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EFFECTS OF 20-HYDROXYECDYSONE AND INSULIN APPLICATION ON REPRODUCTION IN *EPHESTIAKUEHNIELLA* ZELLER (LEPIDOPTERA: PYRALIDAE)

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ABSTRACT

Insulin and 20-hydroxyecdysone (20E) have known roles in insects and mammals, but their interactions are complex. Therefore, the effects of human insulin and 20E have been examined in an insect *Ephestiakuehniella*. These two molecules, administered separately (5 and 10 µg) on newly emerged pupae, were evaluated on the reproduction; insulin and ecdysone have been given to insects by topical acetone application. The primary effects of human insulin were, firstly, specified. Insulin increased glycogen, carbohydrates and proteins amounts compared to 20E, while it decreased the levels of lipids only with the 5µgdose. Insulin and 20E increased the vitellogenin and vitellin amounts but decreased ecdysteroid content in ovaries. Duration of the oviposition period was not affected by the 20E but is shortened by insulin at the two tested doses. Moreover, the fecundity and fertility (egg hatchability) are affected by the two molecules. Conclusively, acetone solutions of 20E or human insulin, applied topically, on insects have similar effects on all considered parameters.

Key words: *Ephestia kuehniella*, Insulin, 20-hydroxyecdysone, Reproduction, Metabolism.

INTRODUCTION

Reproduction and development in insects are based on two main hormones, juvenile hormone (JH) and ecdysteroids (Gäde and Hoffman, 2005; Belles and Piulachs, 2015). Ecdysteroidogenesis, widely documented (Brown *et al*. 2009; Niwa *et al*. 2010; Quinn *et al.* 2012; Yamanaka *et al*. 2013), can also be initiated by insulin or insulin-like peptides (ILP_S), a hormone with a major role in the carbohydrate metabolism and which also act as a nutrition-dependant control of insect development (Hua, 2010; Yamanaka *et al*. 2013; Okamoto and Yamanaka, 2015). Indeed, nutritional signals act in collaboration with other co-factors (rapamycin, protein kinases and nitric oxide), directly on ecdysteroidogenesis (Yamanaka *et al*. 2013). As in Vertebrates, insect ILPs seem to control others functions like growth, lifespan and reproduction (Antonova *et al*., 2012; Puig and Mattila, 2011).Insulin, secreted by insulin-producing cells in the brain, binds to its receptor (insulin receptors) and the complex thus formed acts through the cell signaling cascade *via* the protein kinases and a transcription factor protein named fork head box class O or FOXO (Antonova *et al*., 2012). The same factor is also connected with the regulation of ecdysone signaling (Yamanaka *et al*. 2013).Insulin signaling pathway, conserved in animal evolution, has been studied in insects (Antonova *et al*., 2012;Hou *et al.* 2012); in spite of similar signaling pathways, the Mammals and Insects, present structural differences at the ILP_S and receptors (Garelli *et al.* 2012).

Prior studies indicated that bovine insulin induces a biological response in insects but the physiological effects can be nuanced between endogenous ILP_S and mammalian insulin (Graf *et al*., 1997; Riehle and Brown, 1999; Manière *et al*., 2004; Brown *et al*. 2008); the specificity of the connection between the ligand and insulin receptors could explain the nuanced effects which are noted (Brown *et al*. 2008). The bovine or porcine insulin, compared to endogenous ILP_S, show varying effects on vitellogenesis and ecdysteroids synthesis (Brown *et al*. 2008) in Insects; insulin and 20E act either synergistically (Roy *et al*. 2007) or antagonistically manner (Colombani *et al.* 2005, 2006). Moreover, endogenous ILPs are also involved in ovarian steroidogenesis regulation (Riehle and Brown, 1999; Maniere *et al*., 2004). Physiological and molecular interactions, between insulin and steroid hormones, have been reported in some species of insects *Aedes aegypti* (Graf *et al*. 1997; Roy *et al*.2007), *Diacamma sp*(Okada *et al*. 2010) or *Drosophila melanogaster* (Mirth and Shingleton, 2012); however, these interactions were not well established in mammals. Colombani *et al.* (2005) note that this functional interaction, if is conserved in human, could play an important role in cancerous and metabolic pathologies where these signalisation pathways are involved. The factor FOXO is present in Mammals (Human also), and ecdysteroids (20E) can bind to oestrogenic receptors and cause similar effects to those induced by oestrogens (Jian *et al*. 2013).Bovine or porcine insulin, tested in insect, show or suggest, in cited studies, an interaction with ecdysteroid hormone but what about the effects of the human insulin to insects and

which is its action compared with the 20E? Therefore, the present study aims to examine the impact of human insulin and 20E tested separately in *Ephestia kuehniella* (Lepidoptera: Pyralidae). This biological model has been the subject of several studies related to reproduction and development (Tefler, 2009), profiles of ecdysteroids (El Ouar *et al*. 2010) and the impact of molting hormone agonists (Soltani-Mazouni *et al*. 2012).In lepidopteran species, ILPs have been identified and they are five in *Samia cynthia*, two in *Agruis convolvuli* and two in *Spodoptera littoralis* (Antonova *et al*., 2012). Still, little is known about the secretion of ILPs, receptor interactions, and their direct effects on specific processes in the target tissues of insects (Antonova *et al*., 2012). Then, the objective of this work was to investigate the effects of human insulin and 20E on some indicators of reproduction (vitellogenins, vitellins, ovarian ecdysteroids and reproductive potential) due to the role of these hormones on this process (Riehle and Brown, 1999; Maniere *et al*., 2004; De Loof, 2008; Belles and Piulachs, 2015). Moreover, in order to highlight the primary effect of human insulin (glucogenesis and associated interconversions), the main biochemical components (carbohydrates, proteins and lipids in fat body) were examined; only two studies have reported that synthetic ILPs directly affect metabolic processes in insects (Antonova *et al.,* 2012). Effects of 20E on metabolic aspects were also evaluated due to possible relationships between ILPs and ecdysteroids in metabolism control (Tatar *et al.,* 2003; Colombani *et al.,* 2005). The expected results will allow us to evaluate the separate action of these hormones to better understand their interactions.

MATERIALS AND METHODS

Insect and breeding: *E. kuehniella* Zeller (Lepidoptera, Pyralidae) is reared, to the laboratory, in boxes containing flour at a temperature of 25°C and a relative humidity of 70% in total obscurity (Soltani-Mazouni *et al*. 2012). Pupae are dated according to their age (days) from the pupal molting. Under our laboratory conditions the duration of pupal development is above 9 days.

Hormones and treatment of pupae: 20E (Sigma, USA) and insulin or INS (Sigma) were tested separately by topical application (3 µl/ insect), at two doses (5 µg and 10 µg), according to El Ouar *et al*. (2010) and Roy *et al*., (2007).The compounds are administrated on the abdomen of newly emerged pupae (< 6 h and without cocoon). Controls pupae received 3µl of solvent (acetone); acetone allows a better diffusion throughout the cuticle. Insuline is soluble in organics solvents (Brange, 1987) and solubility in acetone was cited in literature (Bergeron *et al*., 2003). In addition, previous studies, in our laboratory, show that acetone has no significant effect compared with untreated series in *E. kuehniella* (El Ouar *et al*., 2006).

Determination of biochemical composition of fat body: The fat body carbohydrates, glycogen, proteins and lipids amounts were determined, at various times during the pupal stage (0, 1, 3, 5 and 7 days), from control and treated series (20E or INS at 5µg and 10µg). Samples drawn from 6-8 individuals/age were conserved at -20°C in trichloroacetic acid (20%) until analysis. Extraction and quantification of main biochemical constituents were as previously described (Abdellaoui *et al*., 2013). Briefly, carbohydrates, lipids and proteins were extracted according to the procedure of Shibko *et al*. (1966), while glycogen according to Van Handel (1965). The dosage of carbohydrates and glycogen was realized according to the method of Duchateau and Florkin (1959) using anthrone as reagent and glucose or glycogen as standard. The lipids were quantified according to Goldsworthy *et al.* (1972) using vanillin as reagent and lipids as standard. Lastly, the proteins were quantified following the method of Bradford (1976) using blue brillant coomassie (G 250) as reagent and bovine serum albumin (BSA) as standard. The different results are expressed in mg.

Extraction and determination of ovarian free ecdysteroid amounts: The ovaries of controls and treated adult females, newly emerged (<6 hours), are dissected out and conserved in methanol (500µl); for each treatment, six to eight paired ovaries were analyzed individually. The enzyme immunoassay (EIA), of the free ovarian ecdysteroids, was conducted according to Aribi *et al*. (1997). Individual samples were diluted in methanol, sonicated and centrifuged. Supernatants were taken out and evaporated. The obtained extracts were dissolved in phosphate buffer (0.1 M, pH 7.4) and stored at -20°C. Each sample was analyzed in duplicate using, peroxidase as the enzymatic tracer, a polyclonal antibody (L2) and tetramethyl benzidine as color reagent (L2 antibodies have a six- to eightfold higher affinity for ecdysone than for 20E).The rabbit polyclonal antibody and the tracer were kindly supplied by Dr J.P. Delbecque (University of Bordeaux I, France) and Dr. C. Blais (UMPC, Paris, France), respectively. Data are expressed in pg equiv. 20E.

Vitellin and vitellogenin quantification: The vitellogenins quantification was made, on fat body samples collected at different ages (0, 1, 3, 5 and 7 days), in female pupae of controls and treated series. Ovaries, collected on newly emerged adult female controls and treated, were used for vitellin quantification. Extraction and quantification were donne as previously described (Boulahbel *et al*., 2015). The extraction was performed using Tris buffer (0.5 M; pH 7.4) following the procedure of Postlethwait *et al*. (1980) and Fabre *et al*. (1992); then, the quantification was made according to Bradford (1976). Assays were conducted with 6–8 repeats by age and for each series.

Evaluation of reproductive potential: The newly emerged adult females, resulting from control and treated (20E or insulin) pupae, are coupled with untreated males. The different couples were maintained under controlled conditions and regular monitoring was allowed to determine the pre-oviposition and oviposition periods, fecundity (number of eggs per female throughout its lifespan), fertility (hatgheg egg by female) and egg hatchability (percentage of neonates that emerged from eggs). Experiments were conducted with 6–8 repeats by age and for each series.

Statistical analysis: Results are presented as means ± standard deviation (SD). The homogeneity of variances was checked by Levene's test. Data were subjected to Student's *t*-test and analysis of variance followed by a *post-hoc* Tukey at level 5%.

RESULTS

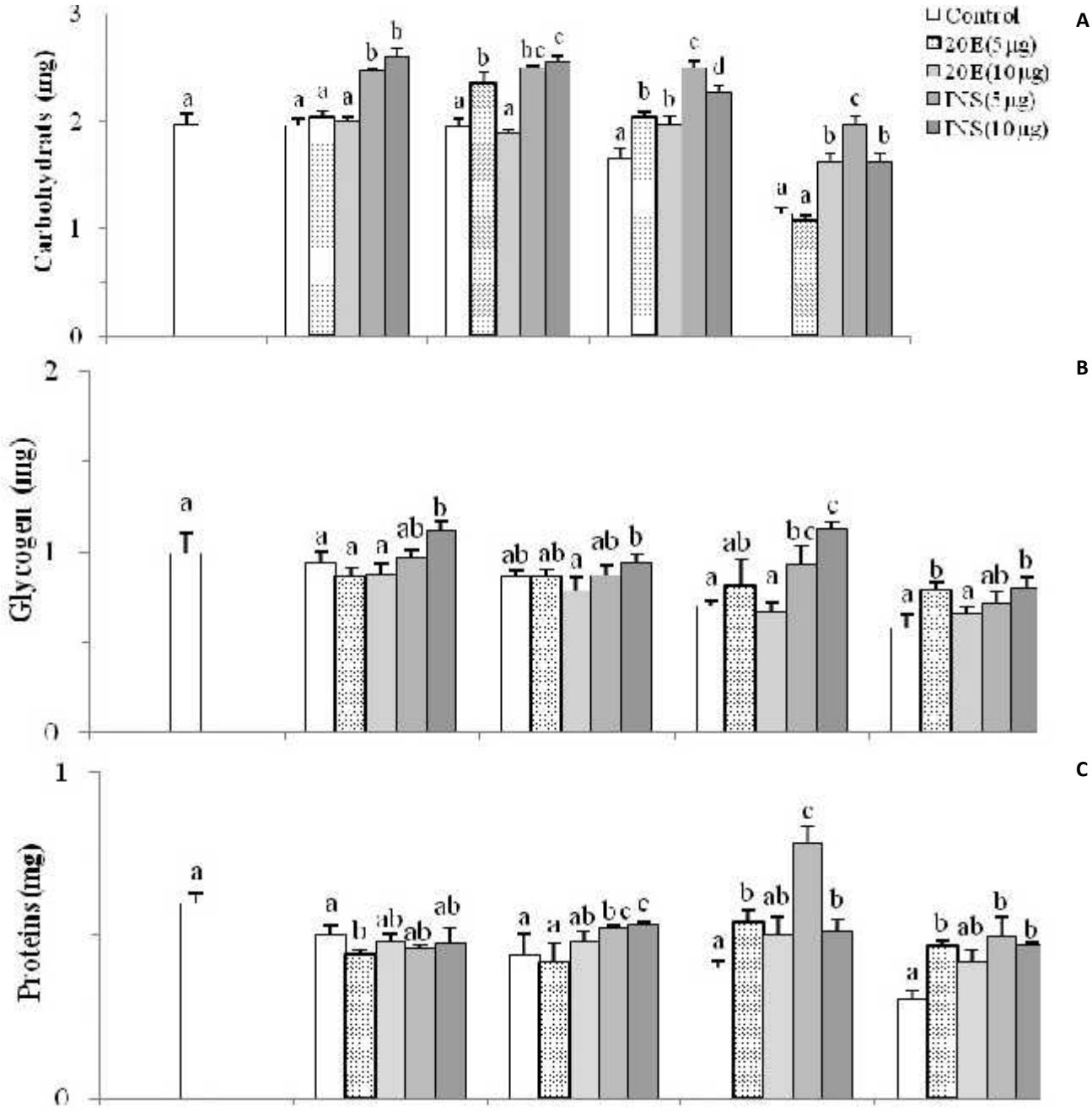
Effects of insulin and 20 E on the fat body biochemical components: In the control of *E. kuehniella*, the carbohydrates amount decreased significantly during the pupal stage at 5 ($p = 0.01$) and 7 days ($p = 0.002$) (Fig. 1A). The comparison between controls and treated series shows that insulin increases the carbohydrates amount at all ages tested with 5 and 10 µg ($p = 0.0001$); however, a dose response relationship with 5µg of insulin shows higher values at days 5 ($p = 0.0001$) and 7 ($p = 0.0001$). Treatment with 20E also increases significantly, on day 3, at the lowest dose ($p= 0.0001$), at day 5 with the two tested doses ($p = 0.0001$) and day 7, only at the highest dose ($p = 0.0001$). Insulin induces a marked effects on the amounts of carbohydrates ($p= 0.0001$), particularly at 1 and 3 days ($p = 0.0001$) (Fig. 1A). In the control series, the glycogen amounts decreased from day 3 ($p = 0.02$) of the pupal stage (Fig. 1B). Treatment with insulin increases glycogen amounts compared to the controls ($p <0.01$); this rise occurs, at day 5 of pupal stage, with both doses 5 and 10 µg ($p < 0.001$) and also at days 1, 3 and 7 in the high dose ($p = 0.02$, $p = 0.03$, $p = 0.0002$ respectively). The 20E increases glycogen amounts, at day 7 ($p < 0.01$), only with 5 µg of the pupal stage. Insulin, in high dose shows an effect more significant on glycogen amounts at all tested ages ($p = 0.006$). Proteins, in the control series, decrease during the pupal stage (Fig. 1C). Insulin application, compared to controls, increases protein amounts, at days 3, 5 and 7 with the two tested doses ($p <0.001$); insulin dose effect is noted, at day 5, with 10 µg ($p = 0.0001$). Treatment with 20E involves a reduction, on day 1 ($p < 0.001$), with only the low dose (5 µg) but an increase at days 5 ($p < 0.001$) and 7 ($p < 0.001$), respectively. Insulin has an effect more significant than 20E on protein amounts ($p = 0.01$), particularly on day 5 of the pupae ($p < 0.0001$). Lipids amount, during the pupal stage, in the control and treated series with 20E and insulin, increases significantly ($p < 0.01$) at days 5 and 7 (Fig. 1D). The comparison, between controls and treated pupae, indicates that only insulin at the low dose (5 µg) causes a decrease in lipid levels at all tested ages ($p < 0.001$). 20E has no effect on lipid amounts.

Effects of insulin and 20E on the ovarian ecdysteroids amounts: In the control series, ovarian hormonal amounts, determined to newly emerged females adults, were 2235±220.90 pg equivalent 20E. Insulin and 20E present, compared to controls, a similar and highly significant effect ($p < 0.0001$) in the ecdysteroid amounts. They decrease after treatment by 20E at 5 µg (161.70 ± 23.93; $p = 0.0007$) and 10 µg (343 ± 7.14; $p=0.001$)**.** Insulin, also, involves a reduction at 5 µg (335.30 ± 51.97; $p=0.001$) and 10 µg (552.10 ± 85.59; $p = 0.002$) (Tab. 1). The high dose of 20E and the two doses of insulin have a comparable effect on hormonal titers, while the 20E at the low dose, causes marked decrease ($p < 0.0001$).

Effects of insulin and 20E on vitellogenin and vitellin amounts: In controls pupae, the vitellogenins show a pic at day 5 of the pupal stage ($p = 0.0002$); then, the vitellogenins values decrease on the eve of the adult emergence ($p = 0.02$). The low doses of insulin involves, during the pupal development, an increase in the amounts of vitellogenins at day 1 and 3 ($p < 0.001$), but a reduction at day 5 ($p < 0.001$) (Fig. 2). The 20E, at two doses, increases significantly ($p < 0.001$) the vitellogenin amounts at days 1, 3, 5 and 7 respectively; a dose-response relationship was observed at day 3 ($p < 0.001$). The 20E induces a more significant effect on the vitellogenin amounts ($p < 0.0001$), compared to insulin treatment, particularly, at days 5 and 7 ($p < 0.0001$). The ovarian vitellin, in females of *E. kuehniella*,has values of 418.01 ± 18.33 µg in controls (Tab. 2). Treatment with two doses of 20E and the low dose of insulin increase the ovarian vitellin amounts; the high dose of insulin has no significant effect. The 20E has a marked effect ($p < 0.0001$) on the vitellin amounts than insulin (Tab. 2).

Effect of insulin and 20E on reproductive events: Treatments were made topically, on newly ecdysed pupae, and their effects were investigated on the reproductive events of adult females, following treated pupae. The results, presented in table 3, indicate that in control series the duration of pre-oviposition period was 1.40 ± 0.24 days. The treatment with 20E or insulin has no significant effect ($p = 0.39$)on this parameter. Concerning the duration of the oviposition period, we note a value of 3.80 ± 0.37 days in control. This period is shorter after insulin treatment with 5 µg (2.60 ± 0.24; $p = 0.02$) and 10 µg(2.50 ± 0.43; $p = 0.03$). The oviposition period is not affected by 20E. Fecundity is 189 ± 3.2 eggs per female in the control series (Tab. 4). The 20E and

insulin, with two tested doses, reduce significantly the fecundity compared to controls ($p<0.0001$). Indeed, the number of eggs per female fall to 155.7 ± 3.37, after treatment, with 5 µg of 20E; an important drop is recorded after treatment with 10 µg of 20E (123.7 ± 2.26) and with 5 and 10 µg of insulin (118.2 ± 1.28; 127.4 ± 1.50). Insulin and 20E, at high doses, have a drastic effect ($p < 0.0001$).The fertility (egg hatched by female), in control series was 175.20 ± 3.89. This value is reduced after 20E treatment with 5 µg (98.33 ± 4.31; $p < 0.0001$) and 10 µg (98.93 ± 3.06; $p < 0.0001$). Insulin has the same effect as the 20E and shows values of 92.80 ± 1.88 ($p < 0.0001$) and 101.20 ± 0.96 ($p < 0.0001$), for the 5 and 10 µg respectively. So, the lowest fertility is noted with the 20E and the low dose of insulin.



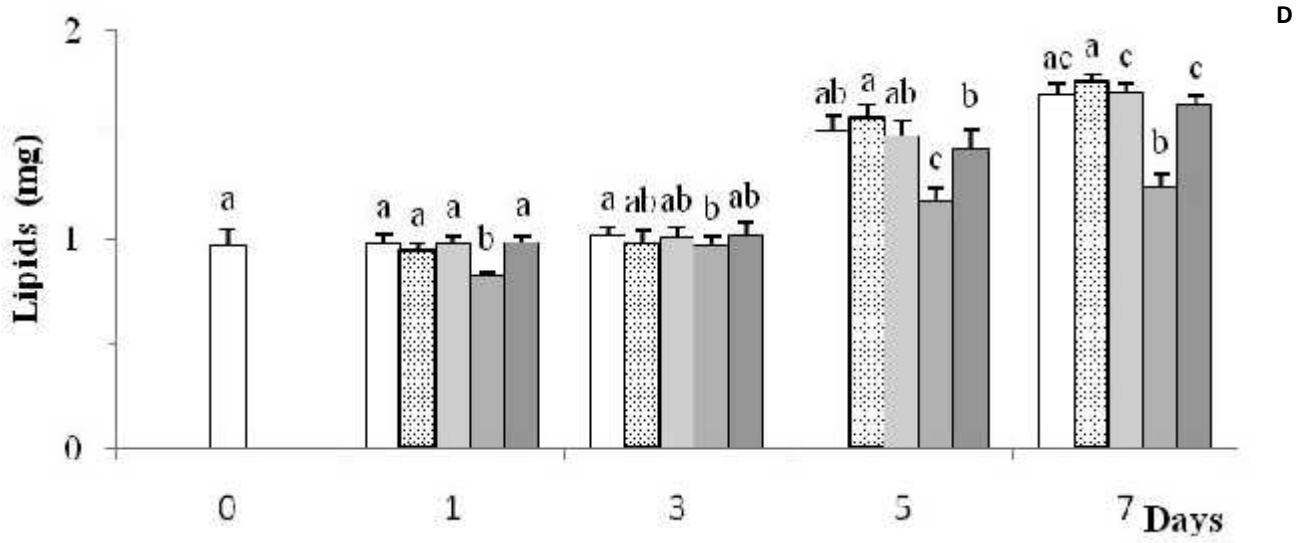


Figure 1. Effects of 20-hydroxyecdysone (20E) and insulin (INS), applied topically and separately, on newly ecdysed pupae (5 and 10 µg), on total biochemical amounts (mg) in the fat body during the pupal stage in females of *E. kuehniella* (m ± SD, n = 6-8): A: carbohydrates; B: glycogen; C: proteins; D: lipids. For each time and for all series, the values followed by the same lowercase letters are comparable to the 5% threshold.

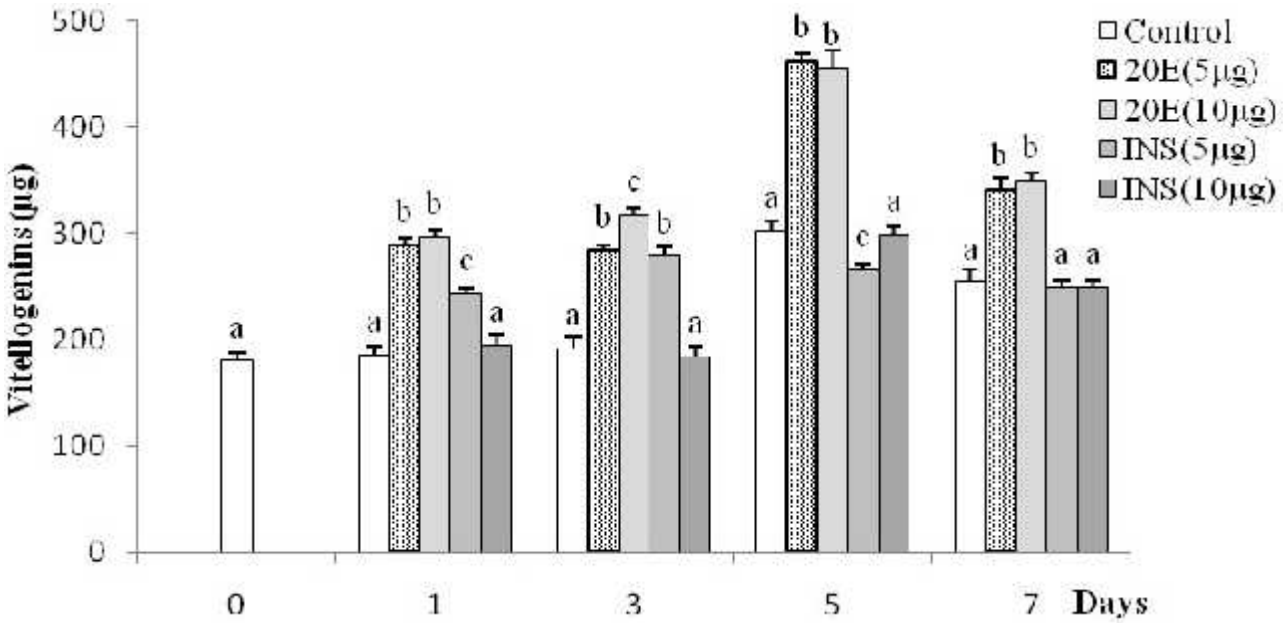


Figure 2. Effects of 20-hydroxyecdysone (20E) and insulin (INS), applied topically and separately on newly ecdysed pupae (5 and 10 µg), on total vitellogenin amounts (µg) in the fat body during the pupal stage in females of *E. kuehniella* (m ± SD, n = 6-8). For each time and for all series, the values followed by the same lowercase letters are comparable to the 5% threshold.

Table 1. Effects of 20-hydroxyecdysone (20E) and insulin (INS), applied topically and separately on newly ecdysed pupae (5 and 10 µg), on total ovarian ecdysteroid amounts (pg equiv 20E) from newly emerged adult females of *E. kuehniella*. (m ± SD, n = 6-8). For all series, the values followed by the same lowercase letters are comparable to the 5% threshold.

Control	20 hydroxyecdysone		Insuline	
	5 µg	10 µg	5 µg	10 µg
2235 ± 220.90[a]	161.70 ± 23.90[b]	343.70 ± 7.14[c]	335.30 ± 51.97[c]	552.10 ± 85.50[c]

Table 2. Effects of 20-hydroxyecdysone (20E) and insulin (INS), applied topically and separately on newly ecdysed pupae (5 and 10 µg), on total ovarian vitellin amounts (µg) from newly emerged adult females of *E. kuehniella*. (m ± SD, n = 6-8). For all series, the values followed by the same lowercase letters are comparable to the 5% threshold.

Control	20 hydroxyecdysone		Insuline	
	5 µg	10 µg	5 µg	10 µg
418.01 ± 18.33[a]	1327.68 ± 48.00[b]	508.84 ± 15.84[c]	447.84 ± 13.34[d]	413.53 ± 8.82[a]

Table 3. Effects of 20-hydroxyecdysone (20E) and insulin (INS), applied topically and separately on newly ecdysed pupae (5 and 10 µg), on the duration (days) of pre-oviposition and oviposition in adult females of *E. kuehniella*. (m ± SD, n = 6-8). For each parameter and for all series, the values followed by the same lowercase letters are comparable to the 5% threshold.

Treatments		Pre-oviposition period	Oviposition period
Control		1.40 ± 0.24[a]	3.80 ± 0.37[a]
20 hydroxyecdysone	5 µg	1.83 ± 0.31[a]	4.00 ± 0.25[a]
	10 µg	2.33 ± 0.33[a]	3.83 ± 0.47[a]
Insuline	5 µg	1.80 ± 0.37[a]	2.60 ± 0.37[b]
	10 µg	1.83 ± 0.31[a]	2.50 ± 0.43[b]

Table 4. Effects of 20-hydroxyecdysone (20E) and insulin (INS), applied topically and separately on newly ecdysed pupae (5 and 10 µg), on the fecundity (number of eggs laid/female), hatchability (egg hatchability in %) and fertility (hatched egg/female) in adult females of *E. kuehniella*. (m ± SD, n = 6-8). For each parameter and for all series, the values followed by the same lowercase letters are comparable to the 5% threshold.

Treatments		Fecundity (eggs/female)	Hatchability (%)	Fertility (hatched eggs/female)
Control		189 ± 3.20[a]	92.70 ± 2.50[a]	175.20 ± 3.89[a]
20 hydroxyecdysone	5 µg	155.70 ± 3.37[b]	63.10 ± 5.32[b]	98.33 ± 4.31[bc]
	10 µg	123.70 ± 2.26[cd]	80.00 ± 6.60[c]	98.83 ± 3.06[bc]
Insuline	5 µg	118.20 ± 1.28[c]	78.34 ± 2.44[c]	92.80 ± 1.88[b]
	10 µg	127.40 ± 1.56[d]	79.43 ± 3.96[c]	101.20 ± 0.96[c]

DISCUSSION

Effects of insulin and 20E on the main biochemical components of fat body: In *E. kuehniella*, the decreased of carbohydrates, during the pupal stage, could be explained by the energy used in the metamorphosis process. Glycogen, essential form of carbohydrates reserves, is reduced on day 3 of the pupal stage, coinciding with the apolysis and is linked to chitin synthesis (El Ouar *et al.* 2010). In *E.kuehniella*, human insulin increases the glycogen amounts, in fat body, similar to synthetic endogenous ILPS (Brown *et al*. 2008). In *E. kuehniella*, our results show that the effect of 20E on carbohydrates is comparable to that of insulin, confirming the previous work in *D. melanogaster* (Hou *et al*. 2012). The 20E can act as an inhibitor of glycolysis during metamorphosis in *Bombyx mori* (Tian *et al*. 2010); indeed, insulin and 20E inhibit the same molecular mechanisms in *D. melanogaster* (Puig and Mattila, 2011) and *Helicoverpa armigera* (Hou *et al*. 2012). In controls pupae of *E. kuehniella*, the reduced protein levels recorded could be related to the synthesis of the new cuticle but, also, with the vitellogenesis process (El Ouar *et al*. 2010); the proteins used for the elaboration of adult tissues may also explained this decrease. The insulin treatment, in *E. kuehniella* pupae, induces an increase in the protein amounts in accordance with known roles of ILP_S in *A. aegypti* (Graf *et al*. 1997). The 20E presents, in low doses, a similar action as the insulin; moreover, the absence of effect in the strongest dose can be linked to a regulatory process. The lipids constitute a mobilizable energy quickly during fasting and this can be explained a gradual increase observed during metamorphosis. Insulin, tested at the high dose, has no effect and this is in agreement with the work of *D. melanogaster* (Ceddia *et al.* 2003) and *A. aegypti* (Brown *et al*. 2008).Otherwise, the insulin signaling pathway is reported as a potential regulator in the modulation of lipid contents (Baumann *et al*. 2013); this may explain the observed decrease in the lipid amounts with the low dose (5µg). After application of 20E in *E. kuehniella*, lipid levels are comparable to those of the control series, confirming the work under taken in other species of Lepidoptera as *Bombyx mori* (Wang *et al*. 2010). Thus, the results show effects of human insulin but, also, the role of ecdysteroids in the metabolism on *E. kuehniella* pupae. Tatar *et al*. (2003) hypothesized that many effects attributed to ILPs, in insects, may be mediated by ecdysteroids. Moreover, in *D. melanogaster*, Colombani *et al*. (2005) noted that ecdysone can exercise its regulation through modulation of the insulin/IGF signalling and the Foxo activity in the fat body.

Effects of insulin and 20E on the reproduction: The endocrine control of reproduction (like the vitellogenesis) is mainly made by juvenile hormone (JH), 20E, neuropeptides and ILPs (Belles and Piulack, 2015). The evolution of vitellogenins amounts in controls pupae of *E. kuehniella* shows a peak, between 3 and 5 days, which coincides with ecdysteroids increase, cited in various studies like in El Ouar *et al.* (2010). Separates application of 20E (5 and 10 µg) and insulin (5 µg) involves an increase in vitellogenins and vitellins amounts. This is in agreement with Parthasarathy and Palli (2011) who report a potentialization of the vitellogenins and vitellins synthesis by the 20E; the works of Roy *et al.* (2007) in *Aedes aegypti* show that insulin involves, also, a rise of the vitellogenins. *In vitro*, a direct stimulatory effect of bovine insulin on protein synthesis of ovaries has been demonstrated for *A. aegypti* (Graf *et al*., 1997). Moreover, the literature specifies that these two signaling pathways are essential in the vitellogenesis and oocyte maturation (Parthasarathy and Palli, 2011). Thus, the increase of vitellogenins and vitellins after the insulin treatment seem to be explained. The decrease of vitellogenins amounts observed at day 5 of the pupal stage, after application of insulin, may also be related to the molecular interaction between insulin and JH (Parthasarathy and Palli, 2011). Insulin/20E or insulin/JH interaction, still under review, is based by the complex relations *via* the factor of transcription FOXO (Antonova *et al*., 2012; Yamanaka *et al*., 2013). Insulin may also act in combination with the amino acids and 20E as a third component which regulate vitellogenins (Roy *et al*. 2007); this regulating effect on vitellogenins could explain the absence of insulin effect at the highest dose (10 µg).

In insects, the ovarian steroidogenesis is a remarkable target of insulin (Riehle & Brown, 1999). Bovine insulin was found to stimulate, *in vitro*, ecdysteroid production by the ovaries in *A. aegypti (*Riehle and Brown, 1999) and *Phormia regina* (Maniere *et al.*, 2004). It was reported that bovine insulin, only to an optimal dose (17 µM), increases ecdysteroids production in *Aedes*'s ovaries (Brown *et al*. 2008) and porcine insulin also stimulates this parameter in the same species (Graf *et al*. 1997). However, Mammals insulin, compared to endogenous ILP_S seems to constitute bad agonists for the ecdysteroids production (Brown *et al*. 2008). In *E. kuehniella*, measurements of ovarian ecdysteroids, before and after enzymatic hydrolysis with porcine liver esterase, show that conjugate ecdysteroids represent only 8.37% of total ecdysteroids (Yezli *et al*., personal communication). In our results, application of 20E and insulin shows similar effects corresponding to a decrease in free ovarian ecdysteroids amounts compared to controls. Ecdysteroids decrease may be explained by a same molecular mechanism cited by Puig and Mattila (2011). The most marked effect noted with the low dose of 20E can suggest an endocrine regulation; indeed,*in vivo*, several factors (hormones, peptides and proteines kinases) can affect the ecdysteroid biosynthesis. In

addition, ecdysteroids themselves play a role in their ownbiosynthesis *via* a feedback regulation of the PTTHaxis in the insect brain, and *via* direct action on the ecdysteroid-producing (prothoracic glands and/or endocrine organs like ovaries) (Belles and Piulack, 2015). In lepidopteran species, recent studies provide evidence for ILP regulation of ecdysteroid production by the PG, perhaps by signaling nutritional state and *via* the prothoracicotropic hormone or PTTH (Antonova *et al*., 2012). Moreover, the role of ILPs relative to ovary ecdysteroidogenic hormone (OEH) that also stimulates ecdysteroid production is unresolved.

The insulin and 20E treatment reduce fecundity and fertility; furthermore, insulin reduces the oviposition period. Fecundity and fertility reduction can be also explained by the decrease in the ecdysteroid amounts. A reduction of fertility was, also, noted after insulin treatment and may be related to a decrease in egg hatchability (Kuo *et al*. 2012; Baumann *et al*. 2013). The insulin signaling pathway can act in the differentiation of the ovary and is essential in the last stages of oogenesis (Okada *et al*. 2010).Otherwise, the same molecular mechanism controls the adjustment of the functions of endocrine system and the insulin signaling pathway (Puig and Mattila, 2011); so, the regulating role would be thus be possible in the process of reproduction where the 20E and the HJ are also involved. In addition, ecdysteroid actions in certain stages of reproduction like oogenesis or the effects of OEH a being studies (Belles and Piulachs, 2015)

In conclusion, the results, on the metabolic aspects, show that human insulin can act in *E. kuehniella*. Insulin signaling pathway/insulin receptors, highly conserved and action of the same molecular mechanisms can be explained this action; in addition, studies suggest that multiple ILPs can activate the insulin receptor and stimulate the signal transduction. Moreover, differences of action between human insulin and endogenous ILPs might be explained by a specificity of binding between ligand and insulin receptor. Besides, the 20E can acts also in metabolism control; consequently, the functional interaction between human insulin and 20E seems to be possible. Acetone solutions of 20E or human insulin, applied topically on insects have similar effects on all considered parameters. The modulations observed, according to doses and age, can be explained by the possible relationships between ILPs and ecdysteroids, cited in the literature; this, in particular, when these hormones are involved in the control of the same functions. In *E. kuehniella*, our results show that human insulin can acts at the level of fat body but cannot stimulate the ovarian steroidogenese; however, these results seem to be in favour of insulin impact at the level of ovaries. Studies show that ILPs can differ in their ability to activate various physiological processes; indeed, at similar doses, ILPs cannot activate the anabolic processes in the fat body, but activated the ovarian production *in vitro* (Antonova *et al*., 2012). Further investigations are needed to understand how do multiple ILPs interact with an insulin receptor and coordinate diverse physiological and behavioral processes in different tissues. Moreover, the mechanisms of action of insulin and 20E remain to clarify; in addition, a combined treatment of insulin and 20E, at different application time, are needed to precise the interaction between these two hormones.

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