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3   **Juvenile hormone, but not nutrition or social cues, affects reproductive maturation in**

4   **solitary alkali bees (*Nomia melanderi*)**

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6   Karen M. Kapheim* and Makenna M. Johnson

7   Utah State University, Department of Biology, 5305 Old Main Hill, Logan, UT 84322, USA

8   *Correspondence: Karen M. Kapheim (karen.kapheim@usu.edu)

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14   **Running title:** Reproductive physiology of a solitary bee

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16   **Key words:** alkali bees, Dufour's gland, nutrition, juvenile hormone, reproduction

17

18   Abstract

19   Eusocial insect colonies are defined by extreme variation in reproductive activity among

20   castes, but the ancestral conditions from which this variation arose are unknown.

21   Investigating the factors that contribute to variation in reproductive physiology among

22   solitary insects that are closely related to social species can help to fill this gap. We

23   experimentally tested the role of nutrition, juvenile hormone, and social cues on

24   reproductive maturation in solitary alkali bees (Halictidae: *Nomia melanderi*). We find that

25   alkali bee females emerge from overwintering with small Dufour's glands and small ovaries,

26   containing oocytes in the early stages of development. Oocyte maturation occurs rapidly,

27   and is staggered between the two ovaries. Lab-reared females reached reproductive

28   maturity without access to mates or nesting opportunities, and many had resorbed oocytes.

29   Initial activation of these reproductive structures does not depend on pollen consumption,

30   though dietary protein or lipids may be necessary for long-term reproductive activity. JH is

31   likely to be a limiting factor in alkali bee reproductive activation, as females treated with JH

32   were more likely to develop mature oocytes and Dufour's glands. Unlike for related social

33   bees, the effects of JH were not suppressed by the presence of older, reproductive females.

34   These results provide important insight into the factors that influence reproductive activity

35   in an important native pollinator, and those that may have been particularly important in

36   the evolution of reproductive castes.

37

38   Introduction

39   Complex social organization, such as that observed among honey bees (*Apis mellifera*), ants

40   (Formicidae), and vespid wasps (Vespidae), is marked by a high degree of variance in

41   reproductive activity among individuals within a colony. This variation is demarcated among

42   reproductive castes, whereby workers do not reproduce, despite engaging in maternal

43   behaviors (e.g., brood feeding or nest defense), and queens reproduce while largely

44   refraining from brood care (Michener, 1974). Workers of many social insect species have

45   similar reproductive anatomy to queens (e.g., ovaries, spermathecae, glands, ovipositor),

46   yet remain functionally sterile. This suggests the factors that influence the function of these

47   structures differ between queens and workers, and that understanding this variation may

48   provide insights into the physiological basis for the origin of social insect castes.

49

50   The factors that differentially influence reproductive activation among social insect castes

51   include nutritional, endocrine, and social cues (Kapheim, 2017). For example, both queens

52   and workers acting as nurses within a honey bee colony consume a protein-rich diet, but

53   this protein contributes to egg-production only in the queens (Winston, 1987). Similarly,

54   treatment with the juvenile hormone (JH) analog methoprene leads to accelerated ovarian

55   development in queen paper wasps (*Polistes canadensis*), but instead increases foraging

56   activity in workers (Giray et al., 2005). Finally, social cues, such as aggression from the

57   queen, can repress endocrine pathways, and thus ovary maturation, in worker bumble bees

58   (*Bombus impatiens*) and social halictid bees (*Megalopta genalis*), but aggression directed

59   from workers toward queens does not have the same effect (Kapheim et al., 2016; Padilla

60   et al., 2016; Smith et al., 2009). Understanding how these differences in sensitivity to

61   physiological and environmental cues arise among females and contribute to variation in

62   reproductive activity is thus key to understanding the origins of social insect castes.

63

64   One approach toward this goal is to investigate how these factors contribute to variation in

65   reproductive development in solitary species representative of the ancestors that gave rise

66   to social castes. We conducted two experiments to determine how variation in nutrition, JH,

67   and the social environment influence reproductive development in a solitary bee that shares

68   similarities with the ancestors of social bees. Alkali bees (*Nomia melanderi*) are semi-

69   managed, native pollinators of alfalfa seed crops that range throughout the western U.S.A.

70   (Cane, 2008). This species belongs to a basal subfamily of Halictidae (Nomiinae) in which

71   eusociality has never evolved, but they are closely related to the Halictinae, in which social

72   behavior is highly variable (Michener, 1974; Michener, 2007), and eusociality evolved at

73   least twice (Brady et al., 2006; Danforth et al., 2008; Gibbs et al., 2012). Bees in the family

74   Halictidae shared a common ancestor with the Apidae (e.g., bumble bees, honey bees)

75   approximately 115 mya, and may thus have important differences in reproductive

76   physiology (Cardinal and Danforth, 2013). Alkali bees are considered solitary, because each

77   female has her own nest, but they often nest in close proximity to other females in large

78   aggregations (Cane, 2008; Wcislo and Engel, 1996). Importantly, these bees exhibit

79   extended maternal behavior, characteristic of that which was a necessary pre-adaptation to

80   sociality (Batra, 1970; Batra and Bohart, 1969; Schwarz et al., 2007). As such,

81   understanding the factors that influence reproductive physiology in this species can shed

82   light on the physiological basis for social evolution.

83

84   Little is known about the factors that influence alkali bee reproductive development.

85   However, a recent study demonstrated that JH accelerates reproductive maturation

86   (Kapheim and Johnson, 2017). It was also recently documented that adult female alkali

87   bees consume pollen on a daily basis (Cane et al., 2016). Pollen is the primary source of

88   dietary protein and lipids for bees (Roulston and Cane, 2000), but whether pollen

89   consumption is necessary for reproduction has not been experimentally tested. We

90   investigated two aspects of reproductive physiology – oocyte growth, which requires

91   proteins for egg-yolk (Badisco et al., 2013) and maturation of the Dufour's gland, which

92   secretes lipids used for nest cell construction (Cane, 1981). Our results reveal that

93   vitellogenesis can occur rapidly among newly emerged females, even without mating or

94   nesting opportunity. The initiation of oogenesis and Dufour's gland maturation does not

95   require dietary protein, and females treated with JH were more likely to reach reproductive

96   maturity. This response to JH was not affected by variation in the social environment (i.e.,

97   co-housing with an older, reproductive female). This provides important insight into the

98   physiological foundation from which social insect castes evolved, as well as the reproductive

99   physiology of an important pollinator.

100

101   **Methods**

102   *Collections*

103   This study took place in Touchet, WA, U.S.A, where alkali bees nest in large soil beds near

104   alfalfa seed fields (Cane, 2008). Alkali bees overwinter as prepupae in below-ground nests,

105   and emerge as adults upon completion of development the following summer. We trapped

106   newly eclosed adult females from 27 May-8 June 2016 by placing emergence traps on 3 bee

107   beds prior to the start of emergence. Traps were checked at least 3 times a day, and new

108   bees were transferred back to the laboratory in individually labeled 15 ml tubes placed

109   inside a cooler with a single ice pack placed under a layer of cardboard.

110

111   *Experiment 1 – nutrition effects on reproductive physiology*

112   Upon arrival to the laboratory, bees were chilled at 4ºC for 5 min and randomly assigned to

113   a treatment group: sugar water only (sterile 35% sucrose solution), sugar water with pollen

114   (2.5 g sterile, finely-ground, honey-bee pollen in 30 ml of sterile 35% sucrose solution),

115   sugar water with pollen plus 4 sprigs of fresh, untripped alfalfa flowers. (Bees were

116   observed manipulating these flowers to release pollen on a regular basis throughout the

117   experiment.) Bees were placed in perforated plastic deli containers (72 mm x 90 mm lower

118   diameter x 113 mm upper diameter), and reared in the lab for 10 days (d). Sugar water or

119   pollen mix and alfalfa flowers were changed daily. Pollen-sugar mixture was shaken

120   vigorously before each feeding to achieve homogeneity, and then pipetted into feeding

121   troughs made from 1.5 ml microcentrifuge tubes with the tapered tip removed. The cages

122   were kept at 22-28ºC, 40-85% RH and full spectrum lighting 13 L: 11 D, as has been

123   previously described (Kapheim and Johnson, 2017). At the end of the 10 d rearing period,

124   bees were chilled for 3 min at 4ºC, placed in individually-labeled tubes, and flash-frozen in

125   liquid nitrogen.

126

127   We also collected newly emerged females and reproductive females of unknown age for

128   comparison to lab-reared females. Newly emerged females were collected from emergence

129   traps as described above, but were flash-frozen immediately upon return to the laboratory.

130   Reproductive females were identified as those returning to a nest hole with pollen on their

131   hind legs. They were captured by net, and flash-frozen immediately upon return to the

132   laboratory.

133

134   *Experiment 2 – social and endocrine effects on reproductive physiology*

135   Newly emerged females were collected as in Experiment 1. Each bee was randomly

136   assigned to a treatment group: sham control, solvent control, or JH. For JH treatments, JH-

137   III (product E589400, Toronto Research Chemicals, Inc., Toronto, Ontario, Canada) was

138   dissolved in dimethylformamide (DMF) at a concentration of 50 µg per µl. Bees in the

139   solvent control group received 1 µl of DMF applied to the thorax with a pipette tip. Bees in

140   the JH group received 50 µg JH in 1 µl of DMF applied to the thorax with a pipette tip. Bees

141   in the sham group were touched lightly on the thorax with a clean pipette tip. Hormone

142   treatments were repeated when bees were 5 d old. Bees in each treatment group were

143   randomly assigned to be caged alone or with an older, reproductive female, defined as

144   above. All bees were paint-marked on the dorsal abdomen with a uniquely colored

145   Decocolor© paint pen (Uchida of America Co, Torrance, CA). All bees were reared in cages,

146   and received 35% sugar water mixed with pollen and fresh alfalfa flowers for 10 d, as

147   described for Experiment 1.

148

149   Upon collection, all bees from both experiments were stored in liquid nitrogen until return to

150   Utah State University, where they were transferred to a -80 ºC freezer.

151

152   *Dissections*

153   Dissections followed previously reported methods (Kapheim and Johnson, 2017). Briefly,

154   bees were dissected under a Leica M80 stereomicroscope fitted with an IC80HD camera

155   (Leica Microsystems, Buffalo Grove, IL, USA). We measured Dufour's gland and terminal

156   oocyte lengths from images, using software in the Leica Application Suite (v. 4.5). The

157   observer was blind to the treatment group of each bee during dissections, and both authors

158   concurred on measurements. Mating status was determined by examination of the

159   spermatheca under a compound microscope. We excluded newly emerged females with

160   sperm and reproductive females without sperm from further analyses.

161

162   *Stages of ovary maturation*

163   Images were further analyzed to classify ovaries into stages of oocyte maturation. Like most

164   halictid bees, alkali bees have three ovarioles in each of two ovaries. The following

165   categories were modified from Duchateau and Velthuis (1989) and Oliveira et al. (2017).

166   Stage I – the oocyte and associated trophocytes occupying a single egg chamber can be

167   distinguished from one another, but the oocyte is much smaller than the trophocytes and is

168   spherical in shape. Stage II – the oocyte is smaller than the trophocytes, and is cylindrical

169   in shape rather than round. Stage III – the trophocytes and oocyte are similar in size, the

170   oocyte occupying 45-55% of the length of the egg chamber. The oocyte is elongated. The

171   trophocytes appears less opaque, and the oocyte appears more solid and full. It is during

172   this stage in which vitellogenesis is initiated. Stage IV – the oocyte is much longer than the

173   trophocytes, and occupies more than 55% of the total egg chamber. The trophocytes

174   appear translucent and small in the anterior portion of the egg chamber. Stage IVr

175   (reabsorbing) – the oocyte has all the characteristics of stage IV, but is misshapen and

176   yellow in color, indicating reabsorption. Stage V – the oocyte is large, robust, and opaque

177   with no associated trophocytes. Vitellogenesis is complete at this stage. Stage Vr

178   (reabsorbing) – oocyte has no trophocytes present, but has the characteristics of being

179   reabsorbed. When possible, we measured the length of the maximum terminal oocyte and

180   its associated trophocytes of both resorbing (if present) and viable oocytes, and used these

181   measurements to classify stage of ovary maturation. We identified the maximum terminal

182   oocyte and maximum stage of oocyte maturation as the longer/more mature from the two

183   ovaries. We used these maxima for statistical analyses.

184

185   *Pollen quantification*

186   To determine whether our diet treatments were effective, we quantified the amount of

187   pollen consumed by lab-reared females receiving pollen, relative to reproductive females, by

188   estimating the number of pollen grains in the hindgut. We followed previously described

189   methods (Cane et al., 2016) to estimate pollen grains in 6 hindguts from each group.

190   Individual hindguts were placed in 0.5 ml microcentrifuge tubes with 50 µl of 70% ethanol

191   and torn apart with forceps. After guts were shredded, the mix was vortexed for 5 sec,

192   using Vortex Genie 2 on highest setting of 10. The shredded gut was then removed using

193   forceps, dabbing tissue on sides of the tube to remove excess ethanol and pollen. Each

194   sample was vortexed on the highest setting for 10 seconds immediately prior to loading 10

195   μl of the solution into one chamber of a hemocytometer for pollen counting. Pollen grains

196   were counted across the entire chamber under a compound microscope at 20X

197   magnification. Three different 10 μl aliquots were counted for each sample, using the entire

198   chamber each time. For each sample, the average of these three counts was divided by

199   0.0009 ml, the volume of each hemocytometer chamber, and then multiplied by the volume

200   of ethanol used per sample (0.05 ml).

201

202   *Statistical analyses*

203   All statistical analyses were performed in R version 3.2.5 (R Core Team, 2016). Visual

204   inspection of a qq-plot (R package "car", (Fox and Weisberg, 2011)) and an Anderson-

205   Darling normality test (R package "nortest", (Gross and Ligges, 2015)) revealed significant

206   departures from normality in the distribution of maximum terminal oocyte and Dufour's

207   gland lengths for Experiment 1, but not Experiment 2. We therefore applied a Box-Cox

208   transformation to the data for Experiment 1 before running the final model (Venables and

209   Ripley, 2002). For both Experiment 1 and 2, we modeled the maximum viable terminal

210   oocyte and Dufour's gland lengths with separate linear mixed effects regressions that

211   initially included intertegular width and treatment (coded as factors: diet for Experiment 1,

212   JH*social for Experiment 2) as fixed effects, with bee bed of origin as a random effect

213   (Bates et al., 2015). In each case, the variance and standard deviation for the intercept of

214   bee bed was zero, so a linear model without random effects was used for subsequent

215   analyses. Intertegular width was removed from the final models in the cases where it was

216   non-significant (p > 0.05) – all except Dufour's gland length in Experiment 2. We used

217   Tukey post-hoc tests to investigate significant differences between treatment groups

218   (Hothorn et al., 2008).

219

220   For Experiment 2, we repeated the analyses after removing cases where the older,

221   reproductive partner had a smaller intertegular width, maximum terminal oocyte, or

222   Dufour's gland than the newly emerged cage-mate to determine whether relative size or

223   reproductive development influenced the outcome of the social treatment.

224

225   We compared stages of oocyte maturation across treatment groups in Experiment 1 with

226   ordinal logistic regression (R package "ordinal", (Christensen, 2015)). However, this model

227   was not appropriate for the data in Experiment 2, due to low representation of some values.

228   We compared the proportion of oocyte maturation stage among JH treatment groups in

229   Experiment 2 using a chi-square test (R package "stats", (R Core Team, 2016)). We also

230   used a chi-square test to compare the proportion of samples with and without resorbed

231   oocytes across groups in Experiments 1 and 2.

232

233   Final estimates of pollen counts in the hindgut were compared between groups

234   (reproductive, nesting females, sugar & pollen mix, sugar, pollen, & alfalfa flowers) with a

235   linear model function (lm), after applying a Box-Cox transformation of the data (Venables

236   and Ripley, 2002).

237

238   **Results**

239   *Patterns of ovary maturation*

240   We used the reproductive and newly emerged females to describe patterns of oocyte

241   maturation in alkali bees. Newly emerged bees activate their oocytes rapidly, even without

242   mating. The modal stage of maturation for newly emerged females was one viable stage I

243   oocyte, with a viable stage I or II oocyte in the other ovary. However, three newly emerged

244   females had viable stage IV or V oocytes in both ovaries. It is possible that these females

245   had spent a day or more in their nests upon eclosion, and were therefore slightly older than

246   the others. Newly emerged females did not show any evidence of resorbing oocytes.

247

248   The modal stage of maturation for reproductive females was one viable stage IV oocyte and

249   one viable stage IV or V oocyte in the other ovary. The maximum viable terminal oocytes in

250   both ovaries were vitellogenic (stage III, IV, or V) for all reproductive females, except one

251   with a pre-vitellogenic (stage II) oocyte in one ovary. Most (70%) reproductive females had

252   at least one resorbing oocyte, and 35% had a resorbing oocyte in both ovaries. Resorbing

253   oocytes were all in either category IVr or Vr, with 58% in stage Vr. The maximum viable

254   terminal oocytes in ovaries with a resorbing oocyte were in stage III or IV, indicating that

255   oocyte maturation is sequential across ovarioles within an ovary.

256

257   *Experiment 1 – nutrition effects on reproductive physiology*

258   Mortality was not significantly different among lab-reared females on different diet

259   treatments (mortality: sugar – 40%, sugar & pollen – 32%, sugar, pollen, & flowers – 46%;

260    $\chi^2 = 1.29$, $p = 0.52$, $n = 92$). There were significant differences in both maximum viable

261   terminal oocyte and Dufour's gland length among treatment groups (oocytes: $F_{4,89} = 30.68$,

262    $r^2 = 0.58$, $p < 4.79 \times 10^{-16}$, Table S1; Dufour's: $F_{4,101} = 45.80$, $r^2 = 0.64$, $p < 2.20 \times 10^{-16}$,

263   Table S2). Among these groups, lab-reared, 10 d old females had significantly longer viable

264   maximum terminal oocytes and Dufour's glands than newly emerged females (Fig. 1).

265   However, actively nesting reproductive females had significantly more developed

266   reproductive anatomy than either newly emerged or lab-reared females (Fig. 1). We did not

267   observe significant differences in maximum viable terminal oocyte or Dufour's gland length

268   among females reared in the lab for 10 d on different diets (Fig. 1).

269

270   The maximum viable terminal oocytes of newly emerged females were at significantly lower

271   stages of development than those of lab-reared 10 d old females or reproductive females,

272   but there were no significant differences between females in the latter groups, all but one of

273   whom had vitellogenic oocytes (stage III or higher) (ordinal logistic regression: $Z = -4.61$, p

274    $= 3.99 \times 10^{-6}$; Fig. 2). Three lab-reared 10 d old females that received a sugar and pollen

275   diet developed viable mature oocytes (stage V). Approximately half of the lab-reared

276   females had at least one resorbing oocyte (sugar only – 50%, sugar & pollen – 50%, sugar,

277   pollen, & flowers – 57%), and all except one (stage Vr) were in stage IVr. The proportion of

278   females with resorbing oocytes among lab-reared females was not significantly different

279   from that of reproductive females ($\chi^2 = 2.10$, $p = 0.55$, $n = 70$ bees). However, most

280   (58%) of the resorbing oocytes in reproductive females were in a more advanced stage

281   (Vr).

282

283   The estimated number of pollen grains detected in the hindguts was not significantly

284   different among reproductive females and the two groups of lab-reared females that

285   received pollen in their diet ($F_{2,15} = 3.32$, $r^2 = 0.31$, $p = 0.06$, Fig. 3).

286

287   *Experiment 2 – social and endocrine effects on reproductive physiology*

288   Mortality was not significantly different among treatment groups in Experiment 2 (mortality:

289   sham/solitary – 19%, sham/social – 19%, DMF/solitary – 20%, DMF/social – 47%,

290   JH/solitary – 35%, JH/social – 44%; $\chi^2 = 7.04$, $p = 0.22$, $n = 101$). There were significant

291   differences in maximum viable terminal oocyte and Dufour's gland length among treatment

292   groups (oocytes: $F_{5,41} = 6.68$, $r^2 = 0.45$, $p = 1.23 \times 10^{-4}$, Table S3; Dufour's: $F_{6,57} = 8.77$, r^2

293    $= 0.48$, $p = 8.97 \times 10^{-7}$, Table S4). Females treated with JH had significantly longer viable

294   terminal oocytes and Dufour's glands than females in control groups, but variation in the

295   social environment did not have a significant effect on these measures of reproductive

296   physiology (Fig. 4).

297

298   Reproductive females and newly emerged females paired in the social treatments were
299   similar in size to one another (mean ratio of intertegular width = 1.03 ± 0.11 s.d.). On
300   average, the reproductive females had longer viable oocytes and Dufour's glands than their
301   newly emerged cage-mates (mean ratio of maximum terminal oocyte length = 1.72 ± 0.62,
302   mean ratio of Dufour's glands = 1.13 ± 0.17). However, there were 12 cases for which the
303   reproductive female was smaller and/or had smaller ovaries or Dufour's glands than their
304   newly emerged female cage-mates. Elimination of these 12 cases from the dataset did not
305   change the results (Table S5-S6).

306

307   There were significant differences in stage of oocyte maturation among JH treatment groups
308   (χ^2 = 23.20, p = 7.31x10^{-4}, n = 40 bees). Only females treated with JH had viable mature
309   oocytes (stage V), and sham treated bees were the only group with pre-vitellogenic (stage
310   II) oocytes (Fig. 5). Most (78%) of the JH-treated females had at least one resorbing
311   oocyte, while 55-58% of the females in the control treatments had a resorbing oocytes.
312   However, this difference was not statistically significant (χ^2 = 2.56, p = 0.28, n = 64).
313   Resorbed oocytes were in stage IVr and Vr in each group.

314

315   **Discussion**
316   Variation in reproductive physiology is a hallmark of the social insect societies, in which just
317   one or a few individuals out of thousands within a colony are reproductively active, despite
318   shared genetic influences. Investigating the factors that contribute to reproductive variation
319   in solitary relatives of social insects can provide clues as to how reproductive castes evolve
320   (Kapheim, 2017). Our results demonstrate that solitary alkali bees do not require dietary
321   protein during the initial stages of reproductive maturation, but that JH enhances this
322   process. We also find that, unlike for social bees, interactions between conspecifics do not
323   influence reproductive physiology. This provides important information about the
324   physiological foundation from which reproductive castes emerged.

325

326   Access to dietary protein did not limit reproductive activation among newly emerged alkali
327   bees, but it was rarely sufficient for reproductive maturation. Most of the lab-reared females
328   in our study did not develop mature (stage V or Vr) oocytes or Dufour's glands during the
329   10 d study period, despite having similar amounts of pollen in their hindguts as actively
330   nesting, reproductive females, indicating they had consumed ecologically relevant amounts
331   of pollen during the experiment. Alkali bees commonly begin laying eggs within a few days
332   of eclosion, and some of the newly emerged bees had mature oocytes, indicating that our

333   study period provided ample time for reproductive maturation (Bohart and Cross, 1955). It

334   is possible that the completion of reproductive maturation is hastened by ecological cues,

335   such as nesting substrate, or access to mates. Seminal fluid is known to trigger oogenesis in

336   several insect species (Avila et al., 2011), and mating limitation is known to influence

337   reproductive activity in other halictid bees (Yanega, 1989; Yanega, 1992). However, lab-

338   reared females of another halictid bee, *Megalopta genalis*, reached reproductive maturity

339   when reared, unmated, in the lab for 10 days (Kapheim et al., 2012). If mating is a

340   reproductive limitation in alkali bees, it can apparently be overridden by JH treatments, as

341   JH treated females in our study were more likely to reach reproductive maturity, even in the

342   absence of mating or ecological cues. Regardless of the role of dietary protein in the

343   initiation of reproductive activation, the fact that alkali bees and other closely related

344   halictid bees consume pollen on a daily basis suggests that protein is likely necessary for

345   sustained reproductive activity throughout the breeding season (Cane et al., 2016;

346   Wuellner, 1999).

347

348   Our results are in contrast to results of similar studies of solitary bees in the family

349   Megachilidae, *Osmia californica* and *Megachile rotundata*, which showed access to dietary

350   protein is essential for reproductive maturation among newly emerged females (Cane,

351   2016; Richards, 1994). Unlike alkali bees, *Osmia* overwinter as adults, begin oogenesis prior

352   to eclosion, and thus eclose with depleted protein stores (Wasielewski et al., 2011). Protein

353   stores have not been measured in newly emerged alkali bees, but remaining reproductively

354   quiescent until eclosion is likely to be less energetically expensive and may be associated

355   with increased availability of nutrient stores for post-eclosion maturation (Hahn and

356   Denlinger, 2007). Alkali bees may therefore be better poised to initiate oogenesis without a

357   dietary protein source. Conversely, pollen is necessary to stimulate vitellogenesis in *M.*

358   *rotundata*, which also remains reproductively quiescent until ecolosion. The apparent

359   difference in nutritional requirements for oogenesis among megachilid and halictid bees

360   indicates that the physiological basis for reproductive activity is highly variable among

361   solitary bees. This suggests that assumptions about reproductive physiology among the

362   ancestors of social bees should be made with caution. Additional research on solitary bees

363   from additional families in which eusociality has evolved (e.g., Apidae) are necessary to fully

364   understand variation in nutritional requirements for reproduction.

365

366   Our results suggest that JH is a limiting factor in reproductive maturation among alkali bees.

367   Females treated with JH were significantly more likely to have developed viable mature

368 (stage V) oocytes and Dufour's glands, though other groups had mature resorbing (stage

369 Vr) oocytes. This is consistent with earlier results (Kapheim and Johnson, 2017), and

370 provides evidence of a conserved gonadotropic role for JH in alkali bees. In most insects,

371 including bumble bees, JH stimulates the synthesis of vitellogenin, an egg-yolk precursor

372 protein necessary for oocyte maturation (Amsalem et al., 2015; Badisco et al., 2013). This

373 suggests the gonadotropic response to JH may depend on a dietary source of protein. All of

374 the females receiving JH treatments in our study also received dietary protein from pollen,

375 and thus had the nutritional resources necessary to complete vitellogenesis. Future studies

376 are needed to determine how nutrition and JH pathways interact in alkali bee oogenesis.

377

378 The path by which JH stimulates Dufour's gland maturation is less clear, as Dufour's gland

379 evolved in the ancestor of Hymenoptera, and secretes chemicals with a wide range of

380 functions within this group (Mitra, 2013). Dufour's gland is likely derived from the colleterial

381 accessory gland in other insects (Mitra, 2013), and reproductive maturity of this gland is

382 induced by JH in cockroaches (*Byrsotria fumigate, Periplaneta americana*) (Bell and Barth,

383 1970; Willis and Brunet, 1966). Moreover, JH influences the chemical composition of

384 Dufour's gland secretions in bumble bee (*B. terrestris*) workers (Shpigler et al., 2014). This,

385 along with our results, suggests that endocrine regulation of Dufour's gland is deeply

386 conserved among insects. Additional research is needed to determine the molecular

387 mechanisms by which JH affects Dufour's gland function.

388

389 Unlike for social bees and some solitary bees, variation in the social environment does not

390 influence reproductive physiology among solitary alkali bees. Most of the 20,000 species of

391 bees are solitary, mass-provisioning, and annual (Michener, 2007). This means that after

392 mating, females build a nest, provision each brood cell with pollen and nectar, lay an egg on

393 top of those provisions, seal the cell, and die before any of her offspring complete

394 development. For many of these species, a single mating event is the only interaction they

395 have with conspecifics in their entire lives. Conversely, highly eusocial insects have evolved

396 sophisticated forms of social communication. Within colonies of honey bees, bumble bees,

397 and sweat bees, behavior and reproductive physiology is dynamically regulated by the

398 behavior and/or pheromones of nestmates (Alaux et al., 2009; Amsalem and Hefetz, 2010;

399 Arneson and Wcislo, 2003; Grozinger et al., 2003; Huang et al., 1998; Kapheim et al.,

400 2016; Le Conte et al., 2001; Li-Byarlay et al., 2014; Padilla et al., 2016; Smith et al.,

401 2009). Research with a facultatively eusocial halictid bee, *M. genalis*, suggests that

402 aggression from older, reproductive females can limit reproductive development via JH-

403   suppression in newly emerged females (Kapheim et al., 2016; Smith et al., 2009; Smith et

404   al., 2013). However, the degree to which solitary bees respond to social cues varies among

405   species. For example, experimental co-housing of otherwise solitary *Ceratina* bees (Family

406   Apidae) results in division of labor and reproductive suppression in species that occasionally

407   share nests in nature (Sakagami and Maeta, 1977; Sakagami and Maeta, 1984; Sakagami

408   and Maeta, 1989; Sakagami and Maeta, 1995), but apparently not in species that never

409   share nests, like alkali bees. Although we did not directly measure behavioral interactions

410   among pairs as part of our study, we routinely observed aggressive exchanges among pairs

411   of females in cages. Our results thus suggest that sensitivity to cues from the social

412   environment observed in social halictid bees are not conserved in their solitary relatives.

413   Alkali bees nest in extremely dense aggregations, with up to 713 nests per square meter in

414   study area (Cane, 2008). At high density, these ground-nesting females are likely to

415   encounter each other regularly as they dig tunnels and build cells, and there is thus likely to

416   be strong selection against physiological sensitivity to social interactions in these

417   populations. Similar studies with additional solitary bees are necessary to identify the

418   circumstances under which sensitivity to the social environment influences reproductive

419   physiology.

420

Conclusions

421   **Conclusions**

422   This study is the first experimental investigation of dietary, endocrine, and social effects on

423   reproductive maturation in a solitary bee closely related to lineages in which sociality

424   evolved, but which is highly divergent (>100 my) from the more commonly studied eusocial

425   honey bees and bumble bees (Cardinal and Danforth, 2013). Our results reveal that the

426   factors contributing to the initiation of reproductive activation and completion of

427   reproductive maturity may be different. Specifically, dietary protein was not essential for

428   the initiation of reproductive activation, but was rarely sufficient for reproductive

429   maturation. JH, however, may be a limiting factor in maturation of both oocytes and

430   Dufour's gland. This provides important insight into how sensitivity to these cues evolved

431   with the origin of reproductive castes in social insects. For example, the effects of JH on

432   ovary and Dufour's gland maturation are apparently conserved between solitary alkali bees

433   and bumble bee workers. However, these JH effects are responsive to social status in

434   bumble bees, but not alkali bees (Amsalem et al., 2014; Shpigler et al., 2014). This

435   suggests that different components of the endocrine networks influencing reproductive

436   physiology were independently modified during social evolution. Also, nutrition and cues

437   from the social environment are some of the most important factors in reproductive

438   suppression of workers among social bees (Amsalem et al., 2013; Kapheim et al., 2016;

439   Lawson et al., 2016; Padilla et al., 2016), but these factors did not have a significant

440   influence on variation in reproductive activation in solitary alkali bees. This suggests that

441   changes in how nutrient-sensing and environment-sensing pathways regulate reproductive

442   physiology were especially important in the evolutionary origins of reproductive castes.

443   Further comparisons of the molecular networks underlying the physiological response to

444   nutritional, endocrine, and social cues across species are likely to provide key insight into

445   how reproductive division of labor evolves.

446

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455

Competing interest

457   The authors declare no competing or financial interests.

458

Author contributions

460   KMK designed the study. KMK and MMJ conducted the experiments and analyses. KMK

461   drafted the initial manuscript. KMK and MMJ revised the manuscript and approved the final

462   version.

463

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631

632   **Figure Legends**

633

634   **Figure 1. Effects of diet on reproductive maturation in alkali bees.** (A) Maximum
635   viable terminal oocyte length and (B) Dufour's gland length were significantly
636   different between newly emerged, lab-reared, and nesting females (oocytes: $F_{4,89}$ =
637   30.68, r^2 = 0.58, $p < 4.79 \times 10^{-16}$; Dufour's: $F_{4,101}$ = 45.80, r^2 = 0.64, $p < 2.20 \times 10^{-16}$;
638   n = individual bees, newly emerged – 36, sugar – 14, sugar & pollen – 22, sugar,
639   pollen & alfalfa – 14, reproductive – 20). Diet did not have a significant effect on
640   reproductive development when newly emerged females were reared in the lab for
641   10 d. Boxes represent the interquartile range, with the line as the median. Whiskers
642   extend to 1.5 times the interquartile range. Circles are outliers. Letters indicate
643   significant differences ($p < 0.001$ in Tukey post-hoc tests). White boxes = newly
644   emerged females, Grey boxes = lab-reared 10 d old females; Dark grey boxes =
645   Reproductive, nesting females; Full model results are available in the supplementary
646   materials.

647

648   **Figure 2. Effects of diet on oocyte maturation in alkali bees.** Newly emerged females

649   had oocytes at significantly earlier stages of maturation than females in the other

650   treatment groups (ordinal logistic regression: $Z = -4.61$, $p = 3.99 \times 10^{-6}$, n =

651   individual bees, newly emerged – 26, sugar – 11, sugar & pollen – 20, sugar, pollen

652   & alfalfa – 10, reproductive – 7). Shading within bars indicates the proportion of

653   maximum viable terminal oocytes in each stage of maturation, with stage I and II as

654   pre-vitellogenic, III and IV as vitellogenic, and V as mature.

655

656   **Figure 3. Estimates of pollen grains in the hindguts of female bees.** There were no

657   significant differences in hindgut pollen content between reproductive females and lab-

658   reared females in posthoc tests ($F_{2,15} = 3.32$, $r^2 = 0.31$, $p = 0.06$; n = 6 bees per group).

659   Boxes represent the interquartile range, with the line as the median. Whiskers extend to 1.5

660   times the interquartile range.

661

662   **Figure 4. Effects of endocrine and social treatments on reproductive development in**

663   **alkali bees.** (A) Maximum terminal oocyte length and (B) Dufour's gland length were

664   significantly different between lab-reared females treated with JH versus controls,

665   but variation in the social environment did not significantly affect reproductive

666   development (oocytes: $F_{5,41} = 6.68$, $r^2 = 0.45$, $p = 1.23 \times 10^{-4}$; Dufour's: $F_{6,57} = 8.77$,

667   $r^2 = 0.48$, $p = 8.97 \times 10^{-7}$); n = individual bees, sham/solitary – 12, sham/social – 12,

668   DMF/solitary – 12; DMF/social – 10; JH/solitary – 10; JH/social – 8). Boxes represent

669   the interquartile range, with the line as the median. Whiskers extend to 1.5 times

670   the interquartile range. Circles are outliers. Letters indicate significant differences (p

671   < 0.05 in Tukey post-hoc tests). Grey bars = social treatment, white bars = solitary

672   treatment; Full model results are available in the supplementary materials.

673

674   **Figure 5. Effects of JH on oocyte maturation in alkali bees.** There were significant

675   differences in stage of oocyte maturation among JH treatment groups ($\chi^2 = 23.20$, p

676   $= 7.31 \times 10^{-4}$, n = individual bees, sham – 16, DMF – 17, JH - 7). Shading within bars

677   indicates the proportion of maximum viable terminal oocytes in each stage of

678   maturation, with stage I and II as pre-vitellogenic, III and IV as vitellogenic, and V

679   as mature.

680