating status (Fig. 3 and 4). Indicating that mating status does not constrain reproduction in the solitary bee *O. bicornis.* Egg laying was not measured in this study due to constraints in experimental design (this species has been succesfully labreared only once before: Sandrock *et al.*., 2014). Hence virgin females might yet show lower egg laying rates and higher rates of oocyte resorption (as seen in D. rapae; Kant *et al.*., 2013).

We have shown that mating status plays no part in reproductive control in the solitary *O. bicornis*. This is in contrast to *A. mellifera* queens, but consistent with workers which cannot mate because they lack a spermatheca (which is in itself considered a form of reproductive constraint: Khila and Abouheif, 2010). We therefore hypothesise that the lack of dependence on mating for oogenesis resembles the ancestral solitary state in bees. *O. bicornis* emerge from hibernation with primed oocytes as shown here, poised to begin egg laying as swiftly as possible. This is in contrast to some eusocial species, like *A. mellifera*, where egg maturation is dependent on mating status (M. quadrifasciata anthidioides: Martins and Serrão, 2004; de Souza *et al.*., 2007; Tanaka *et al.*., 2009; A. mellifera: Tanaka and Hartfelder, 2004; Tanaka *et al.*., 2006). This difference may, at least in part, reflect a heterochronic shift (a shift in the timing) of ovary development associated with the evolution of eusociality. A heterochronic shift has also been reported for cuticle development in bees; solitary species eclose with fully developed cuticle and are immediately exposed to the environment whereas social species exhibit a delay in cuticle tanning, possibly as a result of adaptation to the protective environment of the nest (Elias-Neto *et al.*., 2014). Additionally, the dependence of oogenesis on mating seen in queens of some eusocial species may have evolved as a consequence of increased colony size and

reduced worker fertility, i.e. where queen-worker conflict has shifted towards brood composition (Bourke, 1999).

It is, however, also possible that the lack of dependence of oogenesis on mating status may be a derived characteristic of *O. bicornis* as a result of relaxation of selection pressures on female *O. bicornis* to delay oogenesis until mating has occurred. Nests tend to contain males at the opening of the nest, which hatch up to two weeks prior to females (Seidelmann, 1995). Males then lie in wait for emerging females or seek them out at feeding areas (Seidelmann, 1995; Ayasse and Dutzler, 1998). This leads to high intrasexual competition, making it unlikely for females to end up without a mate.

It is also important to note that *A. mellifera* last shared a common ancestor with *O. bicornis* approximately 100 mya (Branstetter *et al.*., 2017). Therefore, to further test the hypothesis that the lack of dependence on mating for oogenesis resembles the ancestral solitary state in bees it will be important to examine the effect of mating status on oogenesis in a greater range of eusocial and solitary species that span the phylogenetic gap between *O. bicornis* and *A. mellifera,* including other megachilid and corbiculate bees. Evidence suggests that there may be a single origin of eusociality in the corbiculate bees; at the base of the radiation-giving rise to the Apini, Bombini and Meliponini (Cardinal and Danforth, 2011; Romiguier *et al.*., 2016). Therefore the dependence of oogenesis on mating status that we see within these tribes could reflect common descent rather than a specific adaptation associated with the evolution of eusociality. In this respect, investigating this hypothesis in the Euglossini may be informative, as the latest phylogenomic data indicates that they are a sister group to the Apini, Bombini and Meliponini (Bossert *et al.*., 2019). Although the majority of euglossine species are solitary there are some that are considered primitively eusocial and would further inform whether oogenesis was independent of mating status in the last common ancestor of bees.

In eusocial hymenoptera the reproductive division of labour is maintained by pheromones, including pheromones produced by the queen and her brood. It has been hypothesised that these pheromones may have evolved from sex pheromones (Oi *et al.*., 2015). Although more data spanning the phylogeny of bees would need to be examined to address the origin

of queen pheromones, our data that the *O. bicornis* ovary is unresponsive to mating status together with previous data that *O. bicornis* virgins become unattractive after three days (Seidelmann, 2014) is inconsistent with the hypothesis that queen pheromones evolved from sex pheromones. However, in *O. bicornis* mature oocytes are detected in the ovary three days post eclosion (Fig. 3). This coincides with a shift in the CHC-profile of female *O. bicornis*, including a marked transition towards the longer chained C_{27}-alkane (Seidelmann, 2014; Seidelmann and Rolke, 2019), suggesting a link between oocyte maturation and production of this CHC-component. Intriguingly, this linear alkane is also a component of hymenopteran queen pheromones and inhibits reproduction in workers of the common wasp (*Vespula vulgaris*) and the desert ant (*Cataglyphis iberica*) (Van Oystaeyen *et al.*., 2014). That C_{27}-alkane is associated with the production of mature oocytes in *O. bicornis* is consistent with the hypothesis that queen pheromones could be derived from the by-products of ovary development (Oi *et al.*., 2015).

The RGPH predicts that aspects of the ancestral reproductive cycle of a hypothetical solitary ancestor have been co-opted during the evolution of eusociality into controlling division of labour (Amdam *et al.*., 2006). Taken together, our data indicates that the mechanisms underlying QMP-mediated reproductive division of labour in honeybee adults (Duncan *et al.*., 2016) were unlikely to have been co-opted from ancestral mechanisms associated with mating status – but this does not preclude the involvement or co-option of other aspects of reproductive biology in the evolution of reproductive constraint.

This study has implications for our understanding of the evolution of eusociality but also has wider significance for our understanding of hymenopteran reproduction and physiology. That oogenesis is independent of mating status in *O. bicornis* may indicate that female solitary bees need not necessarily mate to achieve full fecundity. This, together with the relatively narrow window for mating before *O. bicornis* females become unattractive to males, and the limited dispersal from the natal nest raises the possibility of inbreeding and inbreeding avoidance in this species (Conrad *et al.*., 2010). Understanding the reproductive biology and behaviour of this and other solitary bee species is crucial for understanding the species' ecology and population dynamics.

Conclusions

We present the first structural analysis of the *Osmia bicornis* ovary. We found no evidence of mating status impacting on ovary structure, ovary activation nor rate of oogenesis in this solitary bee. We suggest that mating may be unnecessary to attaining reproductive capacity in other solitary Hymenoptera, and that ovary repression and degeneration are likely only present in virgin queens of advanced social species. While access to mating is a component of adult reproductive constraint (absence of spermathecae in workers of many social species), QMP-mediated adult reproductive constraint in the honeybee worker is unlikely to have been derived or co-opted from mating status.

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415 **Table 1: Structural differences in the ovarioles of *A. mellifera and O. bicornis.***

Region	*A. mellifera*	*O. bicornis*
Terminal filament	Cells funnel out of the terminal filament into the germarium (Fig. 2C). Region consists mainly of coin shaped cells interspersed with actin (Fig. 2C; Fig. 2C; Büning, 1994; Tanaka and Hartfelder, 2004). Putative germline stem cells are present at the anterior of the terminal filament (Fig. 2C; and Fig. 2C; and Tanaka and Hartfelder, 2004).	A transverse septum establishes an abrupt transition from terminal filament to germarium (Fig. 2A and 2B). No coin shaped cells are present, but an unknown cell type is interspersed with cortical actin close to the germarium (Fig. 2A and 2B). These cells could possibly be germline stem cells (as cystocyte clusters appear immediately beyond the transverse septum; Fig. 2A and 2B).
Germarium	Long germarium: polyfusomes are maintained for some time along the germarium, with clusters of unspecified nurse cells and oocytes (Fig. 2C and 2D). Polyfusomes differentiate into their individual ring canals (Fig. 2D). Cells cluster into a comet-like conformation (not shown; see not shown; see Tanaka and Hartfelder, 2004). Oocyte and nurse cells are specified shortly after.	Short germarium: polyfusomes were undetected in any of the samples (i.e. it is either very transient or absent, Fig. 2E and 2F). Cystocyte clusters with ring canals appear immediately in the germarium and discernible oocytes are present with only a few undifferentiated cystocyte clusters in the germarium (Fig. 2E and 2F). Once the oocyte is formed, rod-like actin elements can be detected in the ooplasm around the nuclear envelope of the oocyte nucleus (arrows in Fig. 2F).
Vitellarium	No structural differences observed.	

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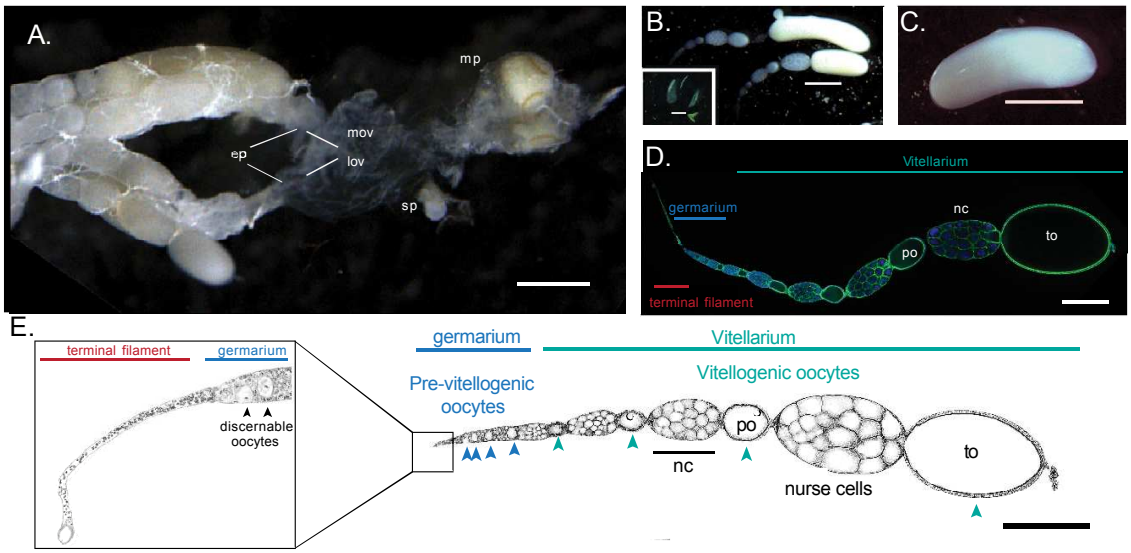

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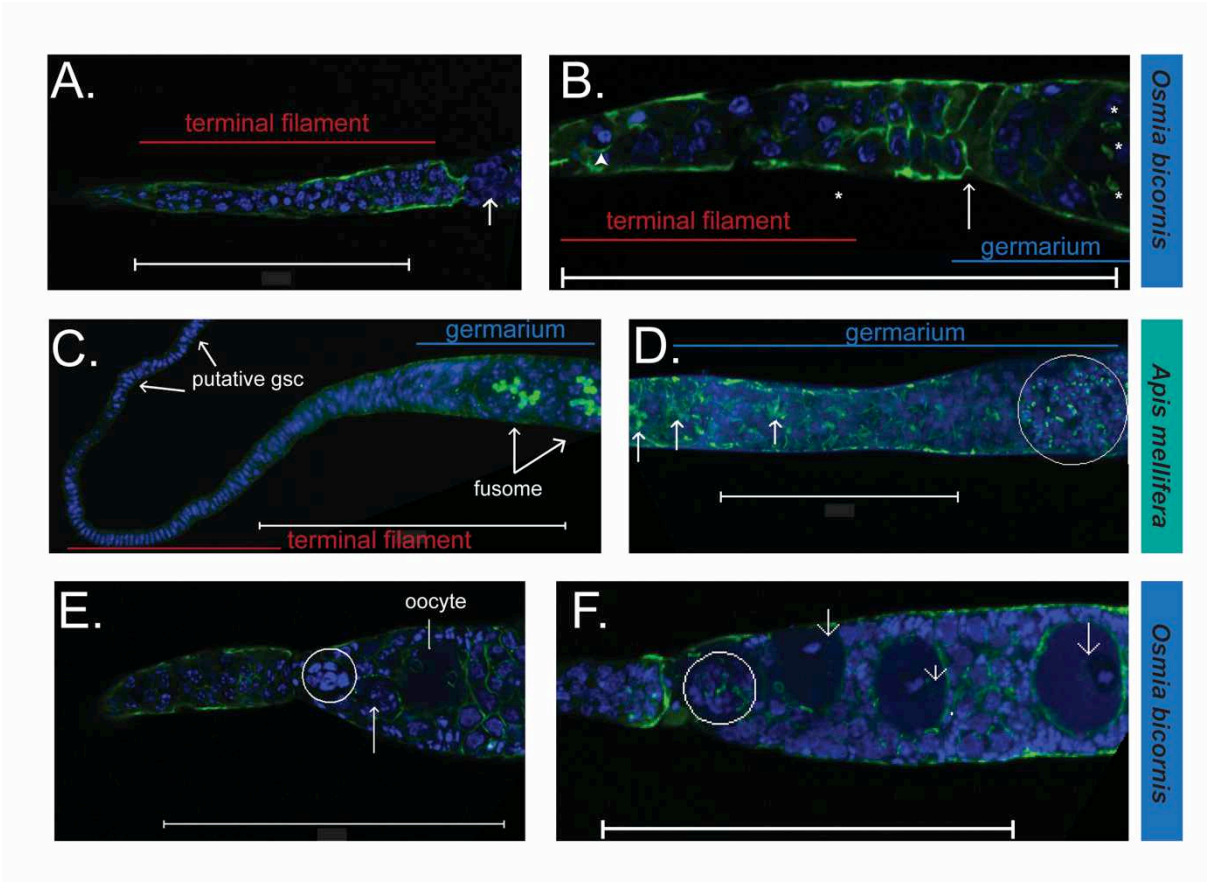
548 **Fig. 1 Overview of the *O. bicornis* ovary.** A) Dissected ovary with accessory
549 structures (ep = epithelial plug, lov = lateral oviduct, mov = median oviduct, mp
550 = mating plug, and sp = spermatheca; with scalebar = 500 µm). B) Two
551 dissected ovarioles with maturing terminal oocytes (scalebar = 750 µm), insert
552 shows corpora lutea (yellow bodies) associated with maturing oocytes
553 (scalebar = 500 µm). C) A fully mature oocyte (scalebar = 1.5 mm). D)
554 Maximum intensity projection of a DAPI (blue) and Phalloidin (green) stained
555 ovariole (scalebar = 500 µm) with key features highlighted (po = penultimate
556 oocyte, nc=nurse cells /trophocytes, to = terminal oocyte). E) Schematic
557 overview of the *O. bicornis* ovariole demonstrating key features and cell types
558 (fc = follicle cell, nc = nurse cell, po = penultimate oocyte, to = terminal oocyte,
559 scalebar = 500 µm)



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562 **Fig. 2 Ultrastructural differences between the *O. bicornis* and *A. mellifera***
563 **ovariole.**(A-F) Maximum intensity projections with DAPI (blue) and phalloidin
564 (green), and scale bars = 200 µm. A & B) *O. bicornis* terminal filament and
565 germarium, with white arrow indicating cystocyte cluster exiting the terminal
566 filament across the transverse septum. Asterisks indicate ring canals. C & D) *A.*
567 *mellifera* terminal filament and germarium respectively. The terminal filament
568 shows the characteristic stack of coin organisation, funnelling out into the
569 germarium. White arrows show polyfusome structures connecting cystocyte
570 clusters progressing along the germarium and dissipating into individual ring
571 canals (white circle) connecting nurse cells and oocyte. E) Optical section
572 showing *O. bicornis* oocyte differentiation, with cystocyte cluster (white circle),
573 losing its dense clustering (white arrow) and separating into nurse cells with
574 oocyte (white line; and ring canals visible). F) Further detail of *O. bicornis*
575 germarium, containing cystocyte cluster (white circle) and rod like actin around
576 the nuclear envelope (white arrows).

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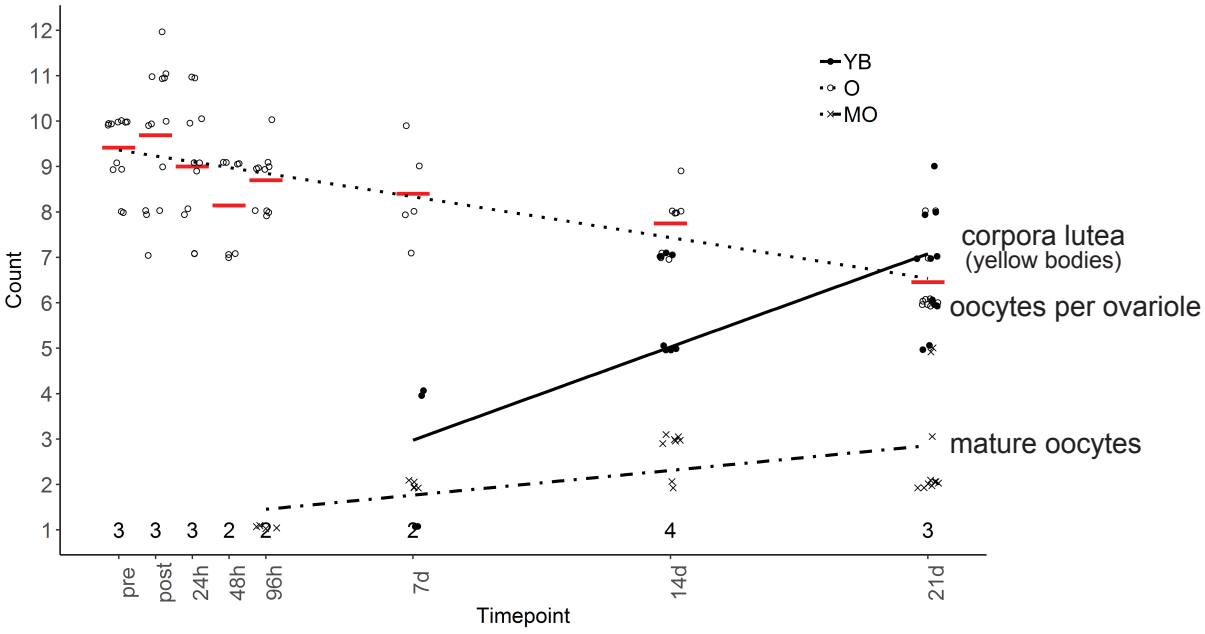

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579 **Fig. 3 Dynamics of oogenesis in *O. bicornis*.** Counts of oocytes per ovariole (O),
580 mature oocytes per individual (MO) and the accumulated corpora lutea (yellow
581 bodies) per individual (YB) pre- and post-eclosion and over the first 21 days of
582 life. The number of oocytes in ovarioles decreased over time, while
583 degenerating oocytes (yellow bodies) accumulated in the ovary. Points are
584 jittered, slopes represent linear regressions, red bars represent means, and
585 numbers along x-axis represent number of individuals.

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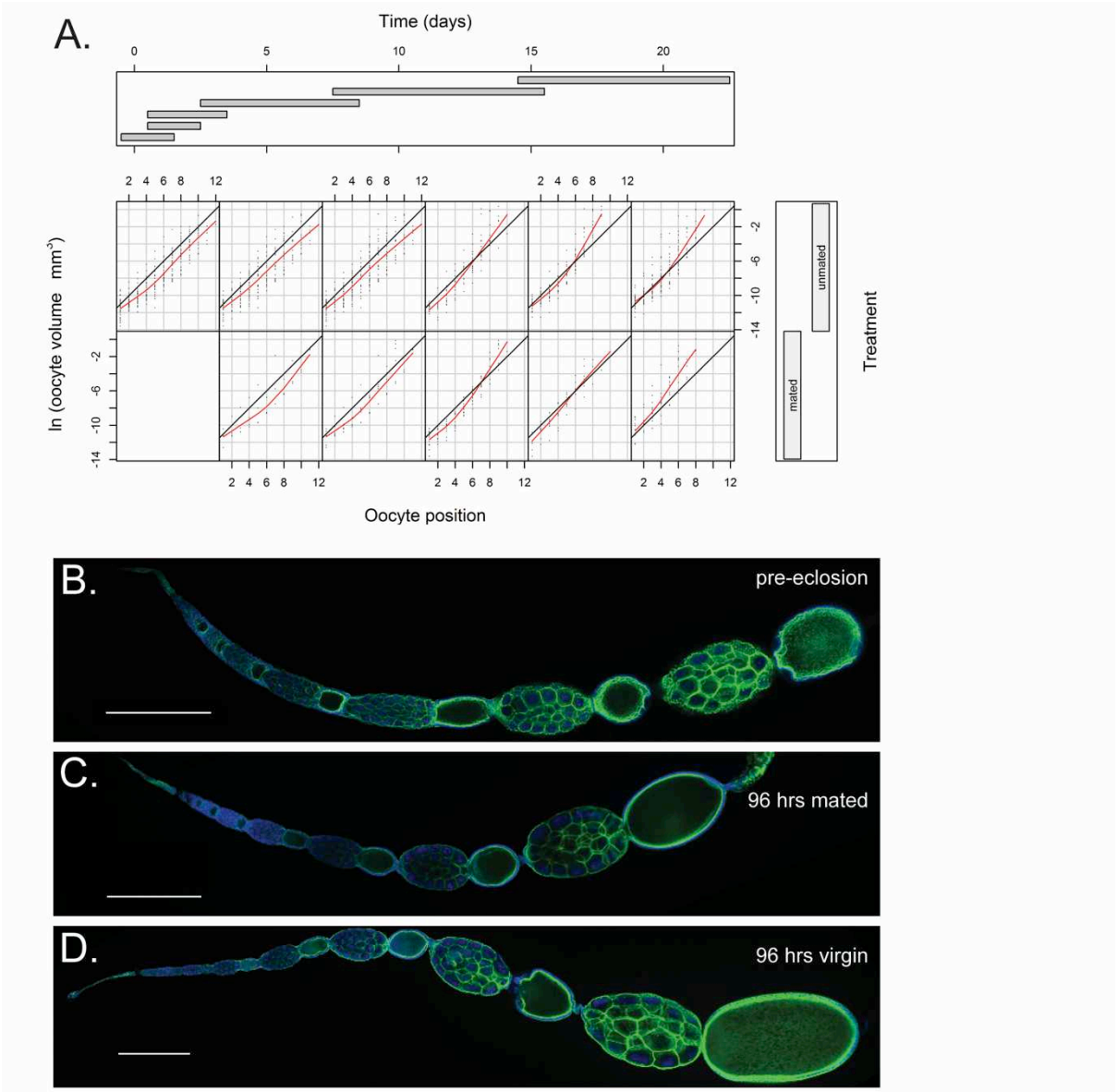
A.
Time (days)
0
5
10
15
20
ln (oocyte volume mm^3)
Oocyte position
Treatment
unmated
mated
B.
pre-eclosion
C.
96 hrs mated
D.
96 hrs virgin

587

Fig. 4 Mating status has no significant effect on ovary activation in *O. bicornis*. A) The rate of oogenesis over time (red slopes left to right) did not differ significantly across mating status (top and bottom rows). Points may overlap and mask one another. Red lines represent LOWESS smoothing, black lines are constant (interc. = -14 and coeff. = 1) to facilitate comparison of red lines. Horizontal bars in top panel represent overlap of time points data used for each plot. B-D) Maximum intensity projections of DAPI (blue) and Phalloidin (green) stained ovarioles of: a pharate, and a mated and virgin female after 96 hours respectively. *O. bicornis* females eclose with primed ovaries, which activate vitellogenesis regardless of mating status, and show no structural differences compared to later stage ovarioles other than the swelling of nutritive chambers and oocytes. All scale bars = 500 μm. (For all time points across treatments, see Fig. C.2)