

Evaluation of pyriproxyfen, a juvenile hormone analog, on *Drosophila melanogaster* (Diptera: Drosophilidae): Insecticidal activity, ecdysteroid contents and cuticle formation

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Abstract. The efficacy of pyriproxyfen, a juvenile hormone analog (JHA), was evaluated using third instar larvae of *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae). Various doses of the compound, ranging from 0.01 to 2 ng/larva, were applied topically to larvae (12 h before pupariation). Treatment did not prevent pupariation but inhibited adult emergence at all the doses tested. In a second series of experiments the ecdysteroid content of pupae was determined following application of pyriproxyfen at two doses, 0.108 and 0.29 ng/larva, corresponding to ID_{25} and ID_{50} , the doses required for 25 and 50% inhibition of adult emergence, respectively. Pyriproxyfen treatment increased the duration of pupal development. In addition, enzyme immunoassay measurements of ecdysteroids in whole body extracts of pupae indicated that pyriproxyfen decreased the ecdysteroid content in a dose-dependent manner. Finally, the effects on the cuticle of pyriproxyfen (ID_{50}) were studied histologically, which revealed that this compound increased the thickness of the new adult cuticle and suppressed the formation of bristles. Biochemical analyzes revealed that an increase in chitin content of the cuticle is only recorded at the highest dose. Thus, a topical application of pyriproxyfen to third instar larvae interfered with the molting hormone and disrupted the normal development of this insect.

INTRODUCTION

In insects 20-hydroxyecdysone (20E) and juvenile hormone (JH), play a central role in the regulation of growth and development (Nijhout, 1994). According to the classical dogma of insect endocrinology, the balance of these two hormones defines the outcome of each developmental transition (Dubrovsky, 2005). 20E initiates all major developmental transitions, but it is an interaction with JH that transduces the 20E pulses into stage-specific responses (Dubrovsky, 2005). Indeed, JH has been shown to modulate the action of ecdysteroids in various insects (Nijhout, 1994; Riddiford, 1996). There is little literature on the physiological and biochemical processes related to molting and metamorphosis in insects compared to other aspects, like reproduction (De Loof et al., 2015).

Recent advances in understanding the mechanism that regulate molting and metamorphosis in insects have relied heavily on using *Drosophila melanogaster* as a model system. However, the effect of JH on 20E and the cross-talk between these two hormones is not well established (Quinn et al., 2012; Yamanaka et al., 2013). In addition to its well-known advantages for molecular and genetic studies, *D. melanogaster* provides an ideal model system for elucidating the mechanisms of hormones, since 20E appears to be responsible for directing the major developmental transitions in the life cycle of this insect, including molting and metamorphosis (Quinn et al., 2012).

As recorded for ecdysone agonists and juvenile hormone analogs, interference with hormonal balance leads to inter-

rupted development and are potential specific targets for use in pest control (Penner & Dhadialla, 2012). However, a better and complete understanding of regulatory processes underlying insect development still imperative for their rational management. Furthermore, JHAs are also excellent tools for studying endocrinological mechanisms in insects (Ramaseshadri et al., 2012).

The insect growth regulator (IGR), pyriproxyfen, is a JHA with a relatively low mammalian toxicity (Miyamoto et al., 1993). It is a broad-spectrum insect growth regulator with insecticidal activity against agricultural, horticultural and public health insect pests (Who, 2008), and has been successfully used to control important pests of many agricultural crops all over the world (Sazo et al., 2008; Moadeli et al., 2014). Pyriproxyfen, the most potent JHA available today (Hatakoshi, 2012), inhibits metamorphosis and embryogenesis in several insect orders, including Diptera (Kawada et al., 1988; Ishaaya et al., 1994; Aribi et al., 2006). In addition, this compound suppresses oviposition, reduces viability of eggs (Ghasemi et al., 2010; Ohba et al., 2013) and reduces fecundity (Singh & Kumar, 2015). Recently, pyriproxyfen was reported to affect some physical and biochemical processes in insects, such as moulting, by influencing the activity of chitinases (Nasr et al., 2010).

Therefore, in the present study we investigated the effect of pyriproxyfen on *D. melanogaster* in order to better understand the interaction between the two principal hormones, JH and 20E. Pyriproxyfen was applied topically to larvae at the end of third instar, when the JH titer drops to

an undetectable level to allow the molting hormone (ecdysteroids) to initiate metamorphosis, in order to firstly determine its insecticidal potency, and then its effects on pupal development, the ecdysteroid profile and the chitin content and structure of the new cuticle, respectively.

MATERIAL AND METHODS

Insects

Canton-S wild-type flies of *D. melanogaster*, a widely used laboratory strain, were reared in glass vials containing a yeast/cornmeal/agar laboratory medium containing an anti-fungal agent, which were kept in a breeding room at $25 \pm 2^\circ\text{C}$ and 70% humidity under a 12L : 12D cycle.

Treatment and bioassay

Pyriproxyfen (>95%, Sumitomo Chemical Company. Ltd., Osaka, Japan) was dissolved in acetone and topically applied (1 μl /insect) to third instar larvae of *D. melanogaster* (12 h before pupariation when the JH titer drop to an undetectable level). Control insects were treated with 1 μl acetone alone.

This compound was tested at five doses (0.01, 0.1, 0.5, 1 and 2 ng/larva) and the percentage inhibition of adult emergence was recorded. For each dose, three replicates of 20 insects were used. The percentage inhibition was corrected following Abbott (1925). The ID_{50} and ID_{25} (doses that caused an inhibition in adult emergence of 50% and 25%, respectively) were determined together with the corresponding 95% fiducial limits (95% FL) and the Hill slope.

Duration of pupal development

The duration of the pupal period was recorded for control and treated (ID_{50} , ID_{25}) insects. Pupae were observed at 4 h intervals until adult emergence. There were seven to eight replicates, each consisting of 20 insects.

Enzyme immunoassay of ecdysteroids

Third-instar larvae were treated as above with two doses (ID_{50} and ID_{25}) and surviving pupae were sampled at various times during pupal development (12, 36, 48, 60 and 84 h after puparium formation (APF)). Samples were extracted individually with methanol by sonication (2–3 min). After centrifugation (5000 g, 10 min), the supernatants were taken and evaporated (60°C). Each body extract was resuspended in 500 μl of phosphate buffer (0.1 M; pH 7.4) and analyzed using an enzyme immunoassay (EIA) as previously described (Aribi et al., 2006) using rabbit polyclonal B antibody against 20E, peroxidase as an enzymatic tracer and tetramethyl benzidine as a colouring agent. Data are expressed in pg 20E equivalents per mg of body weight. Antibody was kindly supplied by J.P. Delbecq (CNRS, Université de Bordeaux I, France) and peroxidase by C. Blaise (Pierre and Marie Curie University, Paris, France).

Chitin quantification

Chitin quantification of a whole pupa was performed as previously described (Farnesi et al., 2012). Chitin content of pupae sampled at various ages (12, 36, 48, 60 and 84 h APF) was evaluated by quantification of glucosamine derivatives obtained by deacetylation, depolymerisation and deamination of N-acetylglucosamine polymer. Chitin undergoes an alkaline digestion with KOH (14 M) at 130°C in order to deacetylate the chitin, thus forming chitosan. A solubilized chitosan solution is then depolymerized using NaNO_2 (10%) and KHSO_4 (10%) in order to release the amine residues from the glucosamine, forming a soluble aldehyde. The aldehydes generated in a reaction with HNO_2 and with the further addition of MBTH and Fe^{+3} develop a blue col-

oration. Absorbance was read at 650 nm and chitin content was expressed as glucosamine equivalents, according to a standard curve of glucosamine (50–1000 μg). Before chitin quantification, the weight of the pupae was determined to normalize the results.

Histology

Histological procedures were performed following Martoja & Martoja (1967). Pupae (60 and 84 h APF, these ages were chosen because adult cuticle deposition begins between 48 and 53 h APF) that developed, from pyriproxyfen-exposed or control larvae were fixed in 10% formol. After dehydration in a series of graded ethanol the samples were passed through three washes in xylene before being embedded in paraffin wax. Transverse sections of the abdomen (4 μm) obtained using a Leica RM2125T (Leica Microsystems Nussloch GmbH, Wetzlar, Germany) manual rotary microtome, were stained with hematoxylin-Eosin. The abdominal region was chosen because JHA causes the development of abnormalities in this region (Zhou & Riddiford, 2002). Observations were made using a Leica DM500 microscope equipped with a Leica ICC50 HD camera and the thickness of the cuticle, based on five measurements, was determined using Las EZ Leica software.

Statistical analysis

Results are given as means \pm standard errors (SE). Data for the toxicity were analyzed using non-linear sigmoid curve fitting, and the activity of the treatment was evaluated in terms of a dose dependent response. The goodness of fit to the curve was evaluated on the basis of R^2 values. The homogeneity of variances was checked using Bartlett's and Brown-Forsythe tests. Data on pupal duration, EIA measurement and chitin quantification were subjected to one-way or two-way analysis of variance (ANOVA) followed by a post-hoc HSD Tukey test. The cuticle measurements were assessed using a Mann-Whitney test. All statistical analyses were performed using Prism v 6.01 for Windows (GraphPad Software Inc., www.graphpad.com).

RESULTS

Insecticidal activity

Pyriproxyfen applied topically to third instar larvae of *D. melanogaster* inhibits adult emergence in a dose dependent way. Partial adult emergence and malformed adults were also classified as dead, since treatment does not prevent pupariation. The corrected inhibition percentages ranged from $8.12 \pm 1.55\%$ at lowest dose (0.01 ng) up to a maximum of $89.69 \pm 1.47\%$ at the highest dose (2 ng) (Fig. 1). The percentage inhibition recorded in controls was $11.66 \pm 1.66\%$. The ID_{50} and ID_{25} values together with their corresponding 95% fiducial limits (95% FL) are given in Table 1.

TABLE 1. Toxicity of pyriproxyfen applied topically to larvae of *D. melanogaster* at the end of the third instar. The data are expressed in terms of inhibiting dose (ID) of adult emergence together with the corresponding 95% fiducial limits (95%FL). R^2 – coefficient of determination.

Doses	Value (ng)	Fiducial limits (95%)	R^2
ID_{50}	0.29	[0.21–0.39]	0.99
ID_{25}	0.108	[0.06–0.17]	
Hill slope	1.11	[0.78–1.43]	

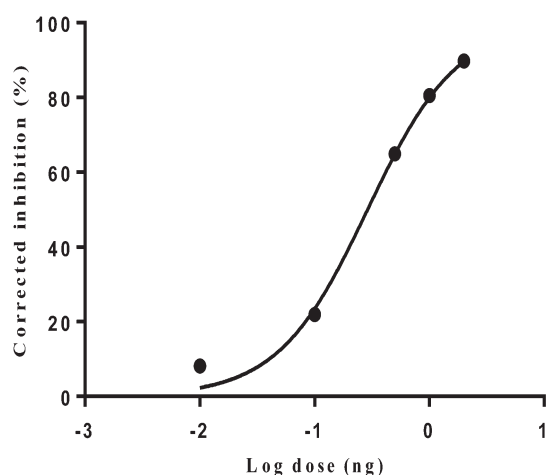


Fig. 1. Effect of pyriproxyfen, topically applied to larvae of *D. melanogaster* at the end of the third instar: sigmoidal dose-dependent response curve of corrected inhibitions (%) of adult emergence as a function of the logarithm of the doses.

Effect on the duration of pupal development

The duration of pupal development is given in Fig. 2. Treatment of third instar larvae increased the duration of pupal development only at the highest dose ID₅₀ (0.29 ng) ($F_{2,20} = 8.543$; $p = 0.0021$). The means values recorded were 88.25 ± 3.01 h for the control and 102.57 ± 1.71 h for the (ID₅₀) treated pupae. Thus, pyriproxyfen delayed adult emergence by approximately 14 h.

Effect on the ecdysteroid content

During pupal development the ecdysteroid titer in the control series increased from 264.89 ± 7.68 pg 20E/mg at 12 h APF to reach a peak of 414.24 ± 2.71 pg 20E/mg at 36 h APF and then decreased ($p < 0.0001$) (Fig. 3). In the treated series, a similar trend was recorded for the two doses tested (ID₂₅ and ID₅₀). However, ecdysteroid titer remained stable after 60 h in the ID₂₅ and after 48 h in the ID₅₀ treatments. EIA measurements revealed that the two doses tested caused significant reductions in the ecdysteroid titers ($p < 0.0001$) at 12 h and 36 h APF. Moreover,

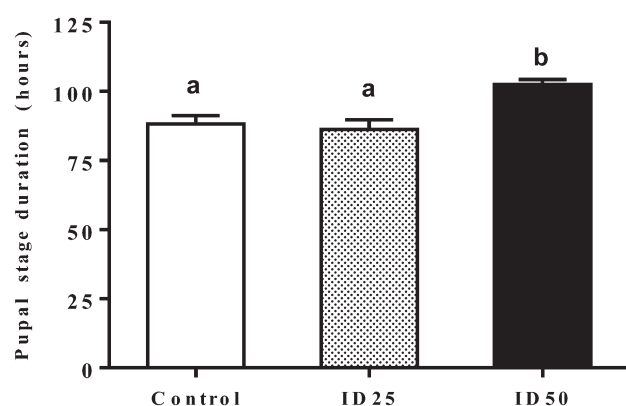


Fig. 2. Effect of pyriproxyfen, topically applied to larvae of *D. melanogaster* at the end of the third instar, on the duration of the pupal stage (h) ($m \pm SE$; $n = 7-8$ replicates, each of 20 insects). Different letters indicate a significant difference ($p < 0.05$).

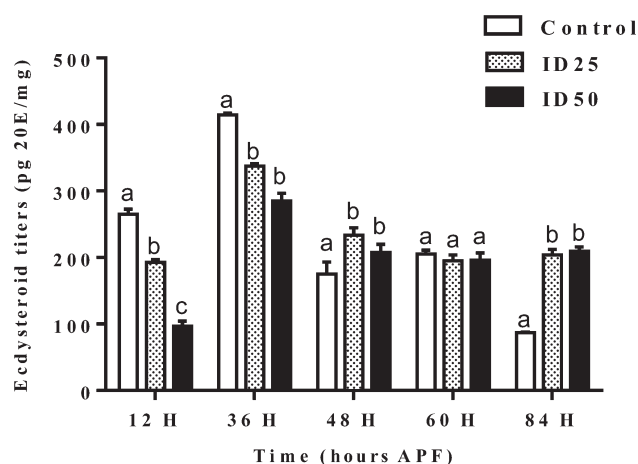


Fig. 3. Effect of pyriproxyfen (ID₂₅, ID₅₀), topically applied to larvae of *D. melanogaster* at the end of the third instar, on the ecdysteroid (pg 20E/mg of weight) titer during pupal development ($m \pm SE$; $n = 3-5$). Mean values for individuals of the same age followed by different letters are significantly different ($p < 0.05$).

an increase in ecdysteroids titer was recorded at 84 h in the treated series because the values recorded between 60 and 84 h remained stable in the treated series but decreased after 60 h in the controls. ANOVA revealed significant effects of dose ($F_{2,36} = 22.25$; $p < 0.0001$), time ($F_{4,36} = 205$; $p < 0.0001$) and the dose-time interaction ($F_{8,36} = 51.92$; $p < 0.0001$).

Effect on chitin content

As shown in Fig. 4, the whole body chitin content of the controls increased during pupal development (36, 48 h APF) and then decreased ($p < 0.0001$) (60 and 84 h). Generally, the trend in the chitin content of the treated series is similar to that in the control and appears to be correlated with the ecdysteroid titers (Fig. 3). Pyriproxyfen treatment resulted in a significant ($p < 0.0001$) increase in the chitin content compared to the control only at the highest dose (ID₅₀). ANOVA revealed a significant effect of dose ($F_{2,119}$

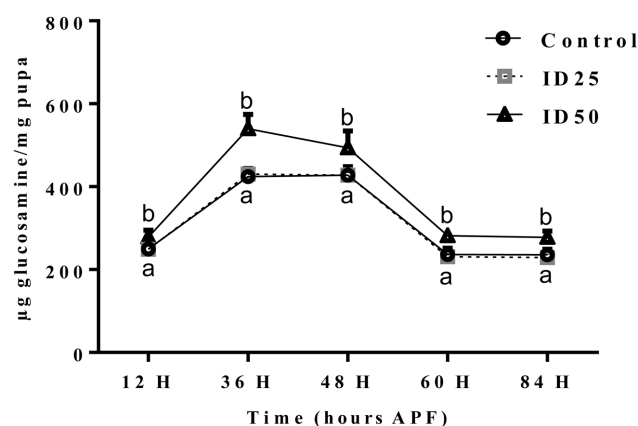


Fig. 4. Effect of pyriproxyfen (ID₂₅, ID₅₀), topically applied to larvae of *D. melanogaster* at the end of the third instar, on the chitin content (μ g of glucosamine/mg) during pupal development ($m \pm SE$; $n = 8-11$). Mean values for individuals of the same age followed by different letters are significantly different ($p < 0.05$).

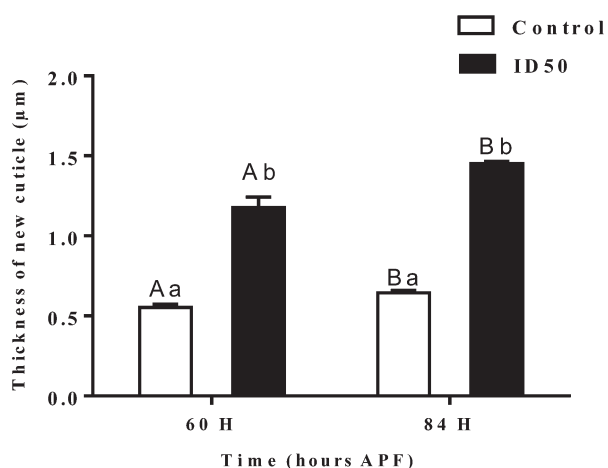


Fig. 5. Effect of pyriproxyfen (ID_{50}), topically applied to larvae of *D. melanogaster* at the end of the third instar, on cuticle thickness (μm) ($m \pm \text{SE}$; $n = 5$). Different capital letters indicate a significant difference between individuals of the same age in the same series; different small letters indicate a significant difference between control and treated individuals of the same age ($p < 0.05$).

= 108.2; $p < 0.0001$), time ($F_{4,119} = 700$; $p < 0.0001$) and the dose-time interaction ($F_{8,119} = 6.085$; $p < 0.0001$).

Structure of new cuticle

As shown in Fig. 5 the thickness of new cuticle in the control series was $0.552 \pm 0.021 \mu\text{m}$ at 60 h APF and increased to a maximum of $0.645 \pm 0.013 \mu\text{m}$ at 84 h APF ($p < 0.01$). Pyriproxyfen (ID_{50}) treatment caused a significant increase in the thickness of the cuticle ($p < 0.01$) recorded at 60 and 84 h APF. Moreover, the increase in cuticle thickness in the treated series (ID_{50}) is much greater whatever the age (mean percentage of 45%) compared to the standard increase with age in the control series, which is 14.41%. As shown in Fig. 6, the new adult cuticle in the control series has normal bristles (Fig. 6A), but lacks bristles in treated series (Fig. 6B).

DISCUSSION

In the present study we topically applied a juvenile hormone analog, pyriproxyfen, to third instar larvae of *D. melanogaster* just prior to pupariation. This did not kill the larvae or prevent them from pupating but did result in a dose-dependent inhibition of adult emergence and caused morphological abnormalities in the adults that did emerge. Zhou & Riddiford (2008) report that unlike Lepidoptera and most other holometabolous insects, higher Diptera, such as *D. melanogaster*, have lost most of their sensitivity to JH. Indeed, topical applications of JH or JHA do not prevent *D. melanogaster* larvae pupating and have no role in initiating metamorphosis (Riddiford et al., 2003). Furthermore, in *Drosophila* and other higher flies, the pupa is derived from imaginal discs, except for the abdominal cuticle, which is produced by persisting larval epidermal cells and histoblasts, which may account for the inability of JH or JHA to prevent the larval-pupal transformation (Zhou & Riddiford, 2002). Nevertheless, applications of JH or JHA before or at the time of pupation prevent normal adult development (Zhou & Riddiford, 2002).

Pyriproxyfen is known to be a potent inhibitor of metamorphosis and adult development. Indeed, Boina et al. (2009) report that the topical application of pyriproxyfen suppress adult emergence in the Asian citrus psyllid. Pyriproxyfen also inhibits the emergence of *Aedes aegypti* (Sihuinchu et al., 2005; Harburger et al., 2011), *Anopheles culicifacies*, *Anopheles subpictus* (Yapabandara & Curtis, 2004), *Anopheles gambiae* (Mbare et al., 2013), *Bradyzia coprophila* (Ludwig & Oetting, 2001), *Lycoriella ingenua* (Erler et al., 2011) and *Culex pipiens* (Al-Sarar et al., 2011). Singh & Kumar (2015) also report a decrease in adult emergence, formation of pupal-adult mosaics and the development of deformed adults in the F1 generation of *Sarcophaga ruficornis* after topically applying pyriproxyfen to the parental generation.

In the current experiments, pyriproxyfen also prolonged pupal development at the highest dose (ID_{50}) compared

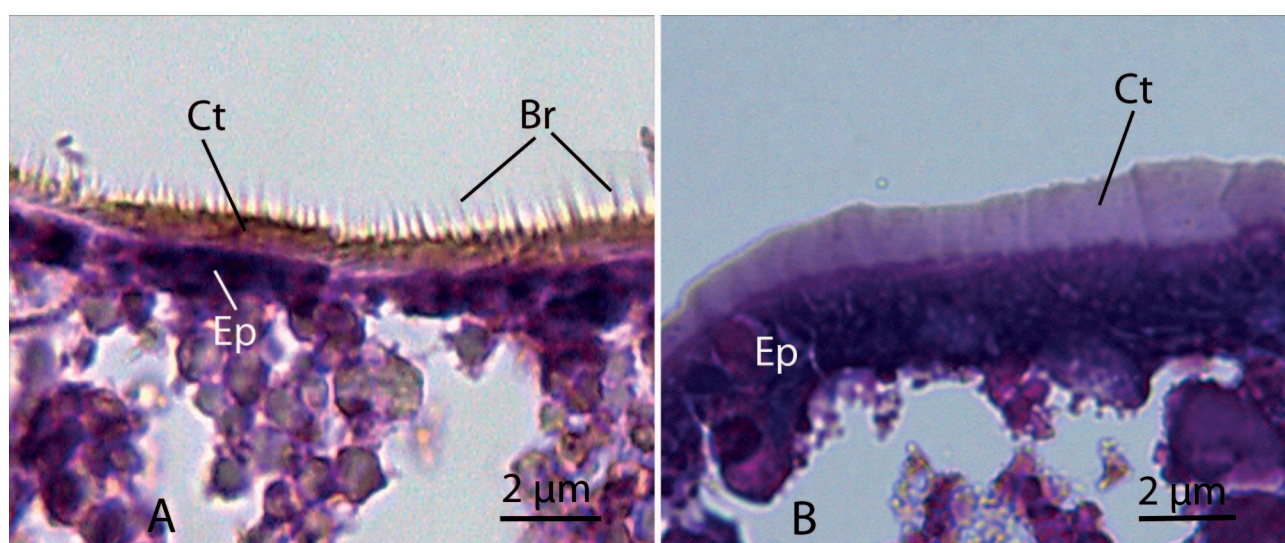


Fig. 6. Effect of pyriproxyfen (ID_{50}), topically applied to larvae of *D. melanogaster* at the end of the third instar. A – control: new adult cuticle with a normal bristles; B – treated: new cuticle with no bristles. Ct – cuticle; Ep – epidermis; Br – bristles.

to the controls, which indicates that the JH analog (here pyriproxyfen) can induce delays in the development of the pupal-adult transition. Alizadeh et al. (2012) previously reported that the larvae and pupae of *Plutella xylostella* treated with pyriproxyfen take longer to complete their development. Similar effects are reported by Singh & Kumar (2011) for *Papilio demoleus* treated with pyriproxyfen. Hamaidia & Soltani (2014) also report an increase in the duration of the larval and pupal stages of *Culex pipiens* treated with Kinoprene, another JHA.

At the beginning of the pupal stage there is JH sensitive period when JH must be absent in epidermal cells for successful adult development (Nijhout, 1994). Hence, the presence of pyriproxyfen at these critical times resulted in a prolonged pupal development, deformed adults and reduction in adult emergence.

In *D. melanogaster*, metamorphosis is controlled by several pulses of 20E secretion, the first occurs at the end of the third instar larva, when the JH titer is very low and initiates puparium formation, the second 12 h after puparium formation (APF) triggering pupation, and finally the onset of adult development requires a large wave beginning approximately 30 h APF in the total absence of JH (Handler, 1982; Riddiford, 1993). In the present study we demonstrated that pyriproxyfen applied at the end of last larval instar significantly reduced the ecdysteroid titer during the pupal stage by inhibiting the production of 20E. Negative effects of JHA on ecdysteroid titers are reported in different orders of insects, especially Lepidoptera and Coleoptera. Aribi et al. (2006) report that pyriproxyfen reduces, in a dose-dependent manner, the ecdysteroid titer in the hemolymph of *Tenebrio molitor* pupae. Similar effects are recorded for other JHA, like methoprene and fenoxycarb, on the larval stages of the Coleoptera, *Tribolium freemani* (Hirashima et al., 1995) and *Zophobas atratus* (Aribi et al., 1999), Lepidoptera, *Bombyx mori* (Monconduit et al., 1998) and *Omphisa fuscidentalis* (Singtriopo et al., 2002) and the adult stage of *D. melanogaster* (Diptera) (Handler, 1982). Indeed, one of the many vital functions of JH in insect development is to modulate the action of the molting hormone (20E) and coordinate insect moulting and metamorphosis (Riddiford, 2008). Moreover, Yamana et al. (2007) demonstrate the direct effect of JH on the prothoracic gland during ecdysterodogenesis.

Chitin has an important role in insect development and its synthesis and degradation must be tightly regulated. Our results indicate that only the highest dose (ID₅₀) of pyriproxyfen significantly increased the chitin content of pupae of *D. melanogaster* compared to the control. It is reported that the molting hormone 20E regulates chitin metabolism by stimulating the secretion and activity of enzymes (Mezendorfer & Zimoch, 2003). Both the secretion and activation of chitinolytic enzymes are clearly controlled by ecdysteroids (Reynolds & Samuels, 1996). In addition, Kimura (1973) showed many years ago that the activity of enzymes in the molting fluid can be stimulated by injecting ecdysteroid. More recently, Nasr et al. (2010) report an inhibition of chitinase activity in larvae

of *Spodoptera littoralis* treated with pyriproxyfen. Indeed, the expression of the gene determining the production of chitinolytic enzymes, which is normally induced by ecdysteroids, is suppressed by juvenile hormone (Cohen, 2010). In *Manduca sexta* expression of chitinase genes is suppressed by the topical application of the juvenile hormone mimic, fenoxycard (Kramer et al., 1993; Zen et al., 1996). The increase in chitin content recorded in our experiments may be related to an inhibition of chitinase activity due to the reduction in the secretion of ecdysteroids recorded following treatment with pyriproxyfen.

The histological study revealed that pyriproxyfen affects the secretion of the adult cuticle resulting in an increase in the thickness of the new cuticle and suppression of the development of bristles. The stimulation of the secretion of the adult cuticle by JHA is reported by Dedos & Fugo (2001) in *B. mori* treated with fenoxycarb. The increase in cuticle thickness recorded in our experiments seems to be correlated with the increase in chitin content, which is an important constituent of cuticles. Moreover, it is reported that in *D. melanogaster* the application of JH or JHA during the final larval instar or at pupariation causes the formation of a pupal-adult mosaic, with a transparent abdomen covered with pupal cuticle, with no or a few bristles (Zhou & Riddiford, 2002). Indeed, the status quo action of JH on the pupal-adult transformation is mediated by the JH-induced re-expression of the broad (br) gene during adult differentiation causing the formation of a second pupal cuticle (Zhou & Riddiford, 2002; Jindra et al., 2013). Br can both activate pupal genes and suppress adult genes, thus, during the crucial period of the commitment to adult development, ecdysone in the absence of JH switches off Br so that the adult-specific program of differentiation can occur (Goodman & Granger, 2005). The presence of JH (here pyriproxyfen) at this critical period may interfere with ecdysone and account for the abnormalities in cuticle structure and inhibition of adult emergence recorded in *D. melanogaster*. Furthermore, as the cuticle of insects is an effective barrier to the evaporation of water (Gibbs, 2011), the increase in cuticle thickness recorded when treated with pyriproxyfen may act as potential protective system against hot temperatures and desiccation, especially in the context of global warming.

In conclusion, our findings indicate that pyriproxyfen negatively affects the hormonal control of metamorphosis causing perturbations in *D. melanogaster* development. Nevertheless, the precise way in which ecdysone is affected by pyriproxyfen requires further research. Moreover, information is needed on the sublethal effects of pyriproxyfen on their offspring.

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