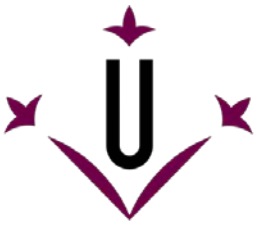

SUBLETHAL EFFECTS OF NEONICOTINOID INSECTICIDE ON CALLING BEHAVIOR AND PHEROMONE PRODUCTION OF TORTRICID MOTHS

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Abstract - In moths, sexual behavior combines female sex pheromone production and calling behavior. The normal functioning of these periodic events requires an intact nervous system. Neurotoxic insecticide residues in the agroecosystem could impact the normal functioning of pheromone communication through alteration of the nervous system. In this study we asses if sublethal concentrations of the neonicotinoid insecticide thiacloprid, that competitively modulates nicotinic acetylcholine receptors at the dendrite, affect pheromone production and calling behavior in adults of three economically important tortricid moth pests [*Cydia pomonella* (L.), *Grapholita molesta* (Busck), and *Lobesia botrana* (Denis & Schiffermüller)]. Thiacloprid significantly reduced the amount of calling in *C. pomonella* females at $LC_{0.001}$ (a lethal concentration that kills only 1 in 10^5 individuals), and altered its calling period at LC_1, and in both cases the effect was dose-dependent. In the other two species the effect was similar but started at higher LCs, and the effect was relative small in *L. botrana*. Pheromone production was altered only in *C. pomonella*, with a reduction of the major compound, codlemone, and one minor component, starting at LC_{10}. Since sex pheromones and neonicotinoids are used together

in the management of these three species, our results could have implications regarding the interaction between these two pest control methods.

INTRODUCTION

In lepidopterans, reproduction shows a periodic pattern related to the duration of the daily light and dark cycles and involves a complex series of behavioral and physiological events including chemical communication mediated by sex pheromones (Groot 2014). Usually females release the sex pheromone and males fly towards females from tens or hundreds of meters (Cardé 2016). Closely related moth species with common phylogenetic origins are under competition for limited communication channels (Roelofs and Brown 1982). Reproductive isolation is instrumental in speciation (Smadja and Butlin 2009), and in the case of pheromone communication is modulated by species-specific differences in sex-pheromone composition and time of release (Byers 2006; Groot 2014)

Several factors influence calling behavior and pheromone production in moths (McNeil 1991; Raina 1993), including age (i.e., Webster and Cardé 1982; Gemeno and Haynes 2000; Kawazu and Tatsuki 2002; Mazor and Dunkelblum 2005; Ming et al. 2007), mating status (i.e., Foster and Roelofs 1994; Delisle et al. 2000; Mazo-Cancino et al. 2004), and pheromone autodetection (Holdcraft et al. 2016). Environmental stressors, such as sublethal doses of insecticides that intoxicate but do not kill the individual, could also affect pheromone production and release (Haynes 1988; Tricoire-Leignel et al. 2012), but this aspect has been tested in relatively few moth species.

Pesticides are often considered a quick, easy, and inexpensive solution to control insect pests. However, pesticides can cause negative effects on the environment and human health (Aktar et

al. 2009). In Integrated Pest Management (IPM) the use of insecticides is often combined with environmentally safer methods (Damos et al., 2015), such as the use of sex pheromones for mating disruption (emitting large amounts of synthetic sex pheromone and so reducing the probability of mate finding), mass trapping (removing from the population individuals attracted to traps baited with pheromone lures), and monitoring the population for precise timing of control procedures (Witzgall et al. 2010). Because semiochemicals exploit insect chemical communication, and neurotoxic insecticides affect the normal functioning of the nervous system, it is plausible that the simultaneous use of semiochemicals and insecticides could affect each other in IPM strategies (Suckling et al. 2016). Indeed, several studies report alterations of the normal perception of and response to chemical signals in insects treated with sublethal doses of insecticides (Haynes 1988; Tricoire-Leignel et al. 2012).

In this context of potential semiochemical and toxicological interactions in the agroecosystem, we explored the effect of sublethal doses of a neonicotinoid insecticide on pheromone production and release (i.e., calling behavior) in three tortricid moths. Our test species, *Cydia pomonella* (L.), *Grapholita molesta* (Busck) and *Lobesia botrana* (Denis & Schiffermüller), are main pests of apple, peach and grapevines, respectively, have a relatively worldwide distribution and are controlled with both semiochemicals and insecticides (Ioriatti et al. 2011; Kirk et al. 2013; Damos et al. 2015). Indeed these three species represent several of the most successful examples of pest control by means of mating disruption (Witzgall et al. 2010). For a toxicant, we used the neuroactive insecticide thiacloprid, a neonicotinoid that competitively modulates nicotinic acetylcholine receptors at the dendrite (Casida 2009). Thiacloprid is recommended for the control of *C. pomonella* and *G. molesta* in stone and pome fruits in Spain (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente [MAPAMA], 2017). Although aimed at eggs and larvae, thiacloprid residues from air blast spraying in fruit orchards could potentially intoxicate adults with residual sublethal doses, and therefore affect semiochemical control (Wise

et al. 2006). Thiacloprid could potentially affect *L. botrana* in vineyards adjacent to fruit orchards treated with this insecticide (Harari et al. 2011). Baseline mortality with thiacloprid has been determined for the three species under laboratory conditions (Navarro-Roldán et al. 2017). *Cydia pomonella* and *G. molesta* belong to the tribe Grapholitinii and *L. botrana* to the tribe Olethreutini, both in the subfamily Oletheutrinae (Regier et al. 2012). By comparing the effect of thiacloprid across phylogenetically related species of diverse ecology we hoped to gain basic background information about sublethal effects of neurotoxic insecticides on sex pheromone signalers.

METHODS AND MATERIALS

Insects. Susceptible laboratory strains of *C. pomonella*, *G. molesta* and *L. botrana* established from individuals collected in Lleida (Spain), Piacenza (Italy), and La Rioja (Spain), respectively, have been maintained under laboratory conditions for more than 5 years without introduction of wild individuals. Larvae were reared in artificial diet (Ivaldi-Sender 1974) at 25 ± 1 ºC under a 16:8 h light:dark photoregime. Females of *G. molesta* and *L. botrana* were separated at the pupal stage and adult emergence was checked daily and always at the same hour. *C. pomonella* was sexed at the adult stage, also in a daily basis. Because adults were collected only once per day, they were 0-24 h old when separated from the pupae, 24-48 h old one day later, and so on.

Insecticide Application and Mortality Estimation. Thiacloprid (PESTANAL®, analytical standard, ≈100% (a.i.), Sigma-Aldrich, Spain), was diluted using acetone (CHROMASOLV®, for HPLC, ≥99.9%. Sigma-Aldrich, Spain) and stored at 7ºC. The same stock of acetone used to prepare the dilutions was also used as the solvent control treatment. The four chosen sublethal

concentrations of thiacloprid were $LC_{0.001}$, LC_1, LC_{10} and LC_{20}, according to Navarro-Roldan et al. (2017), with concentrations shown in Table S1.

Treatments were applied during the first half of the photophase at 0-24 h post-emergence (calling behavior test), or at 16-40 h post-emergence (pheromone gland test). One to three adults were placed in 10 ml clear polystyrene test tubes and received a brief (10 sec) flow of industrial grade CO_2 which quickly anesthetized them. Immediately after being anesthetized they were placed upside down under the field of view of a stereo microscope and a 1 µl test solution was applied to the ventral thoracic region of each individual using a high-precision, positive displacement, repeatable-dispensing micropipette (Multipette®-M4, Eppendorf, Germany). Treated females were transferred immediately to a 150 ml polypropylene non-sterile clinical sample bottle (57 mm diameter x 73 mm-high). Individuals receiving the same treatment were placed in groups of 3 to 10 in the same bottle. The lid of the bottle was punctured to make 10 1-mm-diameter holes to allow gas exchange, and a 1.5 ml Eppendorf tube® containing 10% sugar solution and cotton lid was placed on the bottom to supply nutrients. Bottles with treated insects were placed in the rearing room until test time.

Mortality was determined 24 h post-treatment. Adults were observed with the naked eye and scored as: 1) alive if they flew or walked apparently unaffected; 2) as potentially moribund if they could barely walk or were laying on the floor of the bottle but still moved; 3) or as potentially dead if they laid immobile on the floor of the bottle. Mortality was estimated using the sum of the potentially moribund and dead individuals. The other individuals scored as alive were used in the calling and pheromone tests. No further anesthesia was needed.

Calling Behavior. Females were placed individually in 9 mm-long x 1.5 mm-diameter, 10 mL clear polystyrene test tubes that had both ends covered with 1.5 mm-diameter-mesh galvanized

wire screen (Figure S1). Tubes were placed on a 42 cm-tall platform that could hold up to 13 tubes from bottom to top, leaving 2.5 mm between them (Figures S1 and S2). The platforms were painted white to facilitate observation of calling postures inside of the plastic tubes. Four platforms with test tubes were placed in a chamber with a continuous 0.4 ± 0.1 m s^{-1} air flow. The tubes were aligned with the air flow (flow through the tubes was not measured) to minimize ambient pheromone levels, which could affect moth calling behavior (Holdcraft et al. 2016). Four 18-watt domestic fluorescent lamps (Standard daylight F18W/154-T8, Sylvania) that were placed between 20 and 52 cm above the highest and lowest female positions in the rack provided between 4700 and 1700 lux during photophase, respectively (TES-1330, Tes Electrical Electronic Corp.). During scothophase there was complete obscurity and calling was observed using a 660-nm LED (2.5V, 1.3 candles, 5 mm diameter, 30° view angle, LedTech, part number LURR5000H2D1) which was held manually near each female for observations.

G. molesta females call mainly before the beginning of the scotophase, *C. pomonella* females call mainly during the scotophase (Groot 2014), and *L. botrana* females call during the first hours of scotophase (Harari et al. 2011, 2015). However several factors (i.e., illumination, temperature etc.) could affect the calling period (McNeil 1991), so in order to determine the exact calling period of our laboratory colonies under our experimental conditions we performed preliminary observations on 69-75 untreated (i.e., no acetone or insecticide) individuals of each species over a 12-h period bracketing the expected calling times. The 12-h observation periods of *C. pomonella* and *L. botrana* started 2 h before the onset of the scotophase, covered all the scotophase and continued during the first 2 h of the photophase. The observation period of *G. molesta* started 8 h before the onset of the scotophase and extended 4 h into the scotophase. In order to observe the three species during the same 12 h time period, the photoregimes of *C. pomonella* and *L. botrana* were synchronized with each other and both were observed on the same day, the photoregime of *G. molesta* was delayed with respect to that of the other species.

144 Observations of the two groups were made on alternate days, and were performed at 30 min
145 intervals, except during the last 30 min of the photophase and the first 2 h of the scotophase
146 when they were observed every 15 min to increase sample resolution for the relatively short
147 (about 2 h) calling period of *L. botrana*. Females were placed in the observation setup at least 30
148 min before the first observation. The first observations during scotophase occurred between 5 to
149 10 minutes after lights off.

150 Once the calling period of our laboratory insects was determined (Figure S3), between 61 and 70
151 females treated with sublethal insecticide doses or acetone were observed during the same period
152 as in the preliminary test to determine the effect of the insecticide dose on calling behavior.
153 Calling behavior was categorized as either "weak calling" (the female walks or is slightly
154 agitated, with an intention to adopt, or beginning to adopt, a calling posture consisting in rising
155 its wings and extruding the abdomen tip), "medium calling" (incomplete calling stance: more or
156 less stationary female with partially raised wings and abdomen tip partially extruded), or "strong
157 calling" (full calling stance: mostly stationary female with fully raised wings, and protruded
158 abdomen tip readily visible). The specifics of the calling posture were slightly different and
159 characteristic across species.

160

161 *Pheromone Gland Content*. Pheromone was extracted from females that were 40- to 64-hour-old
162 and had been treated with sublethal insecticide doses, or acetone as control, 24 h earlier.
163 Extractions were restricted to a 1 h period coinciding with peak calling time of each species: 30
164 to 90 min after the onset of scotophase in *C. pomonella*, 120 to 60 min before the scotophase in
165 *G. molesta* and 0 to 60 min after the onset of scotophase in *L. botrana*. The tip of the abdomen
166 containing the sex pheromone gland tissue was excised carefully by pulling it from the abdomen
167 with fine forceps. Abdominal tips were deposited individually in solvent-rinsed and oven-dried

168 conical-bottom glass vials (Total recovery vial, part number 186002805, Waters, USA) with
169 Teflon-lined lids (part number 186000274, Waters, USA) containing 7 µl of a 1ng/µl octadecane
170 internal standard solution (> 99% pure, Sigma-Aldrich, Spain) in *n*-hexane (> 97% pure, VWR
171 Chemicals, BDH-Prolabo, Spain). After 30 min at room temperature the glands were removed
172 from the vial and the extracts were stored at -20°C until analysis (for a maximum of 10 days).

173 The remaining extract (approx. 0.5-3 µl) was injected in a Hewlett Packard 6890 gas
174 chromatograph equipped with a flame ionization detector and a 30 m-long, 0.25-mm I.D., 0.25-
175 µm film-thickness DB-Wax column (Agilent Technologies, Madrid, Spain). The constant helium
176 flow through the column was 1 ml min^{-1}, and the injector and detector temperatures were 250
177 and 270°C, respectively. The oven temperature program stayed at 70ºC during 1 min and then
178 increased to 170°C at 30°C min^{-1}, and from 170°C to 230°C at 10°C min^{-1}, and remained at this
179 temperature for 10 min. Retention time and quantification were estimated with the injection of
180 synthetic standards and with the internal standard, respectively. The pheromone compounds of
181 *C. pomonella* [Dodecan-1-ol (12:OH), (*E*)-9-Dodecen-1-ol (E9-12:OH), Tetradecan-1-ol
182 (14:OH), and (*E*,*E*)-8,10-Dodecadien-1-ol (E,E-8,10-12:OH), Witzgall et al. 2008] eluted at 7.71
183 min, 8.00 min, 9.19 min and 9.36 min, respectively. The pheromone compounds of *G. molesta*
184 [(*E*)-8-Dodecenyl acetate (E8-12:Ac), (*Z*)-8-Dodecenyl acetate (Z8-12:Ac), Dodecan-1-ol
185 (12:OH), and (*Z*)-8-Dodecen-1-ol (Z8-12:OH), Knight et al. 2015] eluted at 7.46 min, 7.55 min,
186 7.71 min and 8.05 min, respectively. The pheromone compounds of *L. botrana* [(*E*)-9-Dodecenyl
187 acetate (E9-12:Ac), (*Z*)-9-Dodecenyl acetate + 11-Dodecenyl acetate (Z9-12:Ac+11-12:Ac),
188 (*E*,*Z*)-7,9-Dodecadienyl acetate (E,Z-7,9-12:Ac), and (*E*,*Z*)-7,9-Dodecadien-1-ol (E,Z-7,9-
189 12:OH), Sans et al. 2017] eluted at 7.52 min, 7.62 min, 8.57 min and 9.12 min, respectively (Z9-
190 12:Ac and 11-12:Ac eluted together). Between 19 and 21 females of each species were analyzed
191 for each treatment. For each individual the quantity of individual compounds and the ratio of the

192 minor compounds to the major compound (E,E-8,10-12:OH in *C. pomonella*, Z8-12:Ac in *G.*
193 *molesta* and E,Z-7,9-12:Ac in *L. botrana*) were calculated.

194

195 *Statistical Analyses*. All the statistical analyses were run in R software (R Core Team 2016).
196 Mortality was analyzed with *Fisher's exact tests* and *Bonferroni* correction. To determine the
197 effect of thiacloprid on the calling period, we calculated the first, mid and final times of calling
198 for calling females. To determine the effect of thiacloprid on the amount of calling behavior we
199 calculated the proportion of observations in which females called out of the total number of
200 observations of the calling period estimated previously. For example, for an 8-h calling period
201 and observations every 30 min there would be 960 observations for 60 insects. If calling
202 appeared in 480 of these observations, then the amount of calling would be 50%. Analyses were
203 performed with generalized linear models (GLM), using Gaussian family functions for
204 continuous variables (calling period and pheromone composition) and binomial family functions
205 for binomial variables (amount of calling). The predictmeans() function performed *Tukey´s*
206 multiple pairwise comparisons and provided parameter estimates and their standard errors and
207 confidence intervals which are shown in tables and figures. Raw data and R scripts are available
208 online (http://hdl.handle.net/10459.1/59531). Whenever the term "significant" is used in the text
209 regarding differences between treatments it indicates a p-value < 0.05.

210

211 RESULTS

212 Mortality in our tests (Table S1) was comparable to the dose-mortality curves used to determine
213 the test concentrations (Navarro-Roldán et al. 2017). Acetone and $LC_{0.001}$ did not induce any
214 mortality, and the maximum mortality with LC_1 was below 2.5 %. LC_{20} mortality ranged

between 7% and 21%, and with LC_{10} it was between LC_1 and LC_{20} in all but one case (Table S1).

Calling Behavior. Under our test conditions, *C. pomonella*, *G. molesta* and *L. botrana* had distinct calling periods. *C. pomonella* called throughout the scotophase, *G. molesta* called from 4 h before the start of the scotophase to 0.5 h into the scotophase, and *L. botrana* called for 2.5 h starting at the beginning of the scotophase (Figure S3). Acetone did not appear to affect the amount or periodicity of calling with respect to untreated females (compare Figure 1 and Figure S3). At least 80% of the acetone-treated females called during peak calling time (all species), but there was a significant dose-dependent reduction of calling in treated females (Figure 1). In *C. pomonella* there was a strong reduction on the amount of calling which was already significant at the lowest concentration ($LC_{0.001}$), in the other two species the reduction started with LC_1 (Table 1), and although significant, the effect was very mild in *L. botrana*. Peak calling reductions with LC_{20} were 70.19 and 75.09 % for *C. pomonella* and *G. molesta*, respectively. In *L. botrana* calling was not reduced beyond LC_1, and reduction with respect to the control treatment was only 10%. A small percentage (< 8 %) of the control females did not call a single time during the entire observation period, but this number increased with thiacloprid doses and peaked at LC_{20} with 53% (*C. pomonella*), 61% (*G. molesta*) and 20% (*L. botrana*) non-calling females, respectively (Table S2). Individual differences in the number of calling observations per female and intensity of calling (weak, medium and strong) were observed (Figures S4, S5 and S6, data not analyzed). In general, “strong” calling coincided with peak calling time, whereas “weak” calling appeared to increase with insecticide dose.

Sublethal doses of thiacloprid modified calling periods (Table 2). LC_1, LC_{10} and LC_{20} advanced the end and midpoint calling times of *C. pomonela*’s (150 min, approx.), and delayed the start

239 and midpoint calling times of *G. molesta* (74 min, approx.). No significant effect in calling
240 period was observed in *L. botrana*.

241

242 *Pheromone Gland Content*. The two highest sublethal doses of thiacloprid, LC_{10} and LC_{20},
243 reduced significantly the quantity of the major pheromone component of *C. pomonella*
244 (codlemone, E,E-8,10-12:OH) from about 5 ng to about 2 ng, and the minor component 12:OH
245 from about 2 ng to about 1 ng, whereas the other two minor components of *C. pomonella* and the
246 pheromone components of the other two species were unaffected (Figure 2). Reduction in the
247 quantity of the major pheromone component of *C. pomonella* resulted in an increase in the
248 relative proportion of two minor compounds, E9-12:OH and 14:OH, relative to codlemone
249 (Table 3). This effect was significant only at the highest pheromone dose, LC_{20}. E9-12:Ac and
250 14:OH were 14 and 16% relative to codlemone in acetone control females, and 56 and 40%
251 relative to codlemone in LC_{20} thiacloprid treated females. No further changes in the proportion
252 of pheromone components were observed in *C. pomonella* or the other two species.

253

254 DISCUSSION

255

256 Thiacloprid persists as surface residue on fruit and leaves (Wise et al. 2006), and has a half-life
257 in the soil of 10 to16 days (Krohn 2001). Therefore, adult moths could be exposed to sublethal
258 doses of thiacloprid even though the application is not aimed at them but to other life stages, or
259 even at other pest species, or from drift by blast sprayers in neighbor fields. In the present study,
260 sublethal doses of thiacloprid producing as low as 0.001 mortality significantly modified female
261 pheromone signaling, but the effect was not the same on the three tortricid species.

In our study *C. pomonella* called throughout the scotophase as previously reported (Castrovillo and Cardé 1979; Weissling and Knight 1996). Reports on the calling period of *G. molesta* are only slightly different from ours (Baker and Cardé 1979; Stelinski et al. 2006, 2014), which could be explained by the effect that variations in light and temperature have on the calling period of moths (Baker and Cardé 1979; Castrovillo and Cardé 1979; Groot 2014). To our knowledge, our study provides the first complete observation on the calling period of *L. botrana*. Its onset of calling coincides with a previous report (Harari et al. 2015). Regarding pheromone gland composition, our estimations are generally similar to what has been previously reported (summarized in Table S3). Minor differences across studies could be attributed to population differences or to methodological aspects related to the extraction and analysis of compounds that are present in very low quantities in the pheromone glands. In general, the mortality caused by thiacloprid was similar to the expected levels of mortality estimated in a previous study (Navarro-Roldán et al. 2017).

The most dramatic phenotypic effect of sublethal thiacloprid doses in our test species was the significant reduction in the amount of calling in *C. pomonella* females treated with $LC_{0.001}$, a remarkably low concentration that kills only one in 10^5 females. The other two species were less sensitive, and the effect on *L. botrana*, although statistically significant was so mild that probably would not have a real effect in the field. The calling curves of the insecticide treatments for the most part fell within the boundaries of the acetone control curves, so the shift in calling period with thiacloprid was not as remarkable as the effect on the amount of calling. A detrimental effect of sublethal insecticide on calling behavior has been observed in other moth species with pyrethroid (Haynes and Baker 1985; Clark and Haynes 1992a; Yang and Du 2003; Shen et al. 2013; Quan et al. 2016) and organophosphate insecticides (Trimble et al. 2004). Insecticides do not always decrease calling behavior, as in the case of *Ostrinia furnacalis* (Güenee) and *Spodoptera litura* (Fabricius) treated with pyrethroids as larvae (Wei and Du 2004;

287 Wei et al. 2004). Yet, sublethal insecticide could increase the percentage of calling females, as in
288 *Trichoplusia ni* (Hübner) treated with chlordimeform (Clark and Haynes 1992b). Regarding the
289 timing of calling behavior, Haynes and Baker (1985) observed that for their highest permethrin
290 dose (15 ng/moth, approx. an LC_{10}) the end of the calling period of *Pectinophora gossypiella*
291 (Saunders) was reduced by 1 h. Surviving adults of *O. furnacalis* (Wei and Du 2004) and
292 *Choristoneura fumiferana* (Clemens) (Dallaire et al. 2004) larvae treated with deltamethrin and
293 tebufenocide, respectively, started to call 1 h later than control females.

294 The calling periods that we have observed in tortricids under laboratory conditions may be
295 different under natural light conditions because our laboratory photoregime did not provide the
296 smooth light:dark transition that occurs at dawn and sunrise in the field, and this factor alone is
297 known to affect the periodicity of locomotor activity of other insects (Vanin et al. 2012).
298 Captures of male *C. pomonella* in pheromone traps show a 4-h activity peak around dusk time
299 under natural conditions (Knight et al. 1994), which suggests that the relatively long calling
300 period of *C. pomonella* observed under artificial conditions could be narrower under more
301 natural light conditions.

302 Unlike calling behavior, thiacloprid only affected pheromone production in one of the three
303 species, *C. pomonella*, and it required higher doses than what was needed to affect calling
304 behavior. The quantity of the major compound, codlemone, and one of the three minor
305 compounds, 12:OH, were approximately halved compared to the acetone control at LC_{10} or
306 LC_{20}, and the ratio with respect to codlemone of two minor compounds, E9-12:OH and 14:OH,
307 increased 4 and 2.5-fold, respectively, at LC_{20}. Detrimental effects on pheromone production
308 have been observed with deltamethrin in *O. furnacalis* (Yang and Du 2003) and with azinphos-
309 methyl in *Choristoneura rosaceana* (Harris) (Delisle and Vincent 2002; Trimble et al. 2004).
310 Changes in component ratios with sublethal doses of deltamethrin have been described in *S.*

311 *litura* (Wei et al. 2004), and with a biopesticide mixture containing *Bacillus thuringiensis*
312 (Berliner) and abamectin in *H. armigera* (Shen et al. 2013). Lack of effect of sublethal doses on
313 pheromone production, as in *G. molesta* and *L. botrana*, has been described also in *T. ni* treated
314 with cypermethrin and chlordimeform (Clark and Haynes 1992a,b).

315 It is interesting that thiacloprid affected calling behavior and pheromone production in *C.*
316 *pomonella* but only calling behavior in *G. molesta* and *L. botrana*. In other species there is also a
317 differential effect of insecticide on calling behavior and pheromone production (Clark and
318 Haynes 1992a,b; Yang and Du 2003; Trimble et al. 2004; Wei and Du 2004; Shen et al. 2013).
319 Pheromone biosynthesis in moths is mediated by a brain-released neurohormone (PBAN) that
320 reaches the pheromone gland through the haemolymph and binds to specific receptors on the
321 membrane of pheromone secretion cells (Jurenka and Rafaeli 2011; Groot 2014). A likely
322 mechanism by which the neurotoxic insecticide thiacloprid could alter pheromone production is
323 by reducing PBAN secretion. In *O. furnacalis* an homogenate of the PBAN-producing tissues
324 from females treated with the pyrethroid deltamethrin, which produced less pheromone than
325 controls, resulted in a reduction of pheromone titer in the glands of the decapitated females in
326 which it was injected, which suggests that deltamethrin reduced PBAN secretion in this species
327 (Yang and Du, 2003). It appears that juvenile hormone (JH) is involved in the regulation of
328 calling behavior (Rafaeli 2009), and therefore insecticides may affect calling behavior and
329 pheromone production differently. Since PBAN, JH and pheromone biosynthesis mechanisms
330 are probably very similar in the three tortricid species (Roelofs and Rooney 2003; Jurenka and
331 Rafaeli 2011), it remains to be determined why similar sublethal doses of thiacloprid resulted in
332 differential effects in pheromone production and calling behavior among the three moth species.

333 Several questions need to be solved in order to determine the impact of our findings in IPM
334 control. Males respond not to the pheromone in the gland but to the volatiles released by calling

335 females, so we need to know if thiacloprid alters the composition of the pheromone blend
336 emitted by females, as has been reported in *T. ni* with chlordimeform (Clark and Haynes, 1992b).
337 Obviously the effect of thiacloprid on male response needs to be determined too, as insecticides
338 are known to affect moth pheromone responses (Linn and Roelofs 1984; Wei and Du 2004; Wei
339 et al. 2004; Zhou et al. 2005; Knight and Flexner 2007; Rahbi et al. 2016). Additionally, it needs
340 to be determined if thiacloprid-treated females are as attractive to males as untreated ones, or less
341 active at mating than untreated ones, as has been shown in other moth species (Delpuech et al.
342 1998; Wei et al. 2004; Knight and Flexner 2007; Reinke and Barrett 2007; Barrett et al. 2013;
343 Quan et al. 2016). Mating in our test species is preceded by a courtship that may include contact
344 chemical cues and short-range pheromones associated with male hair pencil displays (Jurenka
345 and Rafaeli 2011), and these elements of mating behavior could also be affected by thiacloprid.

346 If thiacloprid is detrimental to these elements of mating behavior, its effect on reproduction may
347 be even larger than what our results suggest, with a possible enhancement of semiochemical IPM
348 control. For this reason, basic knowledge of insecticide effects on insect behavior, physiology,
349 and reproductive success could be a critical issue if we want to optimize IPM strategies.

350

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354

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Table 1 Effect of thiacloprid on the percentage of calling observations during the calling period. Different letters within a species indicate significant differences among insecticide treatments (P<0.05, *Tukey* after GLM).

Species	Treatment	N ♀[a]	N Obs.[a]	N Tot.[a]	% calling (95% CI)		
Cydia pomonella	Acetone	64	14	896	67.30	(64.16; 70.29)	a
	$LC_{0.001}$	63	14	882	49.43	(46.14; 52.73)	b
	LC_{1}	61	14	854	39.70	(36.46; 43.02)	c
	LC_{10}	69	14	966	34.16	(31.24; 37.21)	c
	LC_{20}	68	14	952	20.06	(17.64; 22.73)	d
Grapholita molesta	Acetone	65	5	325	81.54	(76.94; 85.39)	a
	$LC_{0.001}$	65	5	325	77.85	(73.00; 82.03)	a
	LC_{1}	65	5	325	67.08	(61.78; 71.97)	b
	LC_{10}	66	5	330	51.21	(45.82; 5657)	c
	LC_{20}	64	5	320	20.31	(16.25; 25.08)	d
Lobesia botrana	Acetone	63	6	378	59.62	(54.22; 64.11)	a
	$LC_{0.001}$	64	6	384	49.74	(44.75; 54.73)	ab
	LC_{1}	62	6	372	48.66	(43.60; 53.73)	b
	LC_{10}	70	6	420	49.29	(44.52; 54.06)	b
	LC_{20}	66	6	396	48.48	(43.59; 53.41)	b

[a] N ♀ = number of females; N Obs. = number of observations into the calling period of each species; N Tot. = total N consider in GLM analysis, which is the product between N♀ and N Obs.

539 **Table 2** Effect of thiacloprid on the start, mid and end calling times relative to the onset of the scotophase (in minutes). Different letters within a

540 column and species indicate significant differences among treatments ($P < 0.05$, *Tukey* after GLM). N = number of females.

Species	Treatment	N	Start (mean ± SEM)		Mid (mean ± SEM)		End (mean ± SEM)	
Cydia pomonella	Acetone	61	37.38 ± 7.08		183.44 ± 10.27	a	329.51 ± 19.38	a
	$LC_{0.001}$	54	43.61 ± 7.52		168.61 ± 10.92	ab	293.61 ± 20.6	ab
	LC_1	48	45.63 ± 7.98		134.38 ± 11.58	bc	223.13 ± 21.85	bc
	LC_{10}	45	25 ± 8.24		130.67 ± 11.96	bc	236.33 ± 22.57	bc
	LC_{20}	32	30.94 ± 9.78		105.23 ± 14.18	c	179.53 ± 26.76	c
Grapholita molesta	Acetone	63	-159.52 ± 6.51	a	-90.48 ± 3.72	a	-21.43 ± 3.61	
	$LC_{0.001}$	62	-162.1 ± 6.56	a	-92.42 ± 3.75	a	-22.74 ± 3.64	
	LC_1	61	-120 ± 6.62	b	-66.15 ± 3.78	b	-12.3 ± 3.67	
	LC_{10}	54	-115 ± 7.03	bc	-67.5 ± 4.01	b	-20 ± 3.9	
	LC_{20}	25	-85.2 ± 10.34	c	-56.4 ± 5.9	b	-27.6 ± 5.73	
Lobesia botrana	Acetone	58	6.98 ± 1.99		35.3 ± 3.24		63.62 ± 6.1	
	$LC_{0.001}$	55	8.45 ± 2.04		38.73 ± 3.32		69 ± 6.27	
	LC_1	51	5.59 ± 2.12		34.56 ± 3.45		63.53 ± 6.51	
	LC_{10}	60	9.5 ± 1.95		35.5 ± 3.18		61.5 ± 6	
	LC_{20}	53	5.94 ± 2.08		33.82 ± 3.39		61.7 ± 6.38	

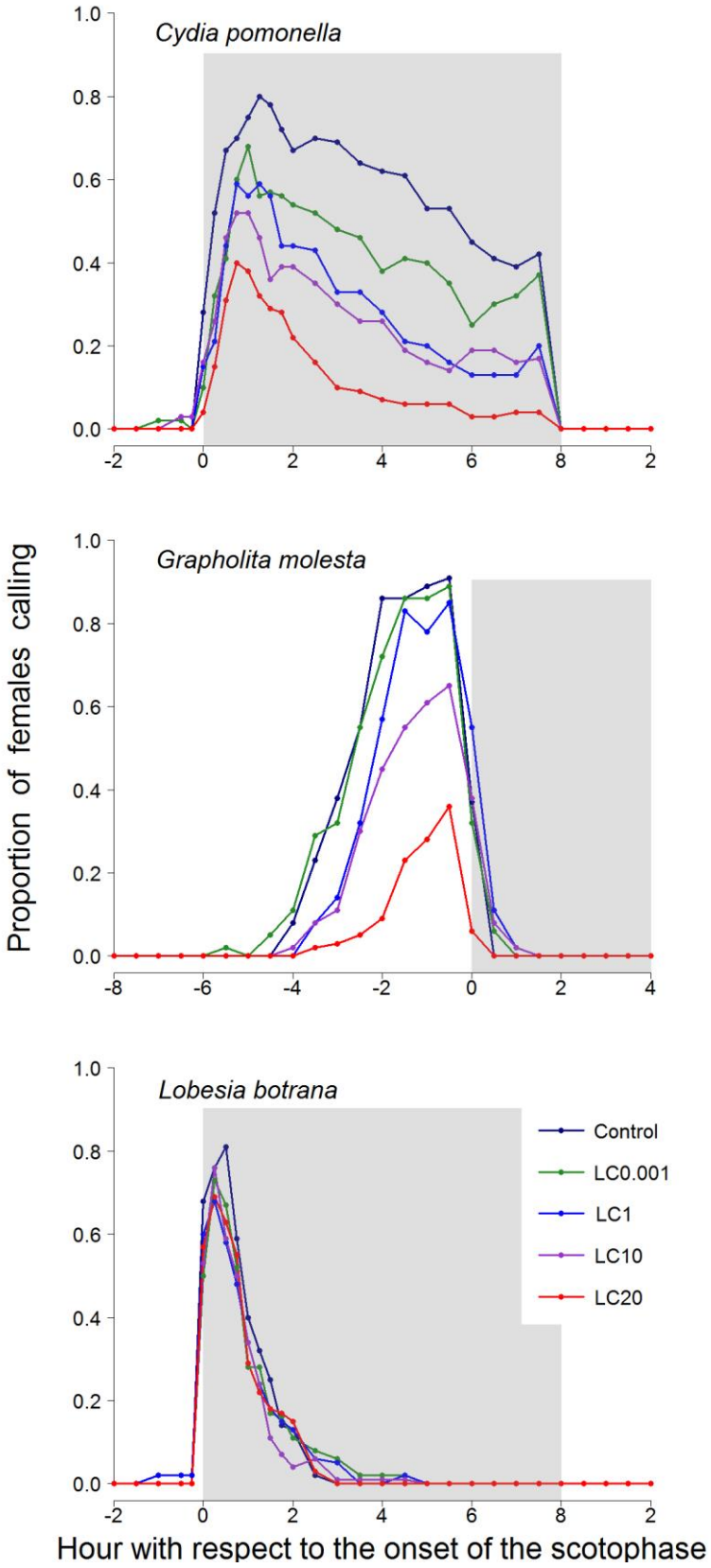
541

Table 3 Effect of thiacloprid on the ratio of minor pheromone components relative to the major pheromone compound. Minor compounds 1, 2 and 3 for *C. pomonella* (12:OH, E9-12:OH, 14:OH), *G. molesta* (12:OH, E8-12:Ac, Z8-12:OH) and *L. botrana* (E9-12Ac, Z9-12Ac + 11-12Ac, E,Z-7,9-12OH). Different letters within a column and species indicate significant differences among thiacloprid treatments ($P<0.05$, *Tukey* after GLM). N = number of females.

Species	Treatment	N	Minor compound 1 % (mean ± SEM)	Minor compound 2 % (mean ± SEM)		Minor compound 3 % (mean ± SEM)	
Cydia pomonella	Acetone	21	31.41 ± 6.63	13.99 ± 7.00	b	15.98 ± 5.86	b
	$LC_{0.001}$	21	39.56 ± 6.63	21.08 ± 7.00	b	17.33 ± 5.86	ab
	LC_1	21	40.86 ± 6.63	23.34 ± 7.00	b	20.81 ± 5.86	ab
	LC_{10}	21	48.57 ± 6.63	39.81± 7.00	ab	28.15 ± 5.86	ab
	LC_{20}	20	45.35 ± 6.80	55.59 ± 7.18	a	40.00 ± 6.00	a
Grapholita molesta	Acetone	20	4.80 ± 0.33	6.06 ± 0.29		35.64 ± 2.04	
	$LC_{0.001}$	21	4.97 ± 0.32	6.89 ± 0.28		33.69 ± 1.99	
	LC_1	21	4.67 ± 0.32	5.94 ± 0.28		31.71 ± 1.99	
	LC_{10}	20	5.22 ± 0.33	6.30 ± 0.29		31.88 ± 2.04	
	LC_{20}	21	4.83 ± 0.32	6.06 ± 0.28		29.33 ± 1.99	
Lobesia botrana	Acetone	21	13.03 ± 1.86	1.42 ± 0.25		20.17 ± 1.90	
	$LC_{0.001}$	20	10.06 ± 1.90	1.36 ± 0.26		22.38 ± 1.95	
	LC_1	19	8.45 ± 1.95	1.09 ± 0.27		16.87 ± 2.00	
	LC_{10}	21	9.48 ± 1.86	1.72 ± 0.25		16.62 ± 1.90	
	LC_{20}	21	9.46 ± 1.86	1.01 ± 0.25		18.36 ± 1.90	

554 **Figure 1** Effect of thiacloprid on the percentage of females calling (N=61-70). The grey area

555 represents the scotophase.



556

Figure 2 Effect of thiacloprid on the quantity of individual pheromone components in the pheromone gland (N=20-21). Different letters indicate significant differences among treatments for each compound and species (P<0.05, *Tukey* after GLM)