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► **To cite this version:**

Anthony G.E. Mathiron, Ryan Earley, Marlène Goubault. Juvenile hormone manipulation affects female reproductive status and aggressiveness in a non-social parasitoid wasp. *General and Comparative Endocrinology*, 2019, 274, pp.80-86. 10.1016/j.yggen.2019.01.006 . hal-02140550

**HAL Id: hal-02140550**

**<https://hal.science/hal-02140550v1>**

Submitted on 22 Oct 2021

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# 1 Juvenile Hormone manipulation affects female reproductive status and 2 aggressiveness in a non-social parasitoid wasp

3  
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13

14 **Abstract.** In vertebrates, titers of androgens such as testosterone are known to upregulate aggressive  
15 behaviors associated with reproduction. In insects, juvenile hormone (JH) is a good candidate for  
16 studying the flexibility of insect endocrine responses because it has important effects on both  
17 reproductive processes and behavior. JH has a gonadotropic effect across a broad range of insect  
18 species, increasing ovarian development in females, and may have a role in the regulation of  
19 aggressive behavior during competition. In Hymenoptera, the functions of JH have been studied in  
20 facultatively eusocial species such as polistine wasps, bumblebees, ants and bees. Surprisingly, no  
21 work has yet focused on the relationship between JH, reproduction and aggressiveness in a non-  
22 social Hymenoptera, although it may help to understand how JH actions have evolved across taxa  
23 with different degrees of sociality. Here, we explored how JH treatment influenced: i) female  
24 reproductive status, and ii) the intensity (aggressiveness) and resolution of conflict, in *Eupelmus*  
25 *vuilleti* (Hymenoptera: Eupelmidae), a solitary ectoparasitoid wasp in which females fight over hosts.

26 We demonstrated that intra-abdominal injections of JH increased the number of mature eggs in  
27 females after 24h. In addition, the number of aggressive behaviors displayed by females was affected  
28 by the interaction between JH treatment and the number of mature eggs in their abdomen, but mature  
29 egg load alone predicted the outcome of staged contests. Wasps were more aggressive when they had  
30 more ready-to-lay eggs, with this effect being stronger when females were injected with JH.  
31 Moreover, females won more frequently when they had higher mature egg load. Our results highlight  
32 how JH affects egg maturation and aggressive behaviors in *Eupelmus vuilleti* females. To our  
33 knowledge, this is the first study showing that hormone manipulation can modulate females'  
34 reproduction status and behavior during intraspecific competition over hosts in a non-social  
35 hymenopteran parasitoid.

36 **Keywords:** juvenile hormone, reproductive maturation, aggressive behaviors, conflict resolution

37

## 38 **1. Introduction**

39 As gaining access to resources brings fitness benefits to individuals, both vertebrate and  
40 invertebrate animals commonly exhibit aggressive behaviors during conflicts over such resources  
41 (Collias, 1944; Breed and Bell, 1983; Briffa and Hardy, 2013). For example, in parasitoid insects,  
42 hosts represent an indivisible resource over which females compete to lay their eggs, and physical  
43 interactions between females for host access have been observed in various parasitoid species  
44 (Hughes et al., 1994; Petersen and Hardy, 1996; Field and Calbert, 1999; Goubault et al., 2007;  
45 Mohamad et al., 2010, Mathiron et al., 2018). In many animal taxa, aggressive conflicts have also  
46 been observed between individuals for sexual partners (West-Eberhard, 1979; Schwagmeyer and  
47 Woontner, 1985; Simmons, 1986), food (Barton, 1993; Vogel, 2005), territories (Rowland, 1989;  
48 Festa-Bianchet et al., 1990), and dominance status (Chase, 1974). However, being constantly  
49 aggressive can be costly, as potential physical injuries can lead to death (Palombit, 1993; Innocent et  
50 al., 2011), and decreased vigilance can increase predation risk (Jakobsson et al., 1995; Díaz-Uriarte,

51 1999). Animals should therefore evaluate the costs and benefits of exhibiting agonistic behaviors and  
52 then behave appropriately (Maynard-Smith and Price, 1973).

53 Endocrine regulation is one important way that animals can match behavioral and  
54 physiological responses to their current environment (Tibbetts and Crocker, 2014). In vertebrates,  
55 testosterone is generally involved in the modulation of aggressive behaviors during conflicts  
56 associated with reproduction, such as the establishment and maintenance of breeding territories, mate  
57 access, and offspring defense (Wingfield, 1984). For example, Salvador et al. (1996) showed that  
58 testosterone supplementation in large males of the territorial lizard *Pseudis algirus* increased  
59 the number of chasing behaviors toward conspecific intruders. Moreover, during mate choice, female  
60 zebra finches treated with testosterone were more aggressive toward conspecific females than control  
61 individuals (Adkins-Regan, 1999). Whilst well- described in vertebrates, the endocrine basis of  
62 behavior is still relatively unknown in insects (Zera, 2007; Tibbetts et al., 2014). Because vertebrates  
63 and insects have similar behaviors but different endocrine systems (Nijhout, 1994; Adkins-Regan,  
64 2005), exploring how hormones modulate insects behaviors can therefore provide insight into the  
65 evolution of endocrine regulation of behavior (Emlen and Nijhout, 2000).

66 In insects, juvenile hormone (JH) is a good candidate for studying the flexibility of endocrine  
67 responses to intraspecific conflict because it has important effects on both reproductive processes  
68 and behavior. Together with nutrition (see the review of Wheeler, 1996), JH plays an important and  
69 diverse role in reproduction in many adult insect species by regulating vitellogenesis in the fat body  
70 and vitellogenin uptake by the ovaries (reviewed by Tobe and Stay, 1985; Nijhout, 1994; Robinson  
71 and Vargo, 1997; Hartfelder, 2000). In Hymenoptera, JH is involved in the regulation of ovarian  
72 development in the facultatively eusocial sweat bee *Megalopta genalis* (Smith et al., 2013) and in  
73 primitively social species like polistine wasps (Barth et al., 1975), halictine bees (Bell, 1973) and  
74 bumblebees (Bloch et al., 2000; Amsalem et al., 2014). However, in the highly social honeybee *Apis*  
75 *mellifera*, JH is apparently involved in the regulation of division of labor but has no clear role in the

76 regulation of reproduction (reviewed in Robinson, 1992; Robinson and Vargo, 1997). In many  
77 eusocial hymenopterans, JH is also associated with displays of behaviors often judged to be  
78 aggressive (Tibbetts and Crocker, 2014). For example, in the epiponine wasp *Polybia micans*, JH  
79 may fuel aggressive behaviors of single queens heading a colony (Kelstrup et al. 2014a). Similar  
80 observations have been made in paper wasps, in which high JH titers are associated with dominance  
81 behaviors (Röseler et al., 1980, 1984; Giray et al., 2005; Tibbetts and Huang, 2010; Tibbetts et al.,  
82 2013; Kelstrup et al. 2016; but see Kelstrup et al., 2015). In honeybees, JH titers are higher in more  
83 aggressive workers and JH treatments cause honeybees to exhibit more frequent guarding behaviors  
84 (Sasagawa et al., 1989; Pearce et al., 2001). However, JH has no effect on aggression in the swarm-  
85 founding epiponine wasp *Synoecca surinama* (Kelstrup et al., 2014b) and may even be negatively  
86 correlated with aggression in the queenless ponerine ant, *Streblognathus peetersi* (Brent et al., 2006).  
87 Surprisingly, no work has yet focused on the relationship between JH, reproduction, and  
88 aggressiveness in strict non-social Hymenoptera species, although it may help to understand how JH  
89 actions have evolved across insect taxa with different degrees of sociality.

90 In the present study, we investigated how JH treatment influences: i) female reproductive  
91 status, and ii) aggressive behaviors exhibited during contests over host access as well as the outcome  
92 of such contests, using *Eupelmus vuilleti* Crawford (Hymenoptera: Eupelmidae). *Eupelmus vuilleti* is  
93 a solitary ectoparasitoid (i.e. only one juvenile can develop per host, with supernumerary offspring  
94 being killed during larval competition). Females parasitize and feed upon larvae and pupae of  
95 *Callosobruchus maculatus* (Fab) (Coleoptera: Bruchidae), which infest cowpea seeds, *Vigna*  
96 *unquiculata* (L.) Walp (Fabaceae; Terrasse and Rojas-Rousse, 1986). When several females are  
97 simultaneously present on a host patch, they tend to protect the host that they exploit by displaying  
98 aggressive behaviors towards conspecific competitors (Mohamad et al., 2010; Mathiron et al., 2018).  
99 Previous studies have investigated the role of different factors (e.g., egg load, host density and host  
100 quality) on aggressiveness and contest outcomes in *E. vuilleti* (Mohamad et al., 2010, 2012, 2013;

101 Mathiron et al., 2018), but nothing is known about how hormones modulate aggressiveness during  
102 competition for host access.

103 While measuring variation in hormone titers is an important first step to understand the  
104 endocrine basis of phenotypic plasticity (Zera, 2007), one method that allows for understanding  
105 individual responses to hormonal change is to administer a similar amount of hormone to numerous  
106 individuals and, subsequently, to test how hormone manipulations affect the phenotype (Tibbetts and  
107 Izzo, 2009). As such, manipulation of JH is one of the most common methods employed to  
108 understand its mode of action and functions in insects (Sláma, 1971; Ramaseshadri et al., 2012). A  
109 first experiment was therefore designed to determine if JH treatment affected female reproductive  
110 status. As for many other insect species, we expected JH to have a gonadotropic effect in females,  
111 i.e. that wasps treated with JH would have more ready-to-lay eggs than control females (i.e. treated  
112 with vehicle). In a second experiment, we studied whether aggressiveness and resolution of conflicts  
113 between females were influenced by a JH treatment. We expected that individuals treated with JH  
114 would display more aggressive behaviors and would ultimately be more likely to win contests for  
115 hosts relative to control females.

116

## 117 **2. Material and Methods**

### 118 *2.1. Laboratory breeding*

119 In 2007, *E. vuilleti* emerged from cowpea seeds collected in Togo (West Africa) and infested  
120 by the bruchid beetle *Callosobruchus maculatus*. Parasitoids were then bred in the laboratory (IRBI,  
121 University of Tours, France) on larval and pupal stages of *C. maculatus*, according to the  
122 methodology described by Jaloux et al. (2004). All experimental procedures were carried out in a  
123 climate room at 30°C and 12:12 h light: dark. Cowpea seeds were dissected to collect hosts for *E.*  
124 *vuilleti*, which were then individually placed in a standard transparent gelatin capsule (length: 2 cm,  
125 diameter: 0.6 cm). This system mimics a cowpea seed without significantly altering the oviposition

126 behavior of females, and facilitates observation of the number of eggs laid by females (Gauthier and  
127 Monge, 1999). Previous studies have shown that after a period of acclimation, parasitoid females  
128 behave in the same manner to the presence of hosts inside seeds and capsules (Gauthier and Monge,  
129 1999; Jaloux et al., 2004), although the number of eggs laid on hosts is slightly lower inside capsules  
130 (Damiens et al., 2001).

131 Females used in the following experiments emerged in isolation, which prevented them from  
132 having any previous experience of oviposition or competition. We placed them individually in petri  
133 dishes (diameter: 8.5 cm; height: 2.7 cm), and provided cotton soaked in water and one pupa of *C.*  
134 *maculatus* located in gelatin capsule that was renewed each day preceding the experiments. *E.*  
135 *vuilleti* is a synovigenic parasitoid species: emerging females possess a few mature eggs and they  
136 mature additional eggs throughout their adult life. Oviposition activity increases over time and peaks  
137 three days after emergence (Jaloux et al., 2004). To stimulate oogenesis (Terrasse and Rojas-Rousse,  
138 1986), we also gave one male to females during the first day.

### 139 2.2. Experiment 1: Effect of JH on females' reproductive status

140 To investigate the influence of JH treatment on female reproductive status, two-day old wasps  
141 were randomly assigned to one of the five following treatments: they received either 0.04  $\mu$ L of  
142 acetone (N=36) or 0.04  $\mu$ L of JHIII (purchased from Sigma-Aldrich, Inc.) dissolved in acetone at  
143 four different concentrations (8, 16, 32 or 64 pg/ $\mu$ l; N=35, N=39, N=39, N=37, respectively). The  
144 final volume was established based on preliminary tests showing that mean haemolymph volume in  
145 *E. vuilleti* females is 0.175  $\mu$ l (but this volume may vary depending on body mass, physiological  
146 state or environmental conditions; Borde et al., unpublished data). Immediately after being  
147 immobilized on a petri dish that was cooled by ice, wasps were injected into the abdomen through  
148 the intersegmental membrane using the nanoliter injector Nanoject III (Drummond Scientific  
149 Company). Females were then individually placed in a petri dish and were frozen at -20°C either 4h  
150 (N=20 for injections of 0, 8, 16, 32 pg/ $\mu$ l and N=18 for injection of 64 pg/ $\mu$ l) or 24h (N=16 for

151 injection of 0 pg/ $\mu$ l, N=15 for injection of 8 pg/ $\mu$ l, and N=19 for injections of 16, 32 and 64 pg/ $\mu$ l)  
152 after injection. Females were deprived of hosts so that no mature eggs were laid during this period.  
153 Finally, all females were weighed using an electronic balance (Ohaus Discovery® model, accuracy:  
154 0.01mg) and then dissected to count mature (i.e. ready-to-lay) oocytes in their abdomen.

155

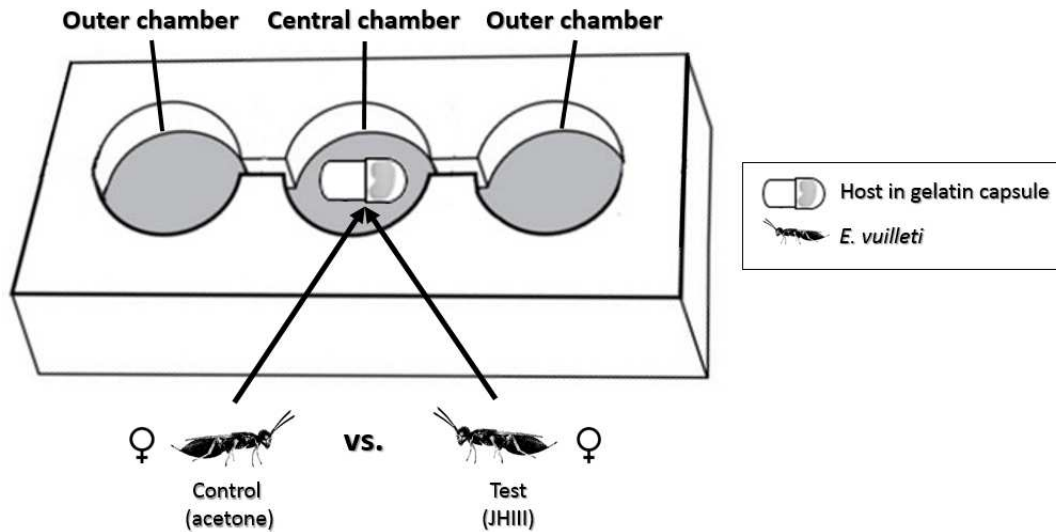
### 156 *2.3. Experiment 2: Effect of JH on females' aggressiveness and contests outcome*

157 To investigate the influence of JH manipulation on female aggressive behavior, three-days  
158 old wasps were randomly assigned to two different injection treatments: females injected with either  
159 0.04  $\mu$ L of JHIII diluted in acetone (concentration = 32 pg/ $\mu$ l) or 0.04  $\mu$ L of acetone alone. Wasps  
160 were marked on the dorsal part of their thorax with a dot of bright yellow or bright red acrylic paint  
161 the day before behavioral observations to allow individual identification during contests. Paint color  
162 did not affect contest outcomes (wasps painted red won 17 of 25 contests; binomial test: P=0.11).  
163 Females were deprived of hosts for at least 2h before the start of the contest so that they were more  
164 ready to oviposit. At the beginning of the observation, two wasps were simultaneously introduced  
165 into an apparatus consisting of a plastic block made of three chambers linked by a narrow channel  
166 (Petersen and Hardy, 1996; Fig. 1.).

167

168





169

170 Figure 1: Experimental apparatus (adapted from Peterson & Hardy, 1996). Three chambers (diameter =1.8 cm, depth =  
 171 0.6 cm) linked by a narrow channel (width = 0.1 cm) were made in a plastic block, decked with Plexiglas. At the start of  
 172 experiments, two *E. vuilleti* females (one control and one test female) were simultaneously introduced into the central  
 173 chamber already containing a 4<sup>th</sup> instar larva of *C. maculatus* within a gelatin capsule mimicking a seed. Wasp behavior  
 174 was observed from above the contest arena.

175

176 All contests were run between one control female (i.e., injected with acetone only) and one  
 177 test female (i.e., injected with JHIII), challenging for a 4<sup>th</sup> instar larva of *C. maculatus* located in  
 178 gelatin capsule and previously placed in the central chamber of the contest arena (Fig.1.). Mathiron  
 179 et al. (2018) showed that females with previous oviposition experience on pupae were more  
 180 motivated to gain access to a 4<sup>th</sup> instar larva. Contests occurred between females of the same age (3  
 181 days old), marked with different colors and visually matched for size; post-experiment data analysis  
 182 confirmed that contestants of the same dyad did not differ significantly in body mass (Wilcoxon  
 183 signed-ranks test: T=147.5, N=25, P=0.7). Both females could freely move throughout the entire  
 184 contest arena. Observations lasted 1h or were stopped when: i) one female pushed her opponent out  
 185 of the central chamber, ii) one female exited the central chamber for at least 2 min, or iii) neither  
 186 female touched the host nor displayed aggressive interactions for at least 2 min.

187 During all tests, we recorded oviposition and aggressive behaviors displayed by each female.  
188 Upon detection of a conspecific (females raised their antennae in the direction of their opponent),  
189 females frequently interrupted their behavioral oviposition sequence (Mohamad et al., 2010). They  
190 either simply kick their opponent with their legs without taking their ovipositor out of the capsule  
191 (defensive behavior) or can escalate to a full attack, in which case one female generally chases her  
192 opponent away from the capsule, hits her with her head or mounts her (Mathiron et al., 2018).  
193 Chasing, hitting and mounting can lead to the loser leaving the central chamber of the arena (8/25  
194 loser females left the central chamber after an opponent attack during this experiment). Only these  
195 behaviors, collectively termed as ‘attacks’, were therefore considered in the rest of the study. When  
196 both females of a dyad stayed in the central chamber during the whole time of observation, the  
197 winning female was determined as the one exhibiting oviposition behaviors.

198 Wasps were immediately frozen at  $-20^{\circ}\text{C}$  after the experiments, weighed using an electronic  
199 balance (Ohaus Discovery® model, accuracy: 0.01mg), and dissected to count the number of mature  
200 and non-mature eggs in their abdomen. Finally, after each contest, we counted the number of eggs  
201 laid on the host by the winning female to determine their mature egg load before the contests (i.e.,  
202 initial mature egg load).

#### 203 *2.4. Statistical analysis*

204 We performed data analyses with the software Rstudio (1.0.143 version – © 2009-2016  
205 RStudio, Inc.), using  $\alpha=0.05$ . Our general approach was to use, when possible, parametric analyses in  
206 which the assumed distribution of residuals was matched to the data rather than transforming data to  
207 fit standard assumptions (Wilson and Hardy, 2002; Briffa et al., 2013). However, we used non-  
208 parametric tests when parametric conditions were not verified.

209 Experiment 1: We first investigated whether the mature egg load of females differed  
210 significantly between JHIII treatments, 4h after injection, by performing a general linear model  
211 (GLM) assuming a Poisson distribution of errors. We then ran a linear model (LM) and a polynomial

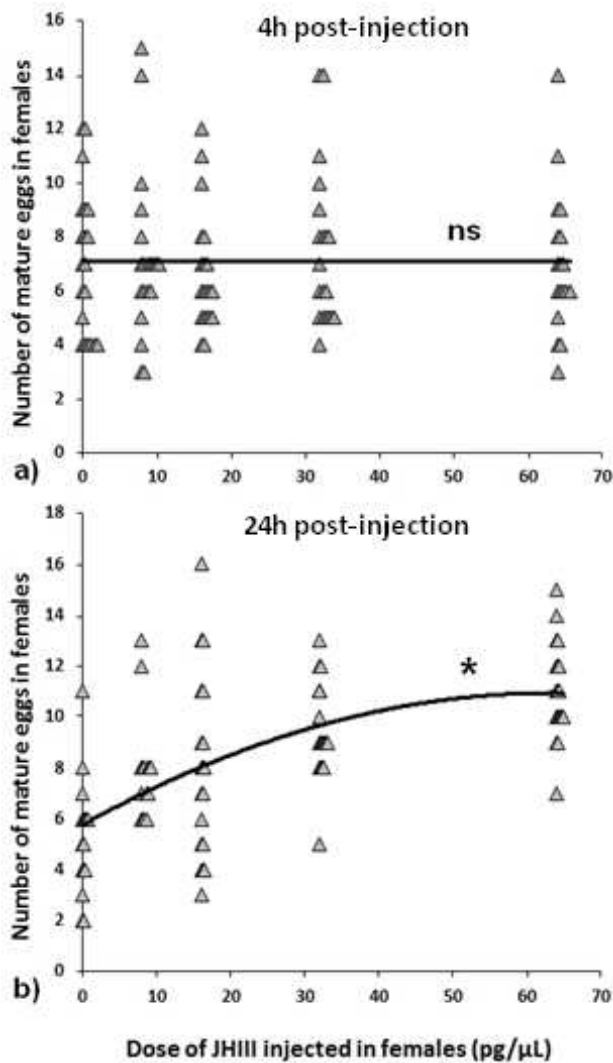
212 linear model (PLM) to explore the effect of JHIII treatments on the number of mature eggs 24h after  
213 injection, and we compared the linear and polynomial models to determine which one provided a  
214 better fit.

215 Experiment 2: We first verified that initial mature egg load did not differ between JHIII  
216 females (mean egg load  $\pm$  S.E.:  $6.2 \pm 0.7$ ) and control females (mean egg load  $\pm$  S.E.:  $5 \pm 0.5$ ;  
217 Student test:  $t_{25}=-1.41$ ,  $P=0.16$ ). We then performed a generalized linear mixed model (GLMM) with  
218 a Poisson error distribution to explore the influence of contestants' initial mature egg load and  
219 injection treatment on the number of attacks (calculated as the sum of chasing, hitting, and mounting  
220 behaviors) displayed by females, with contest identity as a random factor. Lastly, we conducted a  
221 GLMM assuming a binomial error distribution to explore the influence of initial mature egg load of  
222 females and injection treatments on contest outcomes, with contest identity as a random factor. To  
223 run this analysis, we defined contest outcome as a binary response: 0 = the female lost and 1 = the  
224 female won.

### 225 **3. Results**

#### 226 *3.1. Experiment 1: Effect of JH on female reproductive status*

227 The number of mature eggs in females did not vary across treatments 4h after  
228 injections (GLM:  $\chi^2=0.006$ ,  $df=1$ ,  $P=0.94$ ; Fig.2a). However, 24h after injections, mature egg  
229 load was influenced significantly by treatment (LM:  $F_{1,86}=4.05$ ,  $P=0.047$ ,  $R^2=0.335$ ).  
230 Moreover, we found that the polynomial model fitted better than the linear model, with a  
231 dose-dependent effect of JHIII injection on egg maturation reaching a plateau at  $64 \text{ pg}/\mu\text{L}$   
232 (PLM:  $F_{1,85}=4.05$ ,  $P=0.047$ ,  $R^2=0.365$ ; Fig.2b).



233

234 Figure 2: Number of mature eggs of females injected with different doses of JHIII. **a)** 4h after injections, **b)** 24h after  
 235 injections. ns: non-significant; \*:  $P < 0.05$ . Data points have been horizontally displaced from their binary positions to  
 236 show the numbers of observations.

237

### 238 3.2. Experiment 2: Effect of JH on female aggressiveness and contests outcome

239 The number of attacks displayed by females was influenced significantly by the  
 240 interaction between female egg load and injection treatment (Table 1). Wasps attacked  
 241 significantly more during a contest when they had more ready-to-lay eggs, and this effect  
 242 was stronger when females were injected with JHIII compared to control females injected  
 243 with acetone alone (Table 1, Fig. 3). Effect of female egg load x injection treatment interaction

244 was still significant when removing data from the contest with a JHIII female displaying 22 attacks  
 245 and her control opponent displaying 2 attacks (GLMM:  $\chi^2=4.87$ ,  $df=1$ ,  $P=0.03$ ).

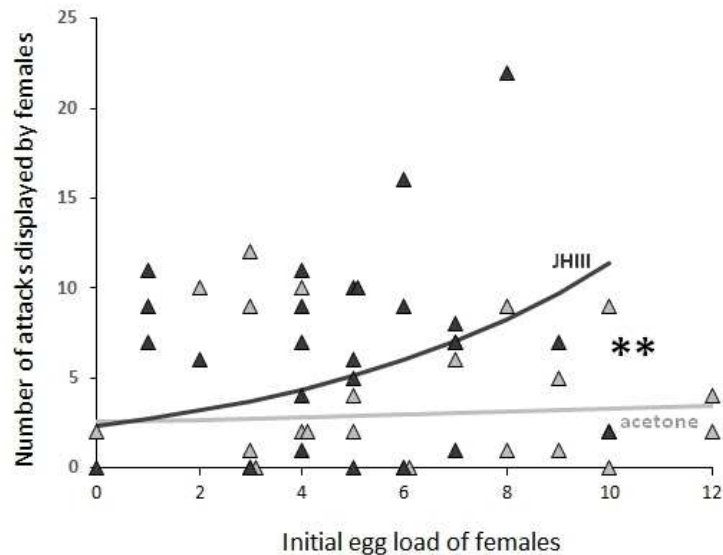
246

247 Table 1. Effect of initial egg load difference and injection on female agonistic behaviors.

Factors affecting female aggressiveness	df	$\chi^2$	P
Egg load	1	4.81	< <b>0.05</b>
Injection	1	20.34	< <b>0.001</b>
Egg load x Injection	1	7.09	<b>0.008</b>

248

*P*-values of significant explanatory variables are shown in bold font



249

250 Figure 3. a) Number of attacks displayed by females treated with acetone alone (light data points, light gray line; N=25)  
 251 or with JHIII (dark data points, dark curve; N=25) when fighting for a 4<sup>th</sup> instar larva of *C. maculatus*. \*\*: P < 0.01. Data  
 252 points have been horizontally displaced from their binary positions to show the numbers of observations. Statistical  
 253 analyses showed that effect of ‘Egg load x Injection’ interaction was still significant when removing data from the  
 254 contest with a JHIII female displaying 22 attacks and her control opponent displaying 2 attacks (GLMM:  $\chi^2=4.87$ ,  $df=1$ ,  
 255  $P=0.03$ ).

256

257 The probability of a female winning a contest was not influenced significantly by  
 258 either the interaction between her initial mature egg load and injection, or the effect of

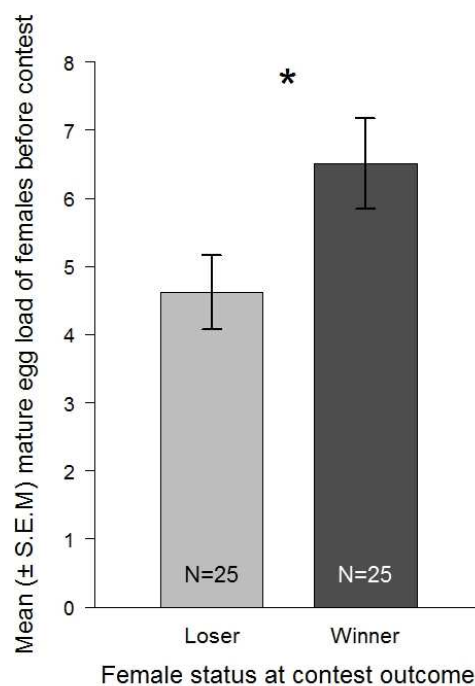
259 injection alone (Table 2). However, females had higher probabilities of winning contest  
260 when they had more ready-to-lay-eggs before contests (Table 2, Fig. 4).

261

262 Table 2. Effect of initial egg load and injection on the winning probability of females.

Factors affecting contest outcomes	df	$\chi^2$	P
Egg load	1	4.62	<b>0.03</b>
Injection	1	0.54	0.46
Egg load x Injection	1	0.22	0.64

263 P-values of significant explanatory variables are shown in bold font



264

265 Figure 4. Mean ( $\pm$  S.E.M) number of mature eggs in females before the contest (i.e. initial egg load) and their winning  
266 status at contest outcome. \*: P < 0.05. Sample sizes are shown as text within each histogram bar.

267

#### 268 4. Discussion

269 The purpose of our study was to test the effect of JH manipulation on female  
270 reproductive status, aggressiveness and conflict resolution in the solitary ectoparasitoid *E.*

271 *vuilleti*. By measuring how JHIII injections affect oocyte maturation, we showed that JHIII  
272 did not influence mature egg load after 4h, but had a dose-dependent gonadotropic effect in  
273 females 24h post-injection. Moreover, JHIII injections and egg load interacted to affect  
274 female aggressiveness during competition for host access.

#### 275 4.1. Effect of JH on females' reproductive status

276 In our first experiment, the reproductive status of the wasps varied after 24h according to the  
277 amount of JHIII injected in females: wasps injected with JHIII had more ready-to-lay eggs than those  
278 injected only with acetone. This result therefore suggests that JH has a gonadotropic effect in *E.*  
279 *vuilleti* females, and was expected given that JH plays an important and diverse role in adult  
280 reproduction of many insect species by regulating vitellogenesis in the fat body and vitellogenin  
281 uptake by the ovaries (reviewed by Tobe and Stay, 1985; Nijhout, 1994; Robinson and Vargo, 1997;  
282 Hartfelder, 2000). In Hymenoptera, JH is involved in the regulation of ovarian development in the  
283 facultatively eusocial sweat bee *Megalopta genalis* (Smith et al., 2013), primitively social polistine  
284 wasps (Barth et al., 1975), halictine bees (Bell, 1973) and bumblebees (Bloch et al. 2000), but not in  
285 the highly social honeybee *Apis mellifera* (reviewed in Robinson, 1992; Robinson and Vargo, 1997).  
286 Because egg maturation was affected by JH in a dose-dependent way, we can reject the hypothesis  
287 that the physiological response to injection was an artefact related to activation of another signaling  
288 pathway such as the stress response. Finally, we found no difference in mature egg load between  
289 control and JHIII treated females 4h after injections. This is consistent with what we found in the  
290 second experiment, where contestants had the same number of mature eggs before contests, whether  
291 they were injected with JHIII or with vehicle alone. Our results therefore suggest that the egg  
292 maturation response to JH injection may happen only after 4h post-injection. Decoupling the effect  
293 of ovarian maturation and JH treatment on aggressive interactions is sometimes difficult (see Barth et  
294 al., 1975 for an example). Observing *E. vuilleti* females fight over hosts between 1h and 4h post-

295 injection permitted us to disentangle the effects of JHIII and mature egg load on aggressiveness and  
296 contest outcomes.

#### 297 4.2. Effect of JH and Subjective Resource Value on female competition

298 In our second experiment, we investigated the role of JH injection and mature egg  
299 load on female aggressiveness and contest outcome. Our results support the idea that  
300 reproductive state influences behavioral decisions during fights (Neat et al., 1998; Stokkebo and  
301 Hardy, 2000; Arnott and Elwood, 2008). As previously demonstrated in *E. vuilleti* (Mohamad et al.,  
302 2010; Mathiron et al., 2018), we found that more gravid females (i.e., females with more mature  
303 eggs) displayed more attacks and had a higher probability of winning contests. This is also  
304 consistent with game theoretical predictions that contest outcome is influenced by differences in the  
305 value that opponents place in the resource ('Subjective Resource Value', SRV; Maynard-Smith and  
306 Parker, 1976; Hammerstein, 1981; Enquist and Leimar, 1987). For instance, parasitoid females with  
307 more developed reproductive tissues are predicted to place a greater value on hosts: they  
308 are more prepared to oviposit and should therefore be more motivated to fight and have a  
309 higher probability of winning contests (Stokkebo and Hardy, 2000; Brown et al., 2006, 2007;  
310 Dissanayake et al., 2009). We therefore added evidence that the initial egg load as a component of  
311 SRV plays a main role in conflict resolution in *E. vuilleti*.

312 More interestingly, we found an interaction between JHIII injection and mature egg load on  
313 the number of aggressive behaviors displayed by females. We observed that the effect of initial egg  
314 load on aggressiveness was more pronounced when contestants were injected with JHIII. We  
315 therefore suggest that JHIII promoted female aggressiveness during fights for host access. This  
316 agrees with previous studies exploring the role of JH on agonistic behaviors in insects (Tibbetts and  
317 Crocker, 2014). Topical application of methoprene (a JH analog) induced strong reactions to  
318 confrontation (i.e., aggression/flight behavior) in individuals of *Schistocerca gregaria* and *Locusta*  
319 *migratoria* (Wiesel et al., 1996). Scott (2006) made the same observation in *Nicrophorus orbicollis*



320 and *Nicrophorus tomentosus*: methoprene application significantly increased the number of injuries  
321 from aggressive interactions in males and females in the absence of a resource. Moreover, in the  
322 cockroach *Nauphoeta cinerea*, JHIII injections sustain males' aggressive posture during intra-sexual  
323 fights (Kou et al., 2008). Here, we highlight that JH treatment upregulated aggressive behaviors  
324 associated with reproduction in *E. vuilleti* females, just as testosterone does in vertebrates  
325 (Wingfield, 1984). This is an important result because vertebrate and insect hormones have different  
326 evolutionary histories and possess different physiological backgrounds, suggesting an evolutionary  
327 convergence of endocrine effects across vertebrates and insects (Tibbetts and Crocker, 2014).

#### 328 *4.3. Evolution of JH functions across hymenopterans with different degrees of sociality*

329 JH has been observed to play two important roles in life history regulation in hymenopteran  
330 societies: gonadotropin and behavioral pacemaker (Robinson and Vargo, 1997). Because regulation  
331 of reproduction is generally the main role of JH in adult insects (Nijhout, 1994), it is assumed that  
332 this was an ancestral function of this hormone in social hymenoptera. So, how the relationship  
333 between JH regulation of reproduction and JH regulation of aggressiveness has evolved across  
334 hymenopteran taxa with different degree of sociality remains an open question. Currently, two  
335 hypotheses have been advanced: i) the 'novel- or single-function hypothesis' (Robinson et al., 1992,  
336 Robinson and Vargo, 1997; Agraharia and Gadagkar, 2003) proposes that the role of JH has changed  
337 from an exclusively reproductive function in primitively eusocial species (those lacking  
338 morphologically distinct queen and worker castes), to an exclusively behavioral function in highly  
339 eusocial societies (those containing morphologically distinct castes), whereas ii) the 'split-function  
340 hypothesis' (West-Eberhard, 1996) proposes that JH affects regulation of both reproduction and  
341 behavior in ancestral non-social species, but that these different effects of JH were then divided  
342 between queens and workers in more social species (i.e. regulation of reproductive maturation in  
343 egg-laying queens and behavioral pacemaker in workers). Previous studies have lent some support to  
344 both hypotheses (Agraharia and Gadagkar, 2003; Giray et al., 2005). To the extent that JH modulates

345 ovarian development and aggressiveness in *E. vuilleti*, a strict non-social hymenopteran, our work  
346 supports the split-function hypothesis, but further investigations in non-social hymenopteran species  
347 will be essential for comparative studies on the evolution of JH function.

348

## 349 **5. Conclusion**

350 By testing the role of JH on both reproductive status and aggressiveness in female *E. vuilleti*,  
351 we have shown for the first time in a non-social hymenopteran parasitoid that JH has a gonadotropic  
352 effect and promotes aggressive behaviors during contests over hosts. Because JH upregulates  
353 aggressive behaviors associated with reproduction, our data support the possibility that JH in insects  
354 and testosterone in vertebrates have parallel functions, suggesting evolutionary convergence of  
355 endocrine systems.

## 356 **Founding**

357 This work was supported by the French Ministry of Research (2016-Bourse Ministérielle-44).

358

## 359 **Acknowledgement**

360 We are grateful to Charlotte Lécureuil and Joël Meunier for their advices and Fabrice Vannier for his  
361 technical assistance. We thank Dr. Mark Sheridan and 2 anonymous reviewers for helpful comments  
362 on the manuscript.

363

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