Regulation of oogenesis in the queen honey bee (*Apis mellifera*)

Sarah E Aamidor[1], Carlos Júnior Cardoso [2], Januar Harianto[3], Cameron J Nowell[4], Louise Cole[5], Benjamin P Oldroyd[1], Isobel Ronai[1]

[1]Behaviour and Genetics of Social Insects Laboratory, Ecology and Evolution, School of Life and Environmental Science, Macleay Building A12, University of Sydney, NSW, 2006, Australia; [2]Departamento de Biologia Celulare Bioagentes Patogênicos, Faculdade de Medicina de Ribeirao Preto,Universidade de Sao Paulo, Ribeirao Preto, Brazil; [3]School of Life and Environmental Science, Macleay Building A12, University of Sydney, NSW, 2006, Australia; [4]Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville 3052, Victoria, Australia; [5]Microbial Imaging Facility, I3 Institute, Faculty of Science, The University of Technology Sydney

Correspondance (email: sarah.aamidor@sydney.edu.au)

Key words: ovary activation; ovariole; nurse cell chamber; honeybee; eusociality; phenotypic plasticity.

Summary statement: Honey bee queens regulate oogenesis using a different combination of 'checkpoints' to workers, but both castes use the same molecular pathways.

Abstract

In the honey bee (*Apis mellifera*), queen and worker castes originate from identical genetic templates but develop into different phenotypes. Queens lay up to 2,000 eggs daily whereas workers are sterile in the queen's presence. Periodically queens stop laying; during swarming, when resources are scarce in winter and when they are confined to a cage by beekeepers. We used confocal microscopy and gene expression assays to investigate the control of oogenesis in honey bee queen ovaries. We show that queens use different combination of 'checkpoints' to regulate oogenesis compared to honey bee workers and other insect species. However, both queen and worker castes use the same programmed cell death pathways to terminate oocyte development at their caste-specific checkpoints. Our results also suggest that the termination of oogenesis in queens is driven by nutritional stress. Thus, queens may regulate oogenesis via the same regulatory pathways that were utilised by ancestral solitary species but have adjusted physiological checkpoints to suit their highly-derived life history.

Introduction

In eusocial insects, females develop into either a highly reproductive queen or a non-reproductive worker (Oster and Wilson 1978). In the honey bee, *Apis mellifera*, queens and workers arise from the same genetic template (genotype), but have radically different morphological and behavioural phenotypes (Snodgrass 1956; Dade 1977; Winston 1987). Queens lack worker-specific characters such as a barbed sting, pollen baskets (corbiculae) and a honey crop, whereas workers generally lack the specialised reproductive organs of the queen such as the sperm-storing spermatheca. Notably, while both queens and workers have ovaries, they differ enormously in the number of ovarioles (the tubes in which oocytes develop). Queens have 150-200 ovarioles per ovary, whereas workers have approximately four (Snodgrass 1956; Rozen 1986; Ronai, Allsopp et al. 2017). The queen's ovaries occupy the majority of her abdominal cavity and can produce up to 2,000 eggs per day (Page and Erickson 1988). Honey bee queens therefore have one of the highest levels of fecundity of any insect (Hodin 2009).

In an activated honey bee ovary, each ovariole contains multiple oocytes (immature eggs) at different stages of development. Oogenesis begins at the tip of the ovariole when a germline cell divides repeatedly to produce what will become an oocyte and a cluster of accompanying nurse cells. The nurse cells provide nutrition and other elements required by the developing oocyte as it travels through the ovariole, in a conveyor-belt fashion, towards the median oviduct (Snodgrass 1956). As the oocyte approaches the oviduct the nurse cells transfer their contents to the oocyte in a process known as nurse cell 'dumping' (Spradling 1993; Cavaliere, Taddei et al. 1998), and die as the oocyte completes its development. Finally, the mature oocyte passes through the median oviduct and is laid.

Worker honey bees, on the other hand, are facultatively sterile (Visscher 1989; Ratnieks 1993). Young workers can switch between 'deactivated' ovaries when the queen is present to 'activated' when the queen is absent (Malka, Shnieor et al. 2007). The mechanisms underlying the reproductive plasticity of workers has been widely investigated (e.g Hoover,

Keeling et al. 2003; Malka, Shnieor et al. 2007; Woyciechowski and Kuszewska 2012; Traynor, Le Conte et al. 2014; Ronai, Oldroyd et al. 2016; Ronai, Oldroyd et al. 2016). Young workers continuously produce new oocytes, but in the presence of a queen, the developing oocytes are aborted during mid-oogenesis via programmed cell death (PCD) (Tanaka and Hartfelder 2004; Tanaka, Schmidt-Capella et al. 2006; Ronai, Barton et al. 2015). The reproductive plasticity of young workers allows them to rapidly activate their ovaries, by switching off the PCD mechanisms, thereby allowing pre-existing immature oocytes to develop. This mechanism likely evolved because there is a fitness premium for the first workers to successfully lay eggs in a queen-less colony (Page and Robinson 1994). A queen signals her presence to the workers via pheromones (Butler 1957; Butler and Fairey 1963; Hoover, Keeling et al. 2003), the most significant of which is queen mandibular pheromone (Butler, Callow et al. 1961). In a colony with a queen, the presence of queen mandibular pheromone and brood pheromones in a worker's environment activates the gene network(s) that initiate PCD in early oocytes of workers (Le Conte, Mohammedi et al. 2001; Ronai, Oldroyd et al. 2016; Hartfelder, Tiberio et al. 2018) .In contrast to workers, the mechanisms underlying the reproductive plasticity of mated queens is unlikely to be regulated by queen mandibular pheromone and brood pheromones, for if they were influenced by these cues, they would be permanently sterile. Nonetheless, queens, like workers, are able to curtail oogenesis in response to environmental cues.

Mated queens exhibit reproductive plasticity under three specific circumstances. First, in temperate zones, queens drastically reduce egg laying in winter (Seeley 1985), becoming reproductively active again in late winter or early spring (Avitabile 1978; Shehata, Townsend et al. 1981). This annual plasticity in oogenesis is likely triggered by decreased availability of fresh pollen combined with declining day length (Mattila and Otis 2007). Second, reproductive swarming; when the queen and approximately half of the colonies workers depart the nest to establish a new colony (Simpson 1959). In preparation for swarming, the workers reduce the frequency at which they feed the queen (Allen 1956; Pierce, Lewis et al. 2007). Probably as a consequence of this reduced feeding, the queen's egg production declines significantly (Allen 1956; Grozinger, Richards et al. 2014). As a result the queen's weight is significantly reduced, which likely allows her to fly more easily when she leaves with the swarm (Seeley and Fell 1981). After the swarm leaves the natal nest, the bees hang in a cluster nearby. While in the cluster the queen's egg production almost ceases (Pierce, Lewis et al. 2007). Only once the swarm moves to its new home and new brood cells are built, does the queen resume egg laying (Ambrose 1976; Winston 1987). Thirdly, commercial queens used in agriculture are often forced to undergo periods when they are prevented from laying due to beekeeping practises (Rhodes, Somerville et al. 2004). Queens produced for sale are sometimes 'banked' within individual cages in a colony for several weeks (Laidlaw and Page 1997). Banked queens are cared for by the colony's workers but lack a comb in which to lay eggs. Queens are often shipped via mail in individual cages with an escort of 5-8 workers that feed and groom the queen, usually for a period of up to 10 days (Laidlaw and Page 1997). Caged queens do not lay eggs, have restricted food, and their abdomens become much reduced in size (Laidlaw and Page 1997). Nonetheless, once the queen is introduced to her new colony she recommences egg laying in a few days (Rhodes, Somerville et al. 2004).

140 In this study, we investigate the mechanisms that enable honey bee queens to switch
141 between activated and deactivated ovaries. We studied oogenesis in queens by examining
142 the morphological changes in their ovaries when they are prevented from laying in two
143 different social environments; (i) within the colony and (ii) when removed from the colony
144 with a few escorting workers. Since queens and workers originate from the same genetic
145 template, we further investigated whether the reproductive plasticity of queens is regulated
146 by differential expression of the same genes that are associated with the reproductive
147 plasticity of workers, and whether such plasticity is driven by nutritional stress. Specifically,
148 we quantified the expression of genes associated with: PCD (*Anarchy, Buffy*, *Apaf-related*
149 *killer* gene [*Ark*], *long non-coding ovary-1* [*Lncov1*], *Tudor-SN*, and *Draper*); nutrition (target
150 of rapamycin [*Tor*], *Forkhead box O* [*FoxO*], elongation growth factor receptor [*Egfr*]);
151 Juvenile Hormone production (*Krüppel-homolog 1* [*Kr-h1*]), and vitellogenesis (*Vitellogenin*
152 [Vg] and *Vitellogenin Receptor* [*VgR*]). We hypothesise that the genetic pathways controlling
153 oogenesis in queens would be similar to those seen in workers (i.e. associated with PCD).
154 We further hypothesised that in contrast to workers, where queen mandibular pheromone
155 and brood pheromones are the cue for workers to stop oogenesis, in queens it is nutritional
156 cues that regulate oogenesis. More broadly, we note that in solitary Hymenopterans,
157 nutritional cues are intimately related to oogenesis (Toth, Varala et al. 2007). Thus
158 nutritional cues may also have a role in regulating oogenesis in the queens of advanced
159 eusocial insects (Hunt and Amdam 2005; Dolezal, Flores et al. 2013).
160
161 **Methods**
162
163 ***Biological material***
164
165 Queens (*n* = 25) were removed from their colonies during summer (December 2018). All
166 individuals were mature (> 6 weeks old), mated, laying queens of standard Australian
167 commercial stock mostly derived from *A. m. ligustica.*
168
169 Queens were randomly assigned to three treatment groups. First, fifteen queens were
170 placed in individual plastic mailing-introduction cages (4 cm x 2 cm, JZ-BZ Queen Cages)
171 used by commercial beekeepers, with five to eight supporting workers and kept in a dark at
172 room temperature. The workers had *ad libitum* access to water and queen candy (a mixture
173 of caster sugar and honey) to feed the queens. Collectively we refer to these treatments as
174 caged 'ex-colony' (EC). Five queens from this EC group were collected on days 4, 7 and 10
175 after caging (which is within standard beekeeping practice). Second, five queens with no
176 escorting workers were suspended in their cages between brood frames above a queen
177 excluder in a strong colony. The queen excluder prevented access by the resident queen to
178 the caged queens. Queens were fed by host workers through the mesh of their cages. These
179 queens were collected 10 days after caging. We refer to these queens as caged 'in colony'
180 (IC). Third, the remaining five queens were collected from their colonies at day 0 as the
181 control group. During the course of these experiments two queens died, one in the 10-day
182 EC group and one in the IC group. Hence there is no data from these two queens.
183
184 All collected queens were immediately frozen on dry ice and stored at -80 °C. Queens were
185 dissected in RNase-free phosphate-buffered saline (PBS) and their paired ovaries were
186 delicately removed. Both ovaries were imaged under a dissecting light microscope (Olympus

187 SZ40 Stereo Zoom) at 8X magnification in order to measure the ovary 2D area. One of the
188 paired ovaries was randomly chosen and halved. One half was set aside for confocal
189 microscopy and the other for gene expression analysis. The remaining ovary was stored but
190 not used for this study. All dissected ovaries were stored at -80 °C until required.
191
192 ***Gene expression in the ovaries***
193
194 Total RNA was extracted from each ovary sample (*n* = 23) using TRIzol (Invitrogen). The
195 amount of RNA present per sample was estimated using a Qubit 2.0 Fluorometer (Invitrogen)
196 and samples were diluted to 200 ng/µL with RNase-free water. DNA was removed using a
197 Turbo DNase Kit (Thermo Fisher Scientific). For each sample, cDNA was synthesised from
198 320 ng of RNA (64 ng/µL) using a SuperScript^{TM} III Reverse Transcriptase Kit (Invitrogen) with
199 Oligo (dT) universal primers with appropriate positive and negative controls. All cDNA
200 samples were diluted to 5 ng/µL with RNase-free water.
201
202 We quantified the expression of 11 genes of interest (*Anarchy*, *Buffy*, *Draper*, *Egfr*, *Lncov1*,
203 *Tudor*, *Tor*, *FoxO*, *Kr-h1, Vg and VgR*) in the queen ovaries using RT-qPCR. Each reaction was
204 run in triplicate with a final volume of 5 µL [1 µL cDNA (5 ng/µL), 2.5 µL SYBR Green (Applied
205 Biosystems, Life Technologies) and 1.25 pmol of each primer] using a CFX384 Real-Time
206 System (Bio-Rad). The sequences of the primers and reaction conditions for the 11 genes of
207 interest and four candidate reference genes (*pRS5*, *RP49*, *Ef1a* and *Actin*) are provided in
208 Supplementary Table S1. The reaction efficiency for each primer set was obtained by a
209 standard curve (serial dilution) fitted for each of the genes (Supplementary Table S1). Of the
210 four candidate reference genes, *Ef1a* and *Rf49* were found to have the most stable
211 expression using the software *BestKeeper* (Pfaffl, Tichopad et al. 2004). These two genes
212 have been previously recommended as appropriate reference genes for gene expression
213 studies in honey bees (Lourenço, Mackert et al. 2008; Reim, Thamm et al. 2013). Relative
214 gene expression was calculated according to the efficiency correction formula considering
215 the efficiency of each gene and normalised with the two reference genes (Brito, McHale et
216 al. 2010).
217
218 ***Fixing, staining and confocal imaging of the ovaries***
219
220 Ovaries were prepared for staining according to Dallacqua and Bitondi (2014). Briefly, each
221 ovary was fixed and permeabilised in heptane fixative (0.83% heptane, 0.067% HEM buffer
222 (pH 6.9, 0.1M HEPES, 2mM MgSO4 and 1mM EGTA), 0.01% paraformaldehyde and 0.0167%
223 DMSO) for 30 minutes on a shaker, followed by three washes in PBS. The ovaries were then
224 dehydrated with increasing concentrations of ethanol (25%, 50%, 75% and 100%) and
225 stored over night at -20 °C. For staining, the ovaries were rehydrated with decreasing
226 concentrations of ethanol in water (75%, 50% and 25%) and then washed three times in
227 PTw (PBS and 0.1% Tween20). Ovaries were refixed in Triton fixative (PBS, 0.1% Triton X-100,
228 4% paraformaldehyde and 10% DMSO) for 20 min and washed three times in PTw. In order
229 to further permeabilise the tissue, samples were incubated for 10 minutes in 20 µg/mL
230 proteinase K followed by two washes in 1% glycine and three washes with PTw. The ovaries
231 were refixed in Triton fixative for 20 minutes and washed six times in PTw to remove all
232 traces of fixative. Ovaries were then stained for actin with ActinGreen 488 (Invitrogen) for
233 30 minutes, washed three times with PTw and counterstained for nucleic acid with DAPI

234 (0.004% v/v) (Sigma) for five minutes followed by three washes in PTw. The ovaries were
235 then split into clusters of one to four ovarioles and mounted on slides in Fluoroshield (Sigma)
236 with glass cover slips. (During fixation one ovary from the control group was lost and
237 therefore not imaged). Slides were imaged using a ZEISS LSM 800 confocal microscope using
238 450 and 488nm excitation wavelengths to image the nucleic acid and actin respectively.
239 Single focal plane and z-stacks were taken using either a 10x or 20x objective. Z-stack
240 images were processed to create a two-dimensional maximum intensity projection (MIP).
241 All images were acquired, stitched and processed using ZEN blue software.
242
243 ***Image analysis***
244
245 Images were analysed using a custom macro in the Fiji distribution of ImageJ (Schindelin *et.*
246 *al.,* 2012). Stereo images were used to calculate total area of both ovaries (one image for
247 each individual ovary). Confocal images were enhanced using the ImageJ gamma function at
248 0.6 to increase contrast. For each stitched image, the oocytes and Nurse Cell Chambers
249 (NCC) in each ovariole were traced by hand and the resulting ROIs saved for future
250 validation if required.
251
252 Confocal image analysis is described in detail in Supplementary Materials and Methods S1
253 and available for download from https://doi.org/10.26180/13557764. Briefly, oocytes and
254 NCC in each string were outlined using the freehand region of interest (ROI) tool on
255 maximum Z projections of each image set from the most developed intact oocyte (oviduct
256 end) until the oocytes and NCC could no longer be distinguished in the late germarium
257 (Büning 1994). Each ROI was then used to calculate the equivalent spherical radius by using
258 the maximum and minimum Feret's diameter to first calculate the oblate volume (due to
259 the 'objects' - oocyte or NCC - being non-spherical) of the object and then to calculate the
260 equivalent spherical radius. The data was logged and then further analysed using Excel and
261 GraphPad Prism. In order to visualise the changes along the entire ovariole a locally
262 weighted smoothing (LOESS) function was added on the scatterplot output using the
263 `loess()` function from the base `stats` package in R (version 4.0.3).
264
265 ***Statistical analysis***
266

267 To determine the effects of treatment group on the morphological features and gene
268 expression of the ovariole, we modelled the data using generalised linear models (GLM)
269 with a normal link function. The dependent variable was unit size or position in the ovariole,
270 or the fold change in gene expression. The independent (fixed) factor was the treatment
271 group (control (Day 0), EC day 4, EC day 7, EC day 10 and IC day 10).

272 All covariates were ordinal, thus model significance was tested with an analysis of variance
273 (ANOVA) using the Wald Chi-square test statistic and Type 3 error. Where statistical results
274 indicated significant differences at $p < 0.05$, estimated marginal means (EMM) we used to
275 develop linear estimates of pairwise comparisons and then tested using Tukey's honestly
276 significantly different (HSD) test to identify significant differences at $p < 0.05$.

Analyses were performed in R (version 4.0.3). GLMs were performed using the `Anova()` function from the `cars` package (version 3.0-10) while post-hoc analyses were performed using the `emmeans()` function from the `emmeans` package (version 1.5.3).

Results

Oogenesis in a laying queen

Below we provide a description of normal oogenesis, obtained from our high-resolution confocal images of the ovaries of queens freshly taken from their colonies (controls; Day 0). Descriptions are also based on the existing honey bee ovary morphology literature (Snodgrass 1956; Gutzeit, Zissler et al. 1993; Büning 1994).

At the apical tip of each ovariole is the terminal filament (Figure 1 Bi) and germline stem cells (Figure 1 B). The early germarium stage is characterised by a single germ cell dividing repeatedly to produce a cluster of cells (also known as cystocyte rosettes) around actin polyfusomes (Figure 1 Bi). Subsequently, one of the cells differentiates into the oocyte, while the remaining cells of the cluster become the nurse cells that will nourish the developing oocyte. Actin fusomes also rearrange into ring canals (Figure 1 Bii), connecting the oocyte to its accompanying nurse cells.

During the mid-germarium stage, oocytes slowly increase in size and are distinguishable from their accompanying nurse cells (Figure 1 A). By the late germarium stage, the oocyte is located towards the oviduct end relative to its accompanying nurse cells (Figure 1 A).

The vitellarium stage is defined by the oocyte becoming encased in a layer of somatic follicle cells (Figure 1 Ci) and the accompanying nurse cells forming a Nurse Cell Chamber (NCC) (Figure 1 A and C). By both qualitative and quantitative analysis of the confocal images, we observed the oocyte and the NCC growing at different rates during the vitellarium stage (Figure 2). Initially the NCC is larger than the oocyte (Fig. 1C) until a point that we define as the 'tipping point' when the developing oocyte first exceeds the size of its accompanying NCC (Figure 1 A and 2). After the tipping point the late vitellarium stage begins and the oocyte grows rapidly (Figure 1A and 2). And the nurse cells undergo developmental programmed cell death (King 1970), ultimately transferring their remaining cytoplasm into the oocyte in a process termed nurse cell dumping (Figure 1 A and D, Figure 2). This nurse cell dumping results in the complete collapse of the NCC ('dnc' in Figure 1A; see also Figure 1D). At the final stage of oogenesis, the oocyte is fully formed and situated at the oviduct end of the ovariole (Figure 1A and 1D). The pedicel (also known as the basal stalk), a constriction at the entry to the oviduct which prevents the oocyte from moving into the oviduct until it is ready to be laid can also be seen clearly by confocal microscopy (Figure 1A and 1D).

Oogenesis in queens prevented from oviposition

Stereomicroscopy showed that caging, both ex-colony (EC) and in-colony (IC), resulted in abnormal ovaries. Overall, the longer a queen was caged EC, the smaller the ovaries became

323 (Figure 3 A). We found that mean ovary area differed significantly among treatments (Wald
324 $\chi^2 = 96.25$, *d.f.* = 4, $P < 0.0001$; Figure 3 B). Queens caged EC had significantly smaller ovary
325 area compared to the controls. Although queens caged IC for 10 days did not show such an
326 extreme reduction in ovary area, they too were smaller than the controls (Figure 3 B).
327
328 Overall, confocal microscopy showed that there was no significant effect of caging
329 treatments on the total number of oocytes (Wald $\chi^2 = 6.04$, *d.f.* = 4, $P = 0.196$; Figure S1).
330 However, the size of the most mature (i.e. maximum-sized) oocyte was significantly reduced
331 by the caging treatments (Wald $\chi^2 = 18.79$, *d.f.* = 4, $P < 0.001$; Figure 2 and S2 A). The size of
332 the most mature oocyte was similar in queens that were caged EC for 10 days and the
333 control queens (Figure S2 A). In contrast, the maximum size of the NCC was not significantly
334 affected by the caging treatments (Wald $\chi^2 = 8.97$, *d.f.* = 4, $P = 0.062$; Figure 2 and S2 B). In
335 addition, the minimum size of oocytes and NCC did not significantly change as a result of
336 caging treatments (Wald $\chi^2 = 5.43$, *d.f.* = 4, p = 0.246; Wald $\chi^2 = 1.48$, *d.f.* = 4, p = 0.830;
337 Figures S2 C and D respectively).
338
339 Although the number of identifiable oocytes per ovariole was unaffected by treatment
340 (Figure S1), we nonetheless observed changes in the developmental gradient of oocytes
341 along the ovarioles between treatments. The cells of the early germarium did not extend to
342 the apical end of the ovariole in the caged queens (Figure 1 E, H, K and N). The early
343 vitellarium stage in the EC caged queens had seemingly healthy NCCs, but the oocytes were
344 misshapen and starting to deteriorate (Figure 4 A, arrows). This deterioration of the oocyte
345 progressively intensified as the days of EC caging increased (Figure 1 I, L and 4 B, arrow). In
346 contrast, the oocytes in the early vitellarium stage in IC caged queens (Figure 1 O) seemed
347 similar to the control queens. The difference between the volume of the oocyte and its
348 accompanying NCC provides a measure of the combined changes in the units (oocytes and
349 NCC) along the ovariole (Figure 2, green line). In all the caged treatments the tipping point,
350 marking the start of the late vitellarium stage, significantly shifted towards the oviduct end
351 of the ovariole relative to control queens (Wald $\chi^2 = 119.43$, *d.f.* = 4, p = 0.0001; Figure 2 and
352 S3). This shift suggests that there was a delay in the maturation of the oocytes in the caged
353 queens, which in turn reduced the number of mature oocytes present in the ovariole. The
354 tipping point for the IC caged queens was at a similar position in the ovariole to those seen
355 in queens that had been caged EC for four days (Figure 2 and Figure S3).
356
357 By the late vitellarium stage several structural abnormalities could be identified as a result
358 of caging. In both EC and IC the cytoplasm bridges (ring canals) that normally connect the
359 oocyte to its accompanying NCC were sometimes lost, resulting in an empty and elongated
360 sheath between them (Figure 4 C arrows). In addition, the link between the oocyte and NCC
361 units appeared to be elongated (Figure 4 C and K, arrows compared to F, arrow). As a proxy
362 for successful maturation of a mature oocyte, we measured the proportion of NCCs
363 dumping by measuring the volume difference between of the maximum and the last NCC.
364 Overall, we found that the proportion of dumping was significantly decreased by caging
365 (Wald $\chi^2 = 18.86$, *d.f.* = 4, p = 0.001; Figure 2 and S4). Interestingly, in EC caged queens,
366 some nurse cell dumping occurred prematurely, just after the tipping point, when the
367 oocyte was still underdeveloped (Figure 4 D arrowhead). As the days of EC caging
368 progressed, the oocyte also appeared increasingly underdeveloped when nurse cell
369 dumping occurred (Figure 4 E and F). By day seven of EC caging, some NCCs were at the

370 oviduct end of the ovariole, but had not yet undergone PCD (Figure 1 J). In addition, there
371 were some NCCs that had not collapsed even though the nurse cells had undergone PCD
372 (Figure 4 B, F and G).
373
374 The oocytes closest to the oviduct undergo degeneration during the late vitellarium stage in
375 EC caged queens. Initially, these degrading oocytes disconnect from their accompanying
376 follicle cells (Figure 4 H arrow). As degradation progresses, the oocyte begins to collapse
377 (Figure 4 I, white arrow) and undergo PCD (Figure 4 J arrow), until it degrades completely
378 (see Figure 1 J). At the oviduct end of the ovariole, an actin aggregation accumulates as the
379 days of EC caging increase (Figure 1G, J and M, arrow). In contrast, in the queens caged IC,
380 the late vitellarium stage oocytes looked similar to those of the control and there appeared
381 to be no actin aggregation (Figure 1 P).
382
383 In summary, confocal microscopy demonstrated that queens caged outside the colony (EC),
384 had abnormal oogenesis as early as the fourth day post caging (Figure 1E, F, G). Throughout
385 the days of EC caging the ovary area decreased, and the length of the germarium increased.
386 This lengthening was a consequence of delayed growth of both the oocytes and NCC, as
387 evidenced by the tipping point shifting towards the oviduct end. This latter shift resulted in
388 underdeveloped, small, oocytes in the late vitellarium stage. By day seven post EC caging,
389 only half of the NCCs completed 'dumping'. In contrast, caging in the colony (IC) did not
390 have such an extreme effect on queen ovaries. Although the size of the ovary slightly
391 decreased as a result of caging IC, it is possible that this decrease was a result of the
392 increase in the length of the germarium. However overall, queens caged IC retained healthy
393 oocytes and may have continued some egg laying.
394
395
396 ***Gene expression***
397
398 The expression level of five (*Anarchy*, *Buffy*, *Tudor*, *Lncov1* and *Draper*) of the six PCD-
399 associated genes investigated increased with the duration of caging EC and IC (Wald χ^2,
400 $p<0.05$, table S2; Figure S5 A-E). The only exception to this pattern was *Ark*, where we found
401 no effect of treatment on its expression levels (Wald $\chi^2 = 5.43$, $d.f = 4$, $p = 0.246$, table S2,
402 Figure S5 F).
403
404 The expression of the four genes associated with nutrition sensitivity pathways and JH (*Kr-*
405 *h1*, *Egfr*, *Tor* and *Foxo*) were significantly affected by the caging treatments (EC and IC)
406 (Wald χ^2, $p<0.05$, Figure S5 G-J, Table S2). These genes significantly increased in expression
407 by day 10 of caging EC. The only gene that differed from this pattern was *Foxo*, which
408 initially increased in expression before returning to levels similar to those seen in the
409 control by day 10 of both EC and IC caging (Figure S5 J).
410
411 Gene expression of *Vg*, one of the two genes associated with vitellogenesis, was not
412 affected by EC or IC caging (Wald $\chi^2 = 4.44$, $d.f = 4$, $p = 0.35$, Figure S5 K), and was extremely
413 low. The expression of its receptor, *VgR*, was also not affected by caging treatments (Wald
414 $\chi^2 = 8.4$, $d.f = 4$, $p = 0.077$, Figure S5 L).
415
416 **Discussion**

417
418 Our study reveals that the control of oogenesis in honey bee queens uses different
419 checkpoints to workers but similar genetic pathways. We identified three putative
420 reproductive 'checkpoints' (also known as 'control points' in Ronai, Vergoz et al. 2016) that
421 regulate oogenesis in honey bee queens. First, there is an absence of visible germline cells.
422 This reduction in germline cells could either be due to suppression of stem cell
423 differentiation (Figure 5 Checkpoint 1) or the early germline cell clusters are undergoing PCD
424 (Figure 5 checkpoint 2).
425
426 PCD in the early germarium has previously been identified in virgin honey bee queens
427 prevented from mating (Berger and Abdalla 2005; Cruz-Landim, Patrício et al. 2006) and in
428 sterile honey bee workers (Tanaka, Schmidt-Capella et al. 2006; Ronai, Barton et al. 2015;
429 Duncan, Hyink et al. 2016). These early checkpoints are also found in *Drosophila*
430 *melanogaster* females that are under nutritional stress (Drummond-Barbosa and Spradling
431 2001; Jenkins, Timmons et al. 2013). Secondly, we found degradation of the oocytes in
432 caged queens at the early vitellarium stage (Figure 1 I, L and 4 A, B). This checkpoint is not
433 known to occur in any other hymenopteran species, or in insects in general, and is
434 potentially unique to honey bee queens (Figure 5 checkpoint 4). Thirdly, mature oocytes
435 degenerate in the late-vitellarium stage in honey bee queens prevented from laying (see
436 also Cruz-Landim, Patrício et al. 2006) (Figure 1 G, J, M and 4 I, J). This checkpoint is also
437 known to occur in another hymenopteran the parasitoid wasp (*Nasonia vitripennis*)
438 (Hopkins and King 1964) but has not been shown in honey bee workers or other insects
439 (Figure 5 checkpoint 5). Thus, it appears that honey bee queens have two additional
440 checkpoints when compared to workers.
441
442 Our results indicate that honey bee queens and workers modulate oogenesis using a
443 different combination of checkpoints to meet their different life history needs. In sterile
444 workers the last checkpoint is at the late germarium stage (Tanaka and Hartfelder 2004;
445 Ronai, Barton et al. 2015). This late germarium checkpoint is also found in nutritionally
446 stressed *Drosophila* females (Drummond-Barbosa and Spradling 2001; Jenkins, Timmons et
447 al. 2013) and other insects (Velentzas, Nezis et al. 2003; Uchida, Nishizuka et al. 2004;
448 Mpakou, Velentzas et al. 2011). For honey bee workers, investing in oogenesis when there is
449 no prospect of producing offspring is wasteful (Berger and da Cruz-Landim 2009). Therefore,
450 it is advantageous for them to abort oocyte production before vitellogenesis, which is the
451 most costly stage of oogenesis (Giorgi and Deri 1976; Drummond-Barbosa and Spradling
452 2001; Ronai, Vergoz et al. 2016). However, when the queen is absent or lost, there is clearly
453 a fitness advantage for workers that are able to quickly switch on oogenesis (Page and
454 Robinson 1994). In contrast to workers, as shown in our study, queens use a combination of
455 checkpoints that act at later stages of oocyte development. These later checkpoints allow
456 the queen to reabsorb the oocytes and therefore preserve invaluable resources if the egg
457 cannot be laid. Therefore, queens slow down the rate of oocyte production while continuing
458 to produce oocytes.
459
460 PCD is an important mechanism for modulating oogenesis in insects and our morphological
461 results support this idea by showing clear evidence of PCD in the ovarioles of caged honey
462 bee queens. Therefore, it was unsurprising to find direct evidence of cell death indicated by
463 the higher expression levels of the phagocytosis engulfment receptor gene *Draper*

464 (Etchegaray, Timmons et al. 2012) in caged queens. Furthermore, we have shown an
465 upregulation of genes from two independent PCD pathways in honey bee queens: one that
466 involves *Anarchy* and *Buffy*, a mitochondrial regulatory gene network associated with PCD
467 (Tanner, Blute et al. 2011) and another, the *Lncov1/Tudor-SN* complex, which interferes
468 with effector caspase activity (Hartfelder, Tiberio et al. 2018). Interestingly, the expression
469 pattern of *Anarchy* and *Buffy* in caged queens is not the same as in virgin queens with non-
470 activated ovaries (Ronai, Oldroyd et al. 2016). Rather, their expression patterns are
471 correlated with the suppression of oogenesis in workers exposed to the queen (Ronai,
472 Oldroyd et al. 2016). Interestingly, expression of *Ark*, a pro-apoptotic gene that initiates the
473 caspase cascade resulting in PCD (Cain, Bratton et al. 2002; Dallacqua and Bitondi 2014),
474 remained constant across our treatments. In virgin queens with non-activated ovaries, *Ark* is
475 downregulated but is not differentially expressed between sterile and laying workers
476 (Rodriguez, Oliver et al. 1999; Dallacqua and Bitondi 2014; Ronai, Oldroyd et al. 2016). Thus,
477 our results suggest that under stressful environmental conditions queens modulate their
478 oogenesis using the same mechanism (PCD) as workers and do not revert to a virgin-like
479 status.
480
481 A critical process for the maturation of an oocyte in insects is the accumulation of
482 vitellogenin, the main egg yolk protein (Raikhel and Dhadialla 1992; Tufail and Takeda 2008).
483 Vitellogenin is mostly synthesised in the fat body and then transferred to the oocyte via the
484 vitellogenin receptor (*VgR*) (Engels 1974). Therefore, it was not surprising that *Vg*
485 expression in the ovary was extremely low (Guidugli, Nascimento et al. 2005; Cardoso‐Jú
486 nior, Oldroyd et al. 2021) and did not change as a result of the treatments. However,
487 previous studies have used *VgR* expression levels as a proxy for the reproductive state of
488 honey bee workers (Guidugli-Lazzarini, do Nascimento et al. 2008; Cardoso‐Júnior,
489 Oldroyd et al. 2021), therefore one may expect *VgR* expression levels to decrease as a result
490 of the reduced oogenesis in caged queens. Our results suggest that under stressful
491 conditions queens potentially keep *VgR* levels constant in the ovaries in order to be able to
492 swiftly provide vitellogenin and restore oogenesis when the environmental conditions
493 improve.
494
495 Our results emphasise the significance of the queens' environment on her reproductive
496 capacity and suggest that nutrition and a normal social environment are necessary for
497 regular oogenesis. We observed a distinct difference in the effects of caging, between EC
498 and IC on oogenesis. Queens caged within the colony had healthier ovaries and oocytes
499 than externally caged queens, in which oogenesis was more severely disrupted. This
500 difference may be linked to the amount of care provided by the workers, particularly the
501 quantity and quality of food fed by the escort workers to the queens EC relative to that
502 which could be provided IC (Szabo 1974). As is standard beekeeping practise, the only
503 source of nutrition available to the escort workers in our external cages was 'queen candy'
504 (Laidlaw and Page 1997). While candy is rich in carbohydrates, it lacks fat and protein, the
505 latter being essential for oogenesis (Fine, Shpigler et al. 2018). Thus, one would expect the
506 ability of escort workers to feed the queen in their care with glandular secretions is severely
507 curtailed because the workers themselves are nutritionally stressed.
508
509 Caging queens both EC and IC influenced the expression of genes associated with the
510 nutrient sensitive pathways Tor and EGFR in our study. We suggest that the upregulation of

511 the nutrition-sensitive genes in the caged and banked queens was due to nutritional stress.
512 *FoxO*, a downstream target gene of *Tor* and *Egfr*, is also upregulated in nutritionally-stressed
513 honey bee workers (Wheeler, Buck et al. 2014; Cardoso‐Júnior, Oldroyd et al. 2021) and in
514 nutritionally-stressed solitary insects such as *Drosophila* (Jouandin, Ghiglione et al. 2014).
515 However, *Tor* has been shown to be downregulated in ovaries of nutritionally-stressed
516 *Drosophila* (Pritchett and McCall 2012; Smykal and Raikhel 2015) and mosquitoes (Hansen,
517 Attardo et al. 2005; Perez-Hedo, Rivera-Perez et al. 2014). The up-regulation of *Kr-h1*, a
518 proxy for juvenile hormone (JH) titres (Minakuchi, Namiki et al. 2009; Belles and Santos
519 2014; Lago, Humann et al. 2016) observed in the queens caged both EC and IC is consistent
520 with higher levels of JH seen in non-reproductive honey bee workers (Robinson, Strambi et
521 al. 1992) and in nutritionally stressed *Drosophila* (Terashima and Bownes 2004) Therefore,
522 JH possibly works as an intermediate regulator of adult ovary activation in both honey bee
523 castes.
524
525 Although more studies need to be conducted to assess the effect of nutrition quantity and
526 quality on honey bee queen oogenesis, our study suggests that nutrition could serve as a
527 cue for the suppression of oogenesis in queens. Our findings accord with behavioural
528 observations (Allen 1956; Pierce, Lewis et al. 2007) in which queens are fed less during
529 swarming. In our particular study, IC queens acquired their nutrition via trophalaxis from
530 colony workers through their cage. Food obtained from free-running nurse bees would be
531 almost certainly of higher quality to that available to the EC queens. Whilst nutrition is not
532 the only environmental difference between queens cages EC and IC, we suggest that food is
533 a major factor that contributed to the nearly-normal ovary morphology of the IC queens,
534 and the loss of oocytes in the EC queens. Nutrition also regulates oogenesis in solitary
535 insects such as *Drosophila* (Drummond-Barbosa and Spradling 2001; Gruntenko, Bownes et
536 al. 2003; Barth, Szabad et al. 2011).
537
538 Our study has practical ramifications for beekeepers. It is clear that queens caged in the
539 colony fare better than queens caged externally to the colony, and that keeping queens
540 within the colony environment should be the preferred method for queen storage. It is
541 suggested that the period of caging should be minimised. Likely, reformulating the candy
542 diet for caged queens and their escorts to include pollen as a source of protein and fat
543 would enhance queen welfare in the mail, and we recommend that this be investigated in a
544 future study.
545
546 In conclusion, although queens and worker honey bees share the same regulatory template,
547 they have diverged in how they regulate oogenesis. Reproductive division of labour
548 between castes is fundamental for the evolution of eusociality in the social insects. One
549 explanation of the creation of castes is the "reproductive ground plan hypothesis" (West-
550 Eberhard 1987) which suggests that the queen and worker castes evolved from a decoupling
551 of the reproductive and foraging stages of a solitary ancestral insect from which
552 contemporary eusocial bees are derived (Amdam, Norberg et al. 2004). This decoupling
553 resulted in the molecular, morphological and behavioural differences between the queen
554 and worker castes but preserved the underling pathways that regulate nutrition and
555 reproduction. It is proposed here that the reproductive divergence of queen and worker
556 honey bees is achieved through different oogenesis checkpoints but the same PCD

pathways in order to modulate oogenesis and to meet the reproductive divergence of the two castes.

Acknowledgements. To Rhiarn Hoban for drawing the detailed ovariole structure; to Mackenzie R. Lovegrove for her assistance in trouble shooting the fixation stages of the ovarioles and to the members the Behaviour and Genetics of Social Insects Lab for their assistance and advice.

Conflict of interests. We have no conflict of interests.

Data archiving. All supporting datasets for this paper are available as part of the electronic supplementary material.

Funding. This work was funded by the Australian Research Council project DP180101696.

571 Figure 1
572 Oogenesis in honey bee queens. (A) A schematic representation of oogenesis in a mated
573 laying queen with the four regions: terminal filament; germarium; vitellarium; and pedicel
574 (nc = nurse cell, oo= oocyte, ncc = nurse cell chamber, s = sheath, dnc = dumped nurse cell,
575 and fc = follicle cell); B-D. Ovaries of a control queen: (B) terminal filament and early
576 germarium; (Bi) magnification of terminal filament and actin polyfusomes; (Bii)
577 magnification of ring canals; (C) early vitellarium; (Ci) magnification of follicle cells; and (D)
578 late vitellarium.
579 E-G. Ovaries of a queen that had been caged EC for four days: (E) terminal filament and
580 early germarium; (F) early vitellarium; and (G) late vitellarium showing actin aggregation
581 (arrow).
582 H-J. Ovaries of a queen that had been caged EC for seven days: (H) terminal filament and
583 early germarium; (I) early vitellarium; and (J) late vitellarium showing actin aggregation
584 (arrow).
585 K-M. Ovaries of a queen that had been caged EC for ten days: (K) terminal filament and early
586 germarium; (L) early vitellarium; and (M) late vitellarium showing actin aggregation (arrow).
587 N-P. Ovaries of a queen that had been caged IC for ten days; (N) terminal filament and early
588 germarium; (O) early vitellarium; and (P) late vitellarium. Ovaries were stained for nuclei
589 (magenta) and actin (green). Scale bars 100 µm and magnified insets 10 µm.
590
591 Figure 2
592 Dynamics of oogenesis in honey bee queens. The mean radius size (µm) of the oocytes (red
593 line) and nurse cell chambers (NCC) (blue line) derived from averaged data for the control,
594 caged EC days 4, 7 and 10 and caged IC for day 10 IC treatment groups. The difference
595 between the size of the paired NCC and oocyte is indicated (green line). The cell position
596 represents the unit number along the chain in the ovariole with the proximal oocyte at the
597 oviduct end designated as position 0 (see Materials and Methods S1). The amount of NCC
598 dumping (purple line) and tipping point (orange circle) are indicated for each group.
599
600 Figure 3
601 Change in ovary area of honey bee queens from the control, caged in-colony (IC) (4, 7 or 10
602 days) or caged Ex-colony (EC) (10 days) treatment groups. (A) Light microscopy image of a
603 representative queen ovary from each treatment group (scale bar 2 mm). (B) Mean area
604 (mm^2) of queen ovaries across treatment groups (n = 4 control, caged IC 10 days and caged
605 EC 10 days; n = 5 caged EC 4 and 7 days). Error bars are SE of the means. Different letters (a-
606 c) represent statistical significance ($p < 0.05$).
607
608 Figure 4
609 Oogenesis abnormalities in honey bee queen ovarioles after caging for 4, 7 and 10 days ex-
610 colony (EC) and 10 days in-colony (IC). (A) EC four days early vitellarium. Misshapen oocytes
611 (arrows) among healthy-looking nurse cell chambers; (B) EC 10 days late vitellarium. Some
612 oocytes are underdeveloped and misshapen (arrow). There is also brightly-stained
613 fragmented nucleic acid, suggesting premature programmed cell death of nurse cells but
614 with no chamber collapse (arrow heads). Mature oocytes undergoing degradation (asterisk);
615 (C) EC 7 days late vitellarium. The cytoplasm bridge is lost, disconnecting the oocyte from its
616 nurse chamber and the connection between the oocyte and the next NCC is deteriorated
617 (arrows); (D) EC 4 days late vitellarium. The nurse cell dumping (arrow head) is premature,

618 occurring for an insufficiently-developed oocyte immediately after the 'tipping point'
619 (yellow arrow). A mature oocyte is collapsing (asterisk); (E) EC 7 days early and late
620 vitellarium. The nurse cell dumping (arrow heads) is premature, occurring immediately after
621 the tipping point (yellow arrow). Mature oocytes are undergoing degradation (asterisk); (F)
622 EC 10 days vitellarium. Premature nurse cell dumping (arrow head) is occurring immediately
623 after the tipping point (yellow arrow) or no nurse cell dumping at all (arrows). The most
624 developed oocytes are degrading (asterisk); (G) EC 4 days late vitellarium. Programmed cell
625 death of the nurse cells has occurred, but the nurse cell chamber has not collapsed (arrow).
626 A mature oocyte is collapsing (asterisk); (H) EC 7 days late vitellarium. The oocyte has
627 disconnected from its surrounding follicle cells (arrow) indicating the beginning of its
628 degradation. Brightly-stained fragmented nucleic acid are being transferred to the oocyte
629 following NCC dumping (arrow head); (I) EC 10 days late vitellarium. Structural collapse of
630 an oocyte (arrow) with premature NCC dumping occurring (arrow head) immediately after
631 the tipping point (yellow arrow). The most mature oocyte is completely degraded (asterisk);
632 (J) EC 10 days late vitellarium. The bright white dots in the mature oocyte are a sign of
633 programmed cell death (arrow) and the most mature oocyte has completely collapsed
634 (asterisk); (K) IC 10 days early vitellarium. The connection between the oocyte and its
635 preceding NCC is deteriorating (arrows). oo = oocyte; NCC = nurse cell chamber. Ovarioles
636 have been stained for nucleic acid (DAPI). Scale bars 100µm.
637
638 Figure 5
639 The oogenesis checkpoints in insects. (A) position of each checkpoint along an ovariole; (B)
640 checkpoints used by the honey bee (*Apis mellifera*) mated queen, virgin queen, worker;
641 solitary insect the fruit fly (*Drosophila melanogaster*); and another social insect the
642 parasitoid wasp (*Nasonia vitripennis*).
643
644 Figure S1
645 Number of visible oocytes along an ovariole of honey gee queens from the control, caged in-
646 colony (IC) (4, 7 or 10 days) or caged Ex-colony (EC) (10 days) treatment groups. n = 4
647 control, caged IC 10 days and caged EC 10 days; n = 5 caged EC 4 and 7 days. Error bars are
648 SE of the means.
649
650 Figure S2
651 Spherical radius (R_{eq}) (µm) of oocytes and NCC of honey bee queens from the control, caged
652 in-colony (IC) (4, 7 or 10 days) or caged Ex-colony (EC) (10 days) treatment groups. (A)
653 maximum oocyte radius; (B) maximum nurse cell chamber radius; (C) minimum oocyte
654 radius; (D) minimum nurse chamber radius. Sample size see Figure S1. Error bars are SE of
655 the means. Bars with a different letter are significantly different ($p < 0.05$).
656
657 Figure S3
658 Position of the oocyte in the ovariole when the tipping point occurs. Honey bee queens
659 from the control, caged in-colony (IC) (4, 7 or 10 days) or caged Ex-colony (EC) (10 days)
660 treatment groups. Sample size see Figure S1. Error bars are SE of the means. Bars with a
661 different letter are significantly different ($p < 0.05$).
662
663 Figure S4

664 Extent of nurse cell dumping in honey bee queens from the control, caged in-colony (IC) (4,
665 7 or 10 days) or caged Ex-colony (EC) (10 days) treatment groups. Sample size see Figure S1.
666 Error bars are SE of the means. Bars with a different letter are significantly different ($p <$
667 0.05).
668
669 Figure S5
670 Gene expression in the ovaries of honey bee queens from the control, caged in-colony (IC)
671 (4, 7 or 10 days) or caged Ex-colony (EC) (10 days) treatment groups. Genes related to
672 program cell death (A) *Anarchy* (B) *Lncov-1* (C) *Tudor* (D) *Buffy* (E) *Draper* and (F) *Ark*. Genes
673 related to nutrition (G) *Kr-h1*; (H) *Egfr*; (I) *Tor* and (J) *FoxO*. Genes related to vitellogenesis (K)
674 *Vg* and (L) *VgR*. Sample size see Figure S1. Boxplot whiskers represent minimum and
675 maximum values, the box is defined by 25th percentile, median and 75th percentile. Bars
676 with a different letter are significantly different ($p < 0.05$).
677
678

679 **References**
680
681 Allen M.D. (1956) The behaviour of honeybees preparing to swarm. The British Journal of
682 Animal Behaviour **4**(1): 14-22.
683 Ambrose J.T. (1976) Swarms in transit. Bee World **57**(3): 101-109.
684 Amdam G.V., K. Norberg, M.K. Fondrk, R.E. Page. (2004) Reproductive ground plan may
685 mediate colony-level selection effects on individual foraging behavior in honey bees.
686 Proc. Natl. Acad. Sci. **101**(31): 11350-11355.
687 Avitabile A. (1978) Brood rearing in honeybee colonies from late autumn to early spring. J
688 Apic Res **17**(2): 69-73.
689 Barth J., J. Szabad, E. Hafen, K. Köhler. (2011) Autophagy in *Drosophila* ovaries is induced by
690 starvation and is required for oogenesis. Cell Death Differ. **18**(6): 915-924.
691 Belles X., C.G. Santos. (2014) The MEKRE93 (Methoprene tolerant-Krüppel homolog 1-E93)
692 pathway in the regulation of insect metamorphosis, and the homology of the pupal
693 stage. Insect Biochem. Mol. Biol. **52**: 60-68.
694 Berger B., F.C. Abdalla. (2005) Programmed cell death during ovarian differentiation in
695 queens of *Apis mellifera* linne, 1758 (Hymenoptera, Apini). Braz. J. morphol. Sci **22**(1):
696 1-4.
697 Berger B., C. da Cruz-Landim. (2009) Ultrastructural analysis of the effect of mating delay on
698 cell death in the ovaries of virgin honey bee (*Apis mellifera* L.) queens. J Apic Res
699 **48**(1): 60-66.
700 Brito R., M. McHale, B. Oldroyd. (2010) Expression of genes related to reproduction and
701 pollen foraging in honey bees (*Apis mellifera*) narcotized with carbon dioxide. Insect
702 Mol. Biol. **19**(4): 451-461.
703 Büning J. (1994) The insect ovary: ultrastructure, previtellogenic growth and evolution, in:
704 Chapman and Hall (Eds.), Tissue and Cell, Elsevier Ltd, New York.
705 Butler C. (1957) The control of ovary development in worker honeybees (*Apis mellifera*).
706 Experientia **13**(6): 256-257.
707 Butler C., E.M. Fairey. (1963) The role of the queen in preventing oogenesis in worker
708 honeybees. J Apic Res **2**(1): 14-18.
709 Butler C.G., R.K. Callow, N.C. Johnston. (1961) The isolation and synthesis of queen
710 substance, 9-oxodec-trans-2-enoic acid, a honeybee pheromone. Proc. Royal Soc. B
711 **155**(960): 417-432.
712 Cain K., S.B. Bratton, G.M. Cohen. (2002) The Apaf-1 apoptosome: a large caspase-activating
713 complex. Biochimie **84**(2-3): 203-214.
714 Cardoso‐Júnior C.A.M., B.P. Oldroyd, I. Ronai. (2021) Vitellogenin expression in the ovaries
715 of adult honeybee workers provides insights into the evolution of reproductive and
716 social traits. Insect Mol. Biol.
717 Cavaliere V., C. Taddei, G. Gargiulo. (1998) Apoptosis of nurse cells at the late stages of
718 oogenesis of *Drosophila melanogaster*. Dev Gen Evol **208**(2): 106-112.
719 Cruz-Landim C., K. Patrício, W.F. Antonialli Jr. (2006) Cell death and ovarian development in
720 highly eusocial bees (Hymenoptera, Apidae): caste differentiation and worker egg
721 laying. Braz. J. Morphol. Sci **23**: 27-42.
722 Dade H.A. (1977) Anatomy and dissection of the honeybee. International Bee Research
723 Association, London.

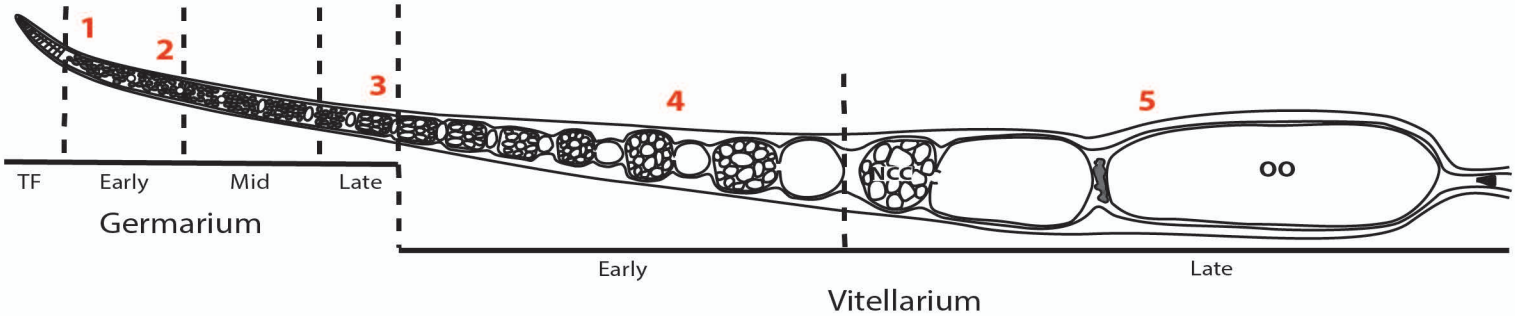
724 Dallacqua R.P., M.M.G. Bitondi. (2014) Dimorphic ovary differentiation in honeybee (*Apis*
725 *mellifera*) larvae involves caste-specific expression of homologs of Ark and Buffy cell
726 death genes. PloS one **9**(5): e98088.
727 Dolezal A.G., K.B. Flores, K.S. Traynor, G.V. Amdam. (2013) The evolution and development
728 of eusocial insect behavior, in: Streelman J.T. (Ed.), Advances in Evolutionary
729 Developmental Biology, Wiley-Blackwell, Hoboken, New Jersey, pp. 37-57.
730 Drummond-Barbosa D., A.C. Spradling. (2001) Stem cells and their progeny respond to
731 nutritional changes during *Drosophila* oogenesis. Dev. Biol. **231**(1): 265-278.
732 Duncan E.J., O. Hyink, P.K. Dearden. (2016) Notch signalling mediates reproductive
733 constraint in the adult worker honeybee. Nat. Commun **7**: 12427.
734 Engels W. (1974) Occurrence and significance of vitellogenins in female castes of social
735 Hymenoptera. Am. Zool. **14**(4): 1229-1237.
736 Etchegaray J.I., A.K. Timmons, A.P. Klein, T.L. Pritchett, E. Welch, et al. (2012) Draper acts
737 through the JNK pathway to control synchronous engulfment of dying germline cells
738 by follicular epithelial cells. Development **139**(21): 4029-4039.
739 Fine J.D., H.Y. Shpigler, A.M. Ray, N.J. Beach, A.L. Sankey, et al. (2018) Quantifying the
740 effects of pollen nutrition on honey bee queen egg laying with a new laboratory
741 system. PloS one **13**(9).
742 Giorgi F., P. Deri. (1976) Cell death in ovarian chambers of *Drosophila melanogaster*.
743 Development **35**(3): 521-533.
744 Grozinger C.M., J. Richards, H.R. Mattila. (2014) From molecules to societies: mechanisms
745 regulating swarming behavior in honey bees (*Apis* spp.). Apidologie **45**(3): 327-346.
746 Gruntenko N., M. Bownes, J. Terashima, M.Z. Sukhanova, I.Y. Raushenbach. (2003) Heat
747 stress affects oogenesis differently in wild‐type *Drosophila virilis* and a mutant with
748 altered juvenile hormone and 20‐hydroxyecdysone levels. Insect Mol. Biol. **12**(4):
749 393-404.
750 Guidugli-Lazzarini K.R., A.M. do Nascimento, E.D. Tanaka, M.D. Piulachs, K. Hartfelder, et al.
751 (2008) Expression analysis of putative vitellogenin and lipophorin receptors in honey
752 bee (*Apis mellifera* L.) queens and workers. J. Insect Physiol. **54**(7): 1138-1147.
753 Guidugli K.R., A.M. Nascimento, G.V. Amdam, A.R. Barchuk, S. Omholt, et al. (2005)
754 Vitellogenin regulates hormonal dynamics in the worker caste of a eusocial insect.
755 FEBS letters **579**(22): 4961-4965.
756 Gutzeit H., D. Zissler, R. Fleig. (1993) Oogenesis in the honeybee *Apis mellifera*: cytological
757 observations on the formation and differentiation of previtellogenic ovarian follicles.
758 Roux's Arch. Dev. Biol. **202**(3): 181-191.
759 Hansen I.A., G.M. Attardo, S.G. Roy, A.S. Raikhel. (2005) Target of rapamycin-dependent
760 activation of S6 kinase is a central step in the transduction of nutritional signals
761 during egg development in a mosquito. J. Biol. Chem. **280**(21): 20565-20572.
762 Hartfelder K., G.J. Tiberio, D.C. Lago, R.P. Dallacqua, M.M.G. Bitondi. (2018) The ovary and
763 its genes—developmental processes underlying the establishment and function of a
764 highly divergent reproductive system in the female castes of the honey bee, *Apis*
765 *mellifera*. Apidologie **49**(1): 49-70.
766 Hodin J. (2009) She shapes events as they come: plasticity in female insect reproduction, in:
767 Whitmen W.D. (Ed.), Phenotypic plasticity of insects: mechanisms and consequences,
768 Science Publishers, Enfield, NH, USA, pp. 423-521.
769 Hoover S.E., C.I. Keeling, M.L. Winston, K.N. Slessor. (2003) The effect of queen pheromones
770 on worker honey bee ovary development. Naturwissenschaften **90**(10): 477-480.

771 Hopkins C., P. King. (1964) Egg resorption in *Nasonia vitripennis* (Walker)(Hymenoptera:
772 Pteromalidae), Proceedings of the Royal Entomological Society of London. Series A,
773 General Entomology, Wiley Online Library, pp. 101-107.
774 Hunt J.H., G.V. Amdam. (2005) Bivoltinism as an antecedent to eusociality in the paper wasp
775 genus Polistes. Science **308**(5719): 264-267.
776 Jenkins V.K., A.K. Timmons, K. McCall. (2013) Diversity of cell death pathways: insight from
777 the fly ovary. Trends Cell Biol. **23**(11): 567-574.
778 Jouandin P., C. Ghiglione, S. Noselli. (2014) Starvation induces FoxO-dependent mitotic-to-
779 endocycle switch pausing during *Drosophila* oogenesis. Development **141**(15): 3013-
780 3021.
781 King R.C. (1970) Ovarian development in *Drosophila melanogaster*. Academic Press, New
782 York.
783 Lago D.C., F.C. Humann, A.R. Barchuk, K.J. Abraham, K. Hartfelder. (2016) Differential gene
784 expression underlying ovarian phenotype determination in honey bee, *Apis mellifera*
785 L., caste development. Insect Biochem. Mol. Biol. **79**: 1-12.
786 Laidlaw H.H., R.E. Page. (1997) Queen rearing and bee breeding. Wicwas Press, Cheshire, CT.
787 Le Conte Y., A. Mohammedi, G.E. Robinson. (2001) Primer effects of a brood pheromone on
788 honeybee behavioural development. Proc. Royal Soc. B **268**(1463): 163-168.
789 Lourenço A.P., A. Mackert, A. dos Santos Cristino, Z.L.P. Simões. (2008) Validation of
790 reference genes for gene expression studies in the honey bee, *Apis mellifera*, by
791 quantitative real-time RT-PCR. Apidologie **39**(3): 372-385.
792 Malka O., S. Shnieor, A. Hefetz, T. Katzav-Gozansky. (2007) Reversible royalty in worker
793 honeybees (*Apis mellifera*) under the queen influence. Behav Ecol Sociobiol **61**(3):
794 465-473.
795 Mattila H.R., G.W. Otis. (2007) Dwindling pollen resources trigger the transition to broodless
796 populations of long‐lived honeybees each autumn. Ecological Entomology **32**(5):
797 496-505.
798 Minakuchi C., T. Namiki, T. Shinoda. (2009) Krüppel homolog 1, an early juvenile hormone-
799 response gene downstream of Methoprene-tolerant, mediates its anti-metamorphic
800 action in the red flour beetle *Tribolium castaneum*. Dev. Biol. **325**(2): 341-350.
801 Mpakou V.E., A.D. Velentzas, P.D. Velentzas, L.H. Margaritis, D.J. Stravopodis, et al. (2011)
802 Programmed cell death of the ovarian nurse cells during oogenesis of the ladybird
803 beetle *Adalia bipunctata* (Coleoptera: Coccinellidae). Dev. Growth Differ. **53**(6): 804-
804 815.
805 Oster G.F., E.O. Wilson. (1978) Caste and ecology in the social insects. Princeton University
806 Press, Princeton
807 Page R., E. Erickson. (1988) Reproduction by worker honey bees (*Apis mellifera* L.). Behav
808 Ecol Sociobiol **23**(2): 117-126.
809 Page R.E., G.E. Robinson. (1994) Reproductive competition in queenless honey bee colonies
810 (*Apis mellifera* L.). Behav Ecol Sociobiol **35**(2): 99-107.
811 Perez-Hedo M., C. Rivera-Perez, F.G. Noriega. (2014) Starvation increases insulin sensitivity
812 and reduces juvenile hormone synthesis in mosquitoes. PLoS One **9**(1): e86183.
813 Pfaffl M.W., A. Tichopad, C. Prgomet, T.P. Neuvians. (2004) Determination of stable
814 housekeeping genes, differentially regulated target genes and sample integrity:
815 BestKeeper–Excel-based tool using pair-wise correlations. Biotechnology letters
816 **26**(6): 509-515.

817 Pierce A.L., L.A. Lewis, S.S. Schneider. (2007) The use of the vibration signal and worker
818 piping to influence queen behavior during swarming in honey bees, *Apis mellifera*.
819 Ethology **113**(3): 267-275.
820 Pritchett T., K. McCall. (2012) Role of the insulin/Tor signaling network in starvation-induced
821 programmed cell death in *Drosophila* oogenesis. Cell Death Differ. **19**(6): 1069.
822 Raikhel A.S., T. Dhadialla. (1992) Accumulation of yolk proteins in insect oocytes. Annu Rev
823 Entomol **37**(1): 217-251.
824 Ratnieks F.L. (1993) Egg-laying, egg-removal, and ovary development by workers in
825 queenright honey bee colonies. Behav Ecol Sociobiol **32**(3): 191-198.
826 Reim T., M. Thamm, D. Rolke, W. Blenau, R. Scheiner. (2013) Suitability of three common
827 reference genes for quantitative real-time PCR in honey bees. Apidologie **44**(3): 342-
828 350.
829 Rhodes J.W., D.C. Somerville, S. Harden. (2004) Queen honey bee introduction and early
830 survival–effects of queen age at introduction. Apidologie **35**(4): 383-388.
831 Robinson G.E., C. Strambi, A. Strambi, Z.-Y. Huang. (1992) Reproduction in worker honey
832 bees is associated with low juvenile hormone titers and rates of biosynthesis. Gen.
833 Comp. Endocrinol. **87**(3): 471-480.
834 Rodriguez A., H. Oliver, H. Zou, P. Chen, X. Wang, et al. (1999) Dark is a *Drosophila*
835 homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death
836 pathway. Nature cell biology **1**(5): 272.
837 Ronai I., M.H. Allsopp, K. Tan, S. Dong, X. Liu, et al. (2017) The dynamic association between
838 ovariole loss and sterility in adult honeybee workers. Proc. R. Soc. B **284**(1851):
839 20162693.
840 Ronai I., D.A. Barton, B.P. Oldroyd, V. Vergoz. (2015) Regulation of oogenesis in honey bee
841 workers via programed cell death. J. Insect Physiol. **81**: 36-41.
842 Ronai I., B. Oldroyd, V. Vergoz. (2016) Queen pheromone regulates programmed cell death
843 in the honey bee worker ovary. Insect Mol. Biol. **25**(5): 646-652.
844 Ronai I., B.P. Oldroyd, D.A. Barton, G. Cabanes, J. Lim, et al. (2016) Anarchy is a molecular
845 signature of worker sterility in the honey bee. Mol. Biol. Evol. **33**(1): 134-142.
846 Ronai I., V. Vergoz, B. Oldroyd. (2016) The mechanistic, genetic, and evolutionary basis of
847 worker sterility in the social hymenoptera. Advances in the Study of Behavior **48**:
848 251-317.
849 Rozen J. (1986) Survey of the number of ovarioles in various taxa of bees (Hymenoptera:
850 Apoidea). Proceedings of the Entomological Society of Washington (USA) **88**: 707-
851 710.
852 Seeley T. (1985) Honey bee ecology Princeton University Press, Princeton.
853 Seeley T.D., R.D. Fell. (1981) Queen substance production in honey bee (*Apis mellifera*)
854 colonies preparing to swarm (Hymenoptera: Apidae). J. Kans. Entomol. Soc.: 192-196.
855 Shehata S., G. Townsend, R. Shuel. (1981) Seasonal physiological changes in queen and
856 worker honeybees. J Apic Res **20**(2): 69-78.
857 Simpson J. (1959) Variation in the incidence of swarming among colonies of *Apis mellifera*
858 throughout the summer. Ins Soc **6**(1): 85-99.
859 Smykal V., A.S. Raikhel. (2015) Nutritional control of insect reproduction. Curr. Opin. Insect.
860 Sci. **11**: 31-38.
861 Snodgrass R.E. (1956) Anatomy of the honey bee. Cornell University Press.

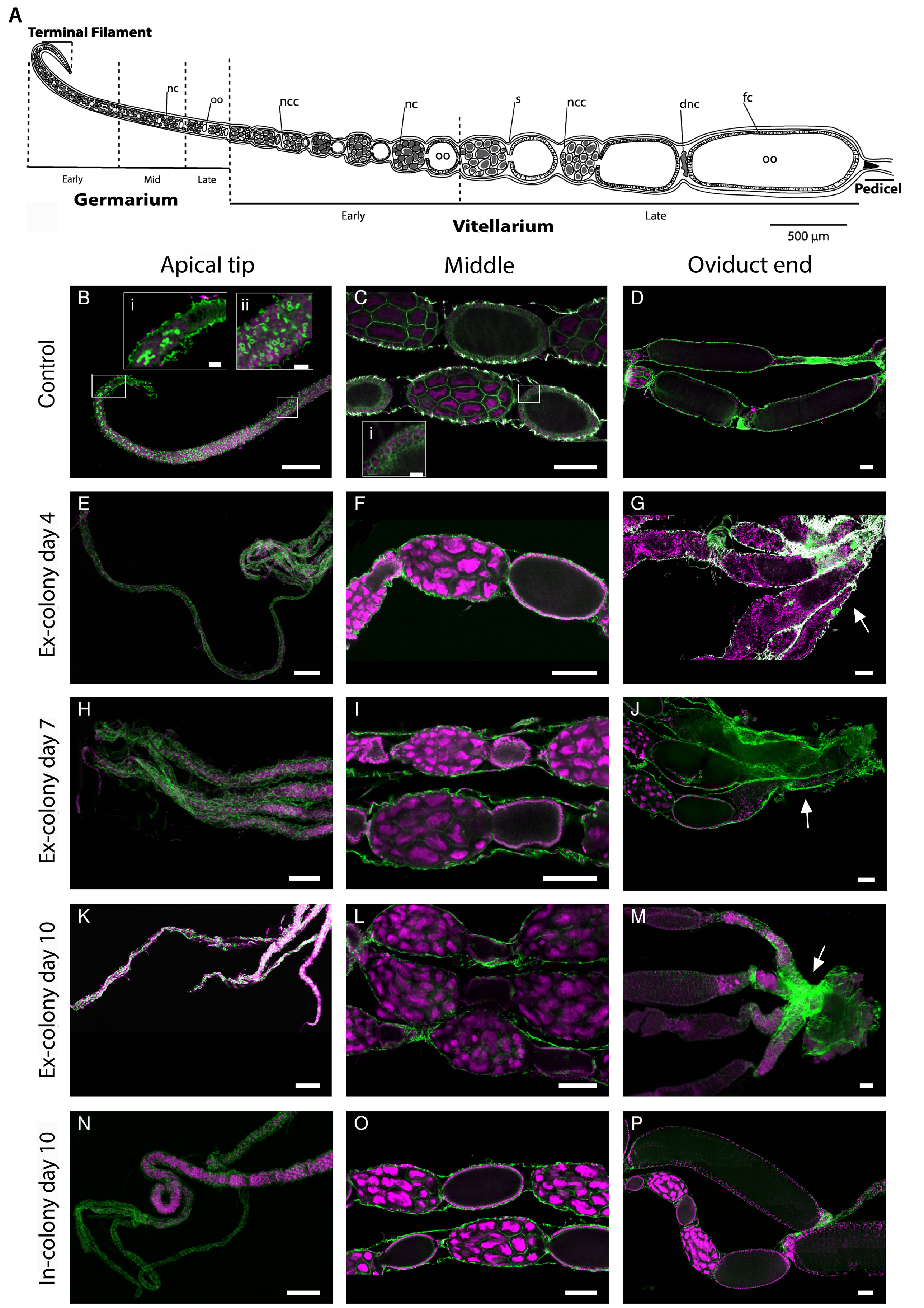
862 Spradling A. (1993) Developmental genetics of oogenesis, in: Bate M. and Arias A.M. (Eds.),
863 The development of *Drosophila melanogaster*, Cold Spring Harbor Lab. Press, New
864 York, pp. 1–70.
865 Szabo T. (1974) Behavioural studies on queen introduction in the honeybee II. effect of the
866 age of workers (from a colony with a laying queen) on their behaviour towards an
867 introduced virgin queen. J Apic Res **13**(2): 127-135.
868 Tanaka E., I. Schmidt-Capella, K. Hartfelder. (2006) Cell death in the germline-mechanisms
869 and consequences for reproductive plasticity in social bees. Braz. j. morphol. sci.
870 **23**(1).
871 Tanaka E.D., K. Hartfelder. (2004) The initial stages of oogenesis and their relation to
872 differential fertility in the honey bee (*Apis mellifera*) castes. Arthropod Struct Dev
873 **33**(4): 431-442.
874 Tanner E.A., T.A. Blute, C.B. Brachmann, K. McCall. (2011) Bcl-2 proteins and autophagy
875 regulate mitochondrial dynamics during programmed cell death in the *Drosophila*
876 ovary. Development **138**(2): 327-338.
877 Terashima J., M. Bownes. (2004) Translating available food into the number of eggs laid by
878 *Drosophila melanogaster*. Genetics **167**(4): 1711-1719.
879 Toth A.L., K. Varala, T.C. Newman, F.E. Miguez, S.K. Hutchison, et al. (2007) Wasp gene
880 expression supports an evolutionary link between maternal behavior and eusociality.
881 Science **318**(5849): 441-444.
882 Traynor K.S., Y. Le Conte, R.E. Page. (2014) Queen and young larval pheromones impact
883 nursing and reproductive physiology of honey bee (*Apis mellifera*) workers. Behav
884 Ecol Sociobiol **68**(12): 2059-2073.
885 Tufail M., M. Takeda. (2008) Molecular characteristics of insect vitellogenins. J. Insect
886 Physiol. **54**(12): 1447-1458.
887 Uchida K., M. Nishizuka, D. Ohmori, T. Ueno, Y. Eshita, et al. (2004) Follicular epithelial cell
888 apoptosis of atretic follicles within developing ovaries of the mosquito *Culex pipiens*
889 *pallens*. J. Insect Physiol. **50**(10): 903-912.
890 Velentzas A.D., I.P. Nezis, D.J. Stravopodis, I.S. Papassideri, L.H. Margaritis. (2003) Stage-
891 specific regulation of programmed cell death during oogenesis of the medfly
892 *Ceratitis capitata* (Diptera, Tephritidae). Int. J. Dev. Biol. **51**(1): 57-66.
893 Visscher P.K. (1989) A quantitative study of worker reproduction in honey bee colonies.
894 Behav Ecol Sociobiol **25**(4): 247-254.
895 West-Eberhard M.J. (1987) Flexible strategy and social evolution. Animal societies. Theories
896 and facts.
897 Wheeler D., N. Buck, J. Evans. (2014) Expression of insulin/insulin‐like signalling and TOR
898 pathway genes in honey bee caste determination. Insect Mol. Biol. **23**(1): 113-121.
899 Winston M.L. (1987) The biology of the honey bee. Harvard university press, Cambridge MA.
900 Woyciechowski M., K. Kuszewska. (2012) Swarming generates rebel workers in honeybees.
901 Curr. Biol. **22**(8): 707-711.
902
903
904
905
906

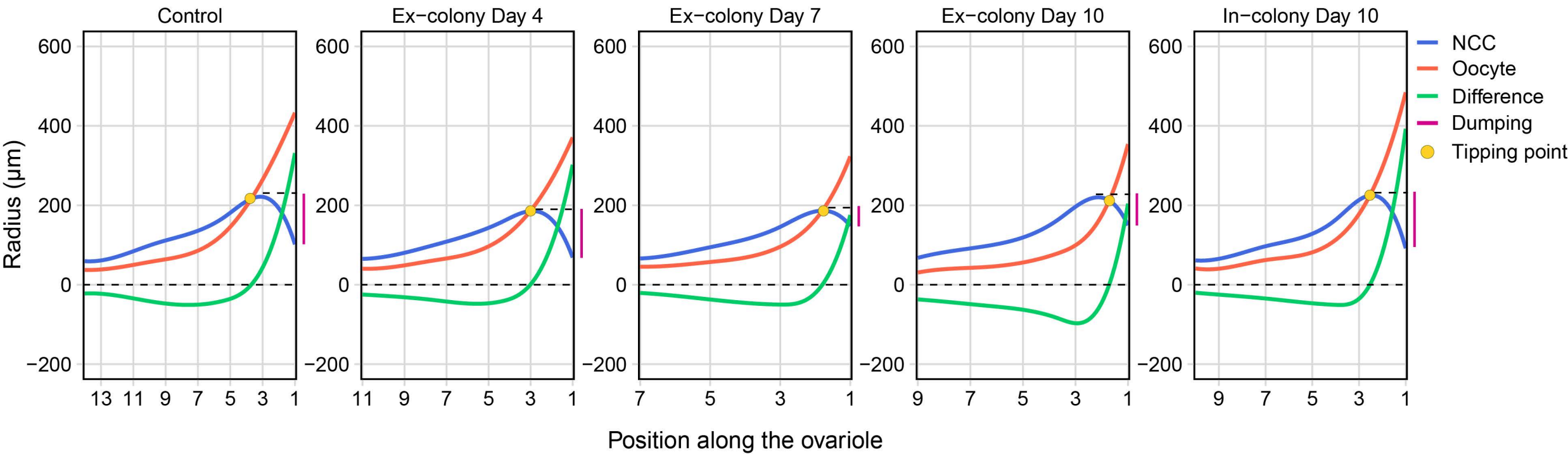
A

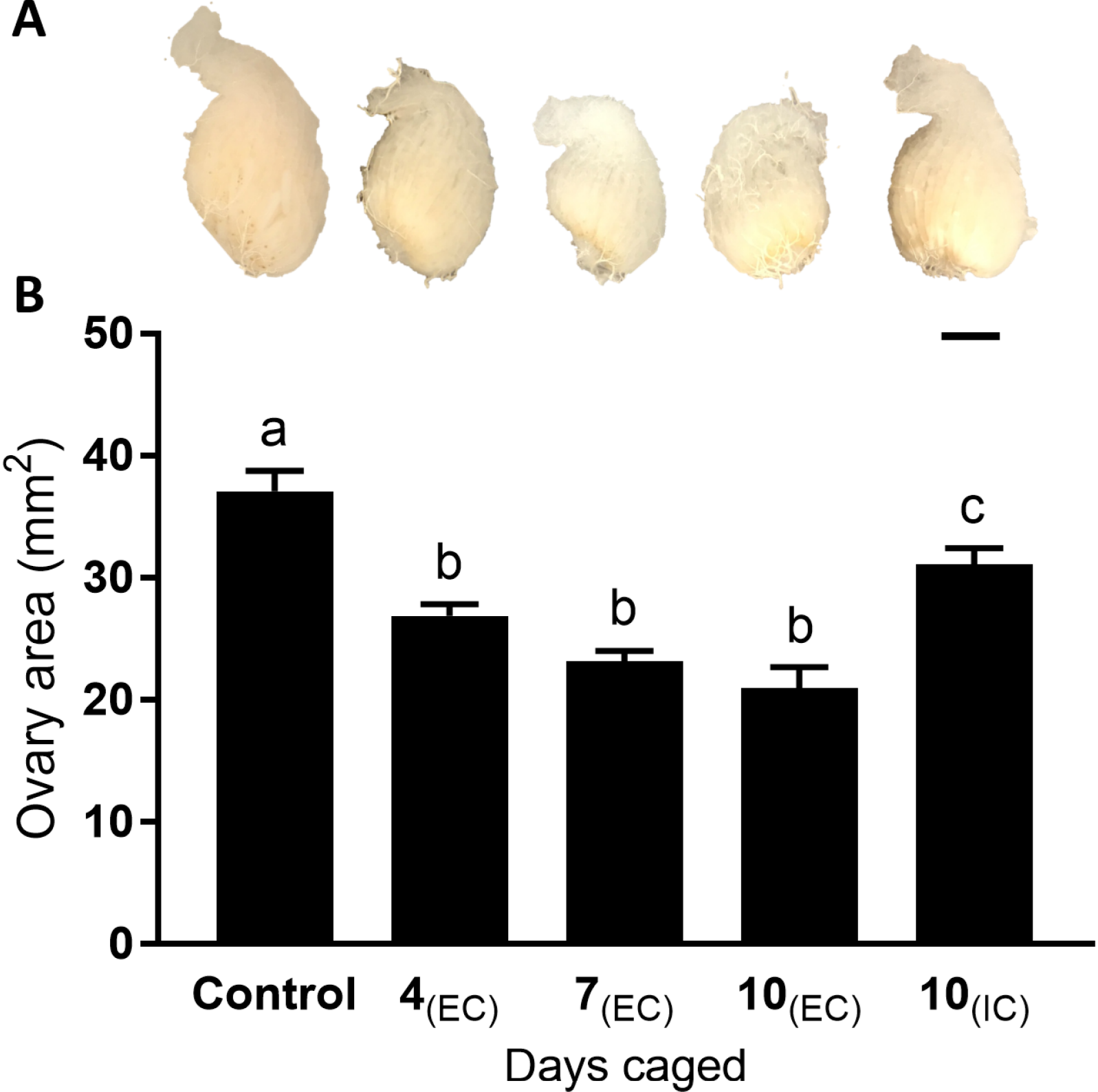


B

	1 Stem cells	2 Early germarium	3 Late germarium	4 Early vitellarium	5 Late vitellarium
Honey bee mated queen	✓		X	✓	✓
Honey bee virgin queen	✓	✓	✓	X	X
Honey bee worker	✓	✓	✓	X	X
Fruit fly	✓	✓	✓	X	X
Wasp	?	?	?	?	✓


A
Terminal Filament
nc
oo
ncc
nc
s
ncc
dnc
fc
oo
oo
Early
Mid
Late
Germarium
Early
Vitellarium
Late
Pedicel
500 µm
Apical tip
Middle
Oviduct end
Control
Ex-colony day 4
Ex-colony day 7
Ex-colony day 10
In-colony day 10
B
i
ii
C
i
D
E
F
G
H
I
J
K
L
M
N
O
P


Control
Ex−colony Day 4
Ex−colony Day 7
Ex−colony Day 10
In−colony Day 10
Radius (µm)
600
400
200
0
−200
13 11 9 7 5 3 1
11 9 7 5 3 1
7 5 3 1
9 7 5 3 1
9 7 5 3 1
Position along the ovariole
NCC
Oocyte
Difference
Dumping
Tipping point


A
B
Ovary area (mm2)
50
40
30
20
10
0
a
b
b
b
c
Control
4(EC)
7(EC)
10(EC)
10(IC)
Days caged

A
NCC
oo
B
oo
C
oo
D
oo
NCC
E
oo
F
NCC
G
H
oo
I
oo
J
K
oo