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## Full title

Effects of phthalate and bisphenol plasticizers on the activity of glycolytic enzymes of the moth *Spodoptera littoralis*

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- BPA and DEHP did not affect the activity of pyruvate kinase and phosphofructokinase

- Phosphoglucose isomerase activity was increased in larvae fed with BPA
- BPA+DEHP-fed larvae had highly variable hexokinase activity

## 1. Introduction

Environmental pollution has grown relentlessly in the recent decades, and the massive release of chemicals and organic wastes has major impacts on human and ecosystems health, as well as economic impacts (Xia et al., 2022). In recent years, novel entities such as plastics and endocrine disruptor compounds have emerged as environmental pollutants of high concern (Persson et al., 2022), in turn prompting an environmental crisis (Dai et al., 2020). In a short time, the extent of plastic accumulation in the environment has become so large that we have most probably reached a non-reversible point (MacLeod et al., 2021). Despite the significant environmental plastic load, the effects on terrestrial ecosystems and organisms have remained poorly explored, even though plastics and their chemical additives may have immeasurable impacts (Chen et al., 2022). The chemical composition, degradation products, and mixtures potentially formed by plastic debris in the environment remain partially known (Lai et al., 2022; Tumu et al., 2023), making it more difficult to appreciate their far-reaching and insofar unsuspected consequences (Persson et al., 2022; Wojnowska-Baryła et al., 2022).

Plastic production was about 391 Mt in 2021 (PlasticsEurope, 2022). Different types of polymers are synthesized, as for example polyethylene, polypropylene, polyvinyl chloride, or polystyrene. Often, plastic garbage collection and management are inefficient; plastics can be incorrectly stored, or discarded in the environment, thus increasing the amounts of plastic transferred into the environment. Then, plastic debris are progressively fragmented into smaller pieces, *i.e.* macroplastics and microplastics, and then in small-sized nanoplastics (Blair et al., 2017; Li et al., 2021). This transformation, and the accompanying degradation process, contributes to the release of their chemical additives, *i.e.* the plastic-associated chemicals (PACs) (Groh et al., 2019). PACs, and more generally plastic-associated organic and inorganic compounds, include metals, metalloids, polyaromatic hydrocarbons, per-fluoroalkyl substances, polychlorinated phenyls, or the commonly used plasticizers bisphenols (such as bisphenol A (BPA)) and phthalates (such as Di(2-ethylhexyl)phthalate, (DEHP)) (Bhagwat et al., 2021; Groh et al., 2019; Sendra et al., 2021).

BPA is extensively produced and used as a plastic monomer in the manufacture of polycarbonates, epoxy resins, and some paper products (Abraham and Chakraborty, 2020; Liu et al., 2021). Many studies have reported the environmental presence of BPA in waste landfills, landfill leachates, dumpsites, surface water, or soils (Corrales et al., 2015; Idowu et al., 2022; Yamamoto et al., 2001). In the field, BPA can be uptake by plants (Lu et al., 2015; Wang et al., 2018), further extending bioaccumulation risks along the food chain (Shi et al., 2022). BPA has also been reported from human food, where it can reach concentrations as high as 711 ng/g food (Almeida et al., 2018; Osman et al., 2018).

DEHP belongs to the family of phthalates, commonly processed as plasticizers in polymers to improve plastic flexibility (Gómez-Hens and Aguilar-Caballós, 2003; Rowdhwal and Chen, 2018). As

phthalates are not chemically bound to polymers, they are easily leached into the environment during manufacturing, use, or after disposal. Consequently, they were detected in surface water, sewage sludge or soil (Abad et al., 2005; Fromme et al., 2002; Sun et al., 2021), with the potential for contaminating plants and food web (Li et al., 2020; Sablayrolles et al., 2005; Sun et al., 2015). DEHP was also detected from human food, with highest recorded concentrations of 3.2 µg/g food (Cao, 2010).

BPA and/or DEHP can have adverse effects on human, wildlife, and biodiversity in general (Wen et al., 2022; Zhang et al., 2021). In some animals, both BPA and DEHP can act as Endocrine Disruptor Chemicals (EDCs) (Golshan and Alavi, 2019; Mathieu-Denoncourt et al., 2015; Oehlmann et al., 2009), with cascading consequences on physiological homeostasis, reproduction, development, or behavior (Kabir et al., 2015). So far, the studies which have documented the BPA-induced or DEHP-induced physiological dysregulations have mainly remained focused on vertebrates (Canesi and Fabbri, 2015; Ito et al., 2019; Kabir et al., 2015). Some investigations have been performed on aquatic invertebrates (Burgos-Aceves et al., 2021; MG et al., 2021; Morales et al., 2011), but information on the impacts of DEHP and BPA on terrestrial invertebrates are very scarce. The available information suggests that plasticizers can impact post-embryonic development, alter body mass in larvae and adults, gonad size, offspring number and clutch size, all of these effects being dose-dependent (Aviles et al., 2019; Cuvillier-Hot et al., 2014; Maria et al., 2019; Rankin and Grosjean, 2010). In the Egyptian cotton leafworm *Spodoptera littoralis*, information on the effects of plasticizers start to accumulate, making it an attractive example model for studying the multiscale and multilevel biological and physiological impacts of plastics. *Spodoptera littoralis* is a highly polyphagous moth species feeding on plants, and an invasive crop pest in Mediterranean countries (CABI, 2022). The voracious larvae of *S. littoralis* develop on cultivated plants whose leaves can contain and accumulate BPA and DEHP (Li et al., 2020; Lu et al., 2015; Sablayrolles et al., 2005; Sun et al., 2015; Wang et al., 2018). Alterations of biological processes and phenotypic changes of individuals of *S. littoralis* result from modifications in the expression of immune genes, levels of ecdysteroid hormones, or metabolomic profiles (Aviles et al., 2019; Maria et al., 2019). In larval instars, metabolic profiles of DEHP-treated larvae highly differed from those of controls (Aviles et al., 2019), and the concentrations of several metabolites involved in amino acid and energetic production, or being part of the glycolytic and Krebs cycle pathways, were altered.

In the present work, we aimed at examining if the impacts of plasticizers on energetic metabolism of the moth *S. littoralis* reported by Aviles et al. (2019) resulted from (a) the increased energetic costs of detoxification and elimination of DEHP, and /or (b) the dysregulation of hormonally-controlled activity of metabolic enzymes. In both cases, it is hypothesized that enzyme activities would be adjusted, with the protein kinetics being increased in the former suggestion, while it would be decreased in the latter one. To test these hypotheses, and get additional understanding on the effects of plasticizers (BPA and DEHP) on the physiology of this terrestrial model insect, the activities of four glycolytic enzymes, hexokinase, phosphoglucose isomerase, phosphofructokinase, and pyruvate kinase, were measured in larvae of *S. littoralis* fed on plasticizer-contaminated or un-contaminated food. Specifically, enzyme activities were measured in the last larval instar of the species which had developed on BPA, DEHP, or the mixture of BPA and DEHP, for several days. As carbohydrate metabolism is tightly controlled by ecdysteroids in insects (Yu et al., 2023), and because plasticizers are suspected to act as endocrine disruptors, a decrease in the activity of the tested enzymes was expected. Following former investigations performed on the moth *S. littoralis* (Aviles et al., 2019; Maria et al., 2019), our results explored the physiological underpinnings shaping the phenotypic effects incurred by exposure to plasticizers.

## 2. Materials and Methods

### 2.1 Insect rearing

The strain and rearing conditions described in the work of [Aviles et al. \(2019\)](#) were used. Briefly, specimens of *S. littoralis* were reared at constant  $23 \pm 1$  °C, relative humidity of 60-80%, and 16:8h light:dark cycle, as recommended by [Hinks and Byers, 1976](#). Prior to the experiments, newly hatched eggs were maintained in plastic boxes (Pro'Jet, St-Ouen, France) until they developed into 3<sup>rd</sup> larval instars, corresponding to the youngest possible instars which can be handled without impacting development. Then, 200 larvae were transferred to one plastic box, and maintained under the same rearing conditions. The day following the transfer of the larvae to the plastic box, developmental progression was followed, and those who had molted into 4<sup>th</sup> instar were kept for the experiment, and transferred and reared individually into small plastic containers of 2 cm of diameter (BCT2, Pro'Jet, St-Ouen, France) ( $23 \pm 1$  °C, relative humidity of 60-80%, and 16:8h light:dark cycle). From the 4<sup>th</sup> larval instar onwards, individuals were fed with food free of plasticizers (control) or contaminated with plasticizers (treated). Consumed food was replaced every two days (see section 2.2).

### 2.2 Exposure of the larvae to bisphenol and phthalate plasticizers

The protocol established by [Aviles et al. \(2019\)](#) and [Maria et al., 2019](#) was followed for the incorporation of Bisphenol A (BPA; CAS No. 80-05-7, Sigma Aldrich) and Di(2-ethylhexyl)phthalate (DEHP; CAS No. 117-81-7, Sigma Aldrich) plasticizers into the food of the larvae. Ethanol was used for the dilution of BPA and DEHP, and concentrations of BPA and DEHP in the food were 1 µg BPA /g food, and 1.1 µg DEHP /g food, respectively [\(Aviles et al., 2019\)](#). These concentrations were used as they represented doses which may be encountered in the environment [\(Lü et al., 2018; Niu et al., 2014\)](#). Moreover, this BPA concentration alter the duration of the larval stage, concentration of ecdysteroids and expression of nuclear receptors responsible of ecdysteroid signalling pathway [\(Maria et al., 2019\)](#). For DEHP, the levels of several metabolites were modified during the prepupal stage at 1.1 µg DEHP/g food [\(Aviles et al., 2019\)](#).

Food was stored in glass jars at 4 °C prior to being given to the larvae. Four experimental groups were established ( $n = 18$  larvae per experimental group): (i) control larvae fed on non-contaminated food, yet containing 0.5% of ethanol, (ii) larvae fed with BPA-contaminated food, (iii) larvae fed with DEHP-contaminated food, and (iv) larvae fed with food contaminated by both BPA and DEHP (mixture). Larval development was daily monitored by examination of the presence of the cephalic capsule of the previous larval instar in the rearing plastic box; this allowed us to accurately determine if the larvae had molted. At the onset of the 7<sup>th</sup> larval instar, the developmental duration was noted. Each individual was collected at the 6<sup>th</sup> day of the 7<sup>th</sup> larval instar as done in [Aviles et al. \(2019\)](#), and stored at -20 °C until the biochemical assays.

### 2.3 Biochemical assays

### 2.3.1 Sample preparation

All 6d-old 7<sup>th</sup> larval instars of *S. littoralis* were weighted individually (Sartorius A200S analytical balance, d = 0.1 mg). Then, the body of each larva was cut at the junction of the 3<sup>rd</sup> thoracic and the 1<sup>st</sup> abdominal segment, and at the junction of the 4<sup>th</sup> and 5<sup>th</sup> abdominal segments. The resulting portion of the body segments, from the 1<sup>st</sup> to the 4<sup>th</sup> abdominal segments (A1-A4) allowed us to keep the foregut, and a portion of the midgut, where a big part of the metabolic activity is assumed to occur (Darvishzadeh et al., 2014; Ioannidis et al., 2022).

The abdominal segments A1-A4 were weighed and transferred into clean 2mL microtubes, each containing two tungsten carbide beads (diameter: 3mm; QIAGEN, USA). Samples were grinded with a bead beater (Retsch MM400, Retsch GbmH, Haan, Germany) at 25 hertz for 1 minute 30 seconds. Then, a volume of 1.5 mL of pH 8.0 Trizma buffer (Sigma-Aldrich, St-Louis, MO, USA) was added to each microtube, and the samples were homogenized at 25 hertz for 1 minute 30 seconds. After a centrifugation at 4,000 *g* at 4 °C for 10 minutes, a volume of 600 µL of the supernatant was pipetted for subsequent protein and enzymatic assays.

### 2.3.2 Measurement of protein amounts and enzymatic activities

The amounts of protein were measured according to Bradford's technique (Bradford, 1976), where Quick Start™ Bradford Dye Reagent (Bio-Rad, USA) reacts with proteins, changing the color of Coomassie (from red to blue when binding to proteins) which can be measured spectrophotometrically at 595 nm. Bovine serum albumin (Thermo Scientific, Waltham, MA, USA) was used for drawing the protein calibration curve (from 0 to 1.25 mg/mL proteins). As protein amounts were highly concentrated in the biological samples, all supernatants had to be diluted 16 times before the assay. Afterwards, 10 µL of each diluted supernatant was transferred into the well of a 96-microplate, and a volume of 240µL of the dye reagent was added to each well. The microplate was homogenized, and incubated at room temperature for 20 minutes, before the absorbance was read at 595 nm. The remaining volume (590 µL) was used for the measurement of enzyme activities.

The activity of four glycolytic enzymes was tested: Hexokinase (HK, E.C. 2.7.1.1), Phosphoglucose Isomerase (PGI, E.C. 5.3.1.9), Phosphofructokinase (PFK, E.C. 2.7.1.11) and Pyruvate Kinase (PK, E.C. 2.7.1.40). All enzyme activities were measured in duplicate by colorimetric assay kits (Sigma-Aldrich, St-Louis, MO, USA). Nine larva extracts per experimental group were used for the measurement of enzyme activities. Assays were performed on the same samples for hexokinase, phosphoglucose isomerase and phosphofructokinase; new samples were prepared for assessing the activity of pyruvate kinase so that all measurements could be done on fresh samples. For each assay, the recommendations of the manufacturer were followed with the below-described minor modifications.

For pyruvate kinase (Assay kit MAK072, Sigma-Aldrich, St-Louis, MO, USA), 50  $\mu\text{L}$  of the supernatant of each sample was diluted with 50  $\mu\text{L}$  of Trizma buffer. Then, 5  $\mu\text{L}$  of the diluted sample was pipetted and homogenized with 45  $\mu\text{L}$  of buffer, 50  $\mu\text{L}$  of reaction mix, and the plate was incubated at room temperature for 1 min. The absorbance was read every 3 min for 42 minutes at 570 nm. Pyruvate standards were prepared for the calibration curve.

For phosphoglucose isomerase (Assay kit MAK103, Sigma-Aldrich, St-Louis, MO, USA), 20  $\mu\text{L}$  of the supernatant was diluted with 300 $\mu\text{L}$  of Trizma buffer; 5  $\mu\text{L}$  of the diluted sample was mixed with 45  $\mu\text{L}$  of Trizma buffer and 50  $\mu\text{L}$  of the reaction mix. The microplate was incubated at room temperature for 2 min, and the absorbance was read every minute for 14 min. NADH standards were prepared for the calibration curve.

For hexokinase (Assay kit MAK091, Sigma-Aldrich, St-Louis, MO, USA), pure extracts (no dilution) were used. A volume of 5  $\mu\text{L}$  of the sample was mixed with 45  $\mu\text{L}$  of Trizma buffer and 50  $\mu\text{L}$  of the reaction mix. The microplate was incubated at room temperature for 5 min. Then, the absorbance was read every 5 min for 32 min. NADH standards were prepared for the calibration curve.

For phosphofructokinase (Assay kit MAK093, Sigma-Aldrich, St-Louis, MO, USA), 20  $\mu\text{L}$  of the supernatant was diluted with 300 $\mu\text{L}$  of Trizma buffer, and this 1/16 diluted sample was further diluted two times with Trizma buffer. Then, a volume of 5  $\mu\text{L}$  of the diluted sample was mixed with 45  $\mu\text{L}$  of Trizma buffer and 50  $\mu\text{L}$  of the reaction mix, and the microplate was incubated at room temperature for 5 min. The absorbance was read every 5 min for 30 min. NADH standards were prepared for the calibration curve.

The activities of the four enzymes were standardized by protein amounts, and values were thus reported as optical density (OD)/ $\mu\text{g}$  protein. For analyses and visualization of the results, the activity of control larvae (those fed on food free of plasticizers) served as a reference, and for each enzyme, changes in the activity in A1-A4 extracts were expressed as percent of the control.

## 2.4. Statistical analyses

Duration of larval developmental was analysed with Log-rank tests. For each experimental treatment, outliers were removed when the mass of the abdominal segments A1-A4, the protein mass, and the specific activity were outside the range mean  $\pm$  1.5\*interquartile range, as defined in (Crawley, 2007). Shapiro and Bartlett tests were applied to check for data normality and homoscedasticity. When data distribution was normal and homoscedastic, one-way ANOVA and Tukey *post-hoc* tests were performed to examine the effects of plasticizers (BPA, DEHP, or DEHP+BPA) on protein amounts and enzyme activities. Distribution of the mass of the abdominal segments A1-A4 was not gaussian in some experimental groups, even after data transformation; in these situations, Kruskal-Wallis tests were computed to compare the masses among the experimental treatments. We observed seemingly high

variability in mass segments A1-A4 in the control group. To compare variability in groups having non-Gaussian distribution, Levene test was performed instead of Bartlett test.

When higher variability of enzymatic activity was measured in an experimental condition, p-value score of Bartlett test was observed. When Bartlett test p-value was  $<0.1$ , research was made to know which specific experimental group demonstrated different variability. To our knowledge, only F-test is capable of comparing the variance between two groups. F-test was applied for the least number of possible comparisons in order to avoid multiple comparison biases. Statistical differences among experimental groups were considered for p-values  $< 0.05$ . Yet, in biochemical or physiological terms, changes in gene expression, hormone concentrations, or enzymatic activities are likely impactful for organismal biology when worthwhile variations are noticed. Several articles have thus proposed to reconcile statistical requirements to biological concepts (see the discussion in [McCarthy and Smyth \(2009\)](#)). As fold-changes are generally more meaningful in biological terms, it has been suggested that both fold-change threshold and reliable p-values must be considered ([McCarthy and Smyth, 2009](#)). In the present study, we followed the suggestion made by [Huggins et al. \(2008\)](#), *i.e.* enzymatic variations having a fold-change of 1.3 and  $p < 0.2$  would be significant and biologically meaningful.

Statistical analyses were performed using R 4.2.2 ([R Core Team, 2022](#)).

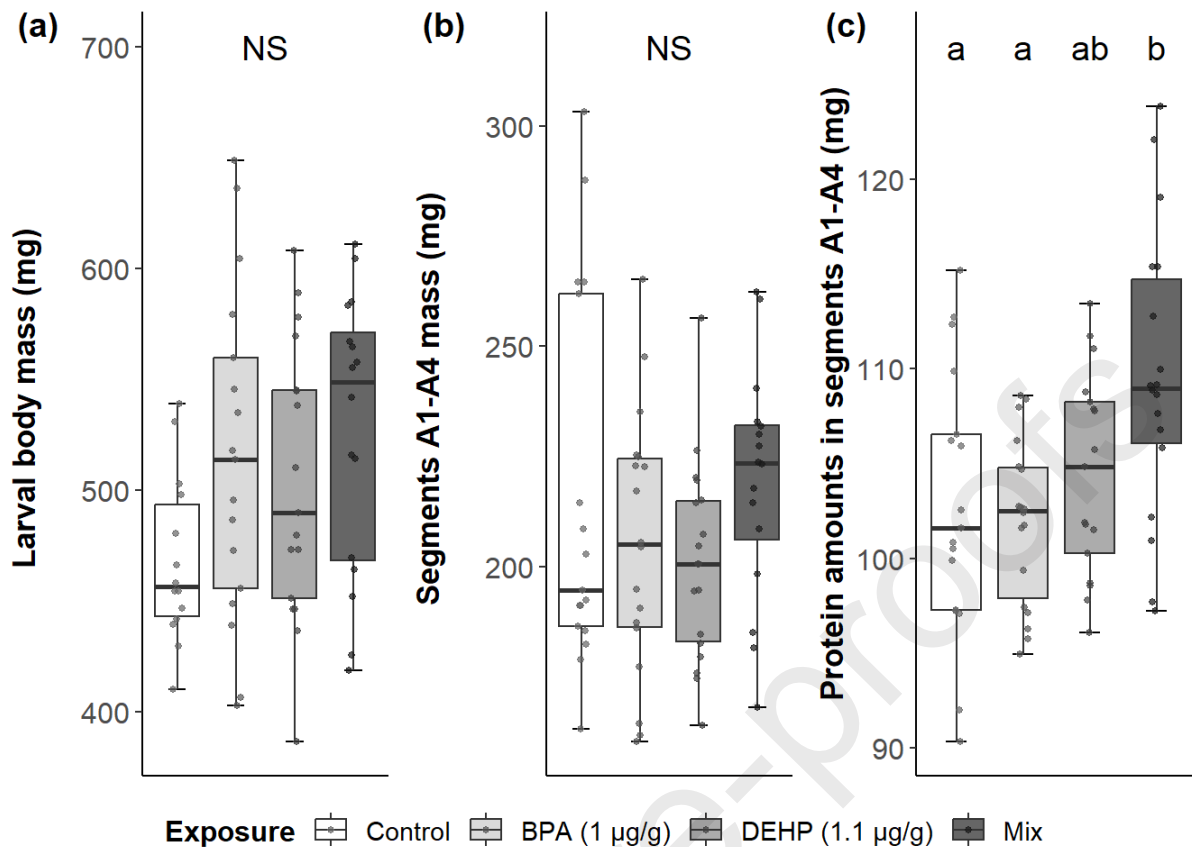
### 3. Results

#### 3.1 Duration of larval development, mass and amounts of proteins

On average, it took 9.41, 9.22, 9.72 and 9.88 days for 4<sup>th</sup> instar larvae to reach the 6<sup>th</sup> day of the 7<sup>th</sup> instar when fed on non-contaminated food, BPA, DEHP and BPA+DEHP-contaminated food, respectively. The duration of development of larvae fed with BPA-contaminated food was significantly quicker than the developmental duration of larvae fed on BPA+DEHP-contaminated food (Log-rank test,  $p = 0.0488$ ).

The mass and amounts of proteins of abdominal segments A1-A4, and larval body mass are shown in **Figure 1**. At day 6 of the 7<sup>th</sup> larval instar, body mass was similar among the experimental groups (one-way ANOVA,  $F = 1.401$ ,  $df = 3$ ,  $p = 0.2517$ , **Figure 1.a**). No statistical difference was found among the experimental groups for the mass of the abdominal segments A1-A4 (Kruskal-Wallis test,  $X^2 = 4.1896$ ,  $df = 3$ ,  $p = 0.2417$ , **Figure 1.b**), or for their variability (Levene test,  $F = 1.365$ ,  $df = 3$ ,  $p = 0.261$ , **Figure 1.b**). The experimental treatments significantly impacted the amounts of proteins (one-way ANOVA,  $F = 5.317$ ,  $df = 3$ ,  $p < 0.01$ , **Figure 1.c**): protein amounts measured from the abdominal segments A1-A4 were significantly higher in larvae fed with BPA+DEHP-contaminated food as compared with control larvae (Tukey test,  $p < 0.01$ ) and BPA (Tukey test,  $p < 0.01$ ).





**Figure 1:** Larval body mass (fresh mass) (a), fresh mass of abdominal segments A1-A4 (b) and protein amounts of abdominal segments A1-A4 (c) of 6-day-old 7th larval instars of *Spodoptera littoralis* fed on non-contaminated (control) or plasticizer-contaminated (BPA, DEHP or BPA+DEHP) food. Since the 4th instar, larvae were grown with food contaminated with 1 µg BPA/g food, or 1.1 µg DEHP/g food, or a mix of the two (n = 16 to 18 for each condition). Statistical differences were reported at the top of the bars: bars with different letters were significantly different (Tukey *post-hoc* test,  $P < 0.05$ ).

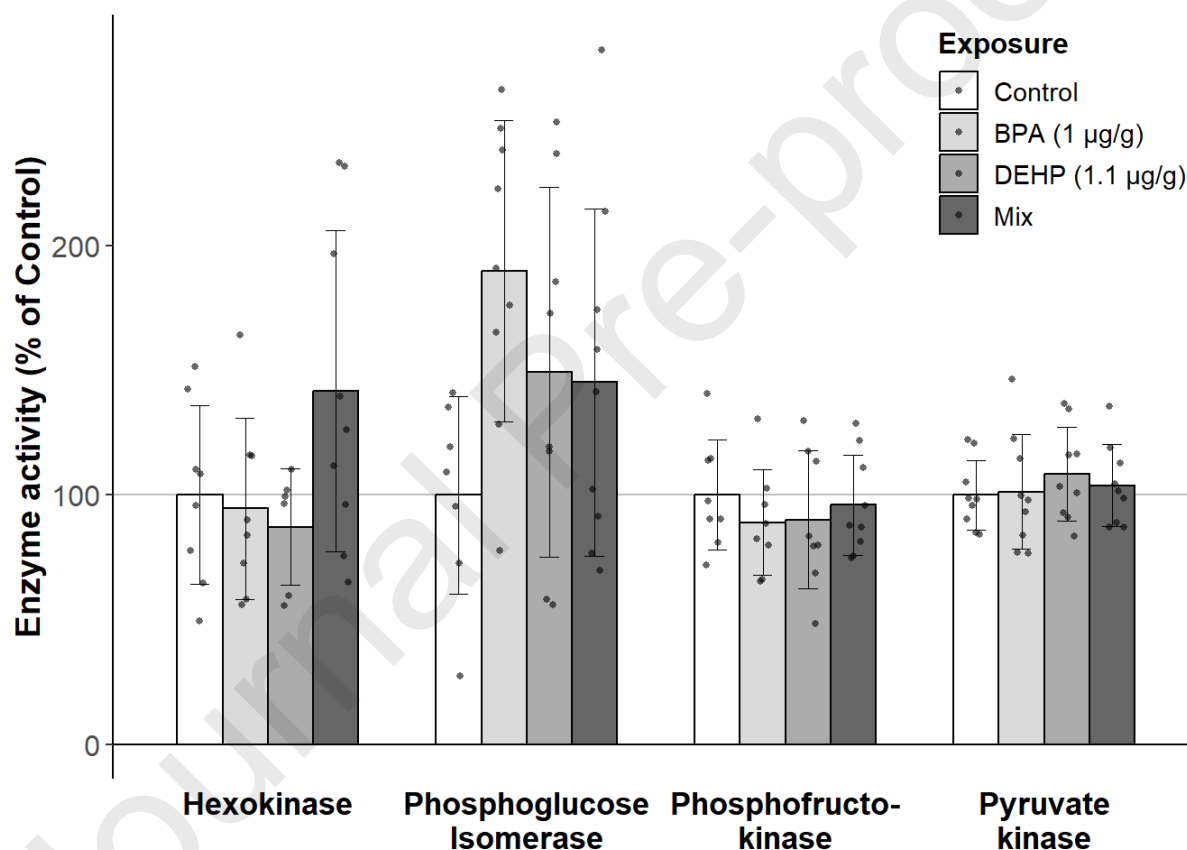
### 3.2 Enzymatic activities

The effects of food contamination by BPA, DEHP, or BPA+DEHP on four enzymes of the glycolysis are shown in **Figure 2**. The activities of phosphofructokinase (**Figure 2**) and pyruvate kinase (**Figure 2**) were not altered by the experimental treatments (one-way ANOVA, phosphofructokinase:  $F = 0.415$ ,  $df = 3$ ,  $p = 0.743$ ; pyruvate kinase:  $F = 0.357$ ,  $df = 3$ ,  $p = 0.784$ ). For the activities of hexokinase (**Figure 2**) and phosphoglucose isomerase (**Figure 2**), the effects of the experimental treatments were nearly significant for both enzymes (one-way ANOVA, hexokinase,  $F = 2.468$ ,  $df = 3$ ,  $p = 0.083$ ; phosphoglucose isomerase,  $F = 2.674$ ,  $df = 3$ ,  $p = 0.065$ ).

In the four experimental groups, activities of hexokinase and phosphoglucose isomerase were also more variable than the activities of the two other enzymes; this high variability likely prevented the obtention of statistical differences at  $p < 0.05$ . Tukey *post-hoc* tests were performed, and revealed

that the activity of phosphoglucose isomerase was increased significantly, by 89%, in larvae which developed on BPA-contaminated food in comparison to control larvae ( $p < 0.05$ ). Despite a 1.49-fold and 1.45-fold elevation in the activity of this enzyme in larvae fed on DEHP- and BPA+DEHP-contaminated food in comparison to controls, differences were not statistically different (Tukey,  $p = 0.444$  and  $0.498$ , respectively).

Different levels of variability in hexokinase activity were suspected among the experimental groups (Bartlett,  $X^2 = 6.3164$ ,  $df = 3$ ,  $p = 0.097$ ). By computing F-tests, further analyses revealed the existence of a higher variance in the activity of hexokinase of larvae fed on BPA+DEHP-contaminated food in comparison to DEHP-fed larvae (F-test,  $F = 0.133$ ,  $num\ df = 5$ ,  $denom\ df = 8$ ,  $p < 0.05$ ).



**Figure 2:** Glycolytic enzyme activities (hexokinase; phosphoglucose isomerase; phosphofructokinase; pyruvate kinase) expressed in percent, and normalized by considering 100% activity in control larvae. Activities of the four enzymes were measured from 6-day-old 7<sup>th</sup> larval instars of the moth *Spodoptera littoralis* fed on BPA, or DEHP or BPA+DEHP-contaminated food. Larvae were exposed to 1 µg/g of BPA, 1.1 µg/g of DEHP or the mix of the two plasticizers, from the fourth larval instar onwards. Data were presented as mean percentage  $\pm$  standard deviation ( $n = 6$  to 9 larvae for each experimental condition, after removal of outliers).

#### 4. Discussion

The number of studies reporting the presence of plasticizers in the environment is growing rapidly, and large amounts of plasticizers have been measured in agricultural soils and plants (Abad et al., 2005; Idowu et al., 2022; Sun et al., 2015, 2021; Wang et al., 2018). Until recently, the effects of these environmental pollutants have remained focused on vertebrates for which hormonal disruption has been reported (Mathieu-Denoncourt et al., 2015). Conversely, the ecological and biological consequences for the terrestrial fauna, and more particularly insects, have remained less investigated. In this study, the impacts of plasticizers' ingestion on the physiology of larvae of *S. littoralis* were examined.

Our results showed that BPA and DEHP had no impact on the mass of the larvae, or on the mass of the abdominal segments of the larvae, while amounts of proteins were increased when larvae were fed with the mixture of BPA and DEHP. This increase suggests that metabolic activities of the larvae were altered, and we hypothesized that the activity of metabolic pathways involved in the synthesis and use of amino acids - the elementary protein bricks - were increased. This assumption is consistent with the findings of Aviles et al. (2019) who reported a decrease in the concentration of several amino acids in larvae of *S. littoralis* fed on DEHP; yet these authors did not consider the potential effects of the plasticizer mixture on the larvae. Further studies should be performed to confirm the suspected increased use in amino acids for protein synthesis when larvae of *S. littoralis* are exposed to DEHP and BPA.

In the study conducted by Aviles et al. (2019), the authors hypothesized that the lower metabolite concentrations measured in larvae of *S. littoralis* resulted from (a) the increased energetic demand for the detoxification and elimination of DEHP, and of its degradation product (MEHP), and / or (b) depressed enzyme activity / expression. Our results showed that the activities of phosphofructokinase and pyruvate kinase remained similar in the four experimental conditions, i.e. plasticizers and plasticizer mix did not alter the activity of these two enzymes. Also, the activity of the four enzymes was similar in control and DEHP-fed larvae, even if a 1.49-fold increase in the activity of phosphoglucose isomerase was found in larvae fed with DEHP-contaminated food in comparison to controls. Conversely, signs of altered activities of hexokinase and phosphoglucose isomerase were measured when larvae were grown on the mixture of BPA+DEHP and BPA, respectively. Increasing the number of samples would probably comfort this pattern. This finding suggests that, depending on the concentration of the plasticizer, exposures to BPA and/or DEHP may modify carbohydrate metabolism in insects. Lower concentrations of fructose (glycolysis pathway), adonitol and sorbitol (pentose phosphate pathway), and citric, fumaric, malic, and succinic acids (Krebs cycle) were previously measured from larvae of *S. littoralis* fed on DEHP-contaminated food (Aviles et al., 2019). In sum, our results tend to support the former hypothesis proposed by Aviles et al. (2019), i.e. higher energetic requirements in larvae fed with plasticizer-contaminated food.

Glycolysis is central for the production of energy in insects and animal in general, and metabolic performance is often associated with glycolytic enzyme capacities, in particular with activity of phosphoglucose isomerase. The activity of this enzyme often positively correlates with flight performance and fitness in insects (Wheat, 2010). As such, phosphoglucose isomerase has been proposed as a valuable biomarker of environmental adaptation in insects (Wheat, 2010). For instance,

genes encoding the enzyme were found as being under positive selection in *Australasian lanceocerata* stick insects (Dunning et al., 2013). The crucial role of phosphoglucose isomerase likely results from its position in the glycolytic pathway: it is located at the branch point where metabolites can be diverted to the pentose phosphate pathway whose activity is fairly important for insects subjected to oxidative stress. As the redox potential is often challenged in stressed insects (Lalouette et al., 2011), it is expected that the fluxes towards the pentose phosphate pathway would be increased, a phenomenon which is made possible by increased activities of hexokinase and phosphoglucose isomerase. The lower concentrations of glucose-6-phosphate and fructose-6-phosphate (glycolysis), and the similar glucose concentrations reported by Aviles et al. (2019) in DEHP-fed larvae in comparison to controls further supports the idea of increased oxidative stress in plasticizer-exposed larvae.

By branching glucose-6-phosphate, the pentose phosphate pathway produces NADPH, and generates fructose-6-phosphate which can be converted again to glucose-6-phosphate by phosphoglucose isomerase, or consumed in the subsequent steps of glycolysis. Because pentose phosphate and glycolytic pathways share similar substrates, in a short-term (within seconds), the up-regulation of one of them would inhibit the other (Stincone et al., 2015). Here, activities of phosphofruktokinase and pyruvate kinase remained unchanged, in line with the little to moderate changes in the concentrations of metabolite intermediates from the Krebs cycle (Aviles et al., 2019). This body of results suggested that physiological adjustments have occurred in order to compensate for the increased oxidative stress caused by the exposure to plasticizers. Other studies support this idea, as for instance the recent work performed on the fruit fly *Drosophila melanogaster* which has evidenced DEHP-provoked oxidative stress, with lower expression of *keap1* genes, and higher expression of *mef2* and *debcl* genes (Frat et al., 2023). *Keap1* genes encode a protein that interacts with the product of the cap-n-collar gene, and regulate the activation of genes by oxidative stress (FlyBase, 2023a). *debcl* is involved in apoptosis (FlyBase, 2023b), and *mef2* reflects the MAPK pathway, with potential consequences on metabolism (Frat et al., 2023). Short-term exposure to BPA and DEHP can additionally incur genotoxic effects, as reported by comet assay in *Chironomus riparius* (Aquilino et al., 2018; Martínez-Paz et al., 2013). Prolonged BPA-exposure (96h) revealed that damages to DNA were decreased, most likely suggesting that DNA repair activities were elicited (Martínez-Paz et al., 2013). Future investigations should examine the level of oxidative stress experienced by insects when they are exposed to BPA and DEHP alone or in mixture.

Comparisons of the impacts of single or combined plasticizer exposure remain uncommon in the literature. In the present work, different physiological impacts were measured on protein amounts and activities of glycolytic enzymes when BPA and DEHP were provided alone or when they were mixed in the contaminated food. For instance, higher phosphoglucose isomerase activity was measured in larvae fed with BPA-contaminated food, while the activity of the enzyme did not differ from the control and when the larvae were fed with BPA and DEHP-contaminated food. Similarly, single and combined exposures resulted in distinct gene expression of *Drosophila melanogaster* (Frat et al., 2023), or in the sex-ratio and vitellogenin synthesis in the fish *Gobiocypris rarus* (Jin et al., 2012). Conversely, survival rates of *Eisenia fetidia* remained similar (Oliveira et al., 2021). Collectively, these findings highlighted the crucial need for further investigations on the potential agonist/antagonist interactions of BPA and DEHP so that the hazards caused by these environmental pollutants could be delineated. A better comprehension of the impacts of the plasticizers on the physiology of insects will also require time-series, and consideration of a wider range of plasticizer concentration. Indeed, Frat et al. (2023) found different degree of gene expression alteration when *Drosophila melanogaster* were exposed to different doses of single and mixture of pollutants of high environmental concern.

Former studies demonstrated that many endocrine disruptor compounds, such as BPA and Bisphenol S (a BPA substitute) can exert 20-hydroxyecdysone agonistic activities, as for instance in *Sesamia nonagrioides* and *Chironomus riparius* (Herrero et al., 2018; Kontogiannatos et al., 2015; Planelló et al., 2008). Conversely, DEHP can have antagonist effects (Aviles et al., 2019; Herrero et al., 2017; Planelló et al., 2011). In arthropods, 20-hydroxyecdysone and juvenile hormone play major roles in insect growth, metamorphosis, and reproduction (Truman, 2005). As 20-hydroxyecdysone can have inhibitory effects on glycolytic gene expression and activities (Tian et al., 2010), both DEHP and BPA could have impacted glycolytic activities in larvae of *S. littoralis* by interfering with the endocrine regulation of the insects. The higher activity of phosphoglucose isomerase in BPA-exposed larvae could have been triggered by a range of factors, including BPA-induced reduction in the concentration of 20-hydroxyecdysone, lower ecdysteroid receptor expression, and/or increasing juvenile hormone concentration. As a potential EDC, these outcomes may have resulted from BPA's ability to mimic endogenous hormones, affecting receptors availability, or leading to different signalization in the cascade reaction, thus affecting endogenous hormonal levels and receptors synthesis/catabolism (Cuvillier-Hot and Lenoir, 2020). These hypotheses are supported by the lower titrations of 20-hydroxyecdysone measured from *S. littoralis* (Maria et al., 2019), and the higher concentrations of juvenile hormones observed in *Musca domestica* (Izumi et al., 2008), when the insects were exposed to BPA. Also, the downregulation of *jheh1*, which is responsible for the deactivation of juvenile hormone in *Drosophila melanogaster* further supports this idea (Frat et al., 2023). Alternatively, altered expressions of EcR and/or USP, as reported in BPA-exposed specimens of *Sesamia nonagrioides* and *Chironomus riparius* cannot be excluded (Kontogiannatos et al., 2015; Planelló et al., 2008).

## Conclusion

The study brought evidence on the effects of the plasticizers BPA and DEHP on the activity of glycolytic enzymes of the moth *S. littoralis*. In particular, a highly variable hexokinase activity was observed when *S. littoralis* larvae were jointly exposed to BPA and DEHP, and the activity of phosphoglucose isomerase was increased in insects fed with BPA. Since no disruption in the activity of glycolytic enzymes was observed, our work tended to demonstrate that exposure to bisphenol and phthalate plasticizer could increase the level of oxidative stress experienced by the insects, and their energetic requirements. Interestingly, different effects were observed when larvae of *S. littoralis* were exposed to BPA, DEHP or to the mix of the two (BPA+DEHP). This finding indicated that the physiological impacts of the plasticizers in interactions should be considered in future studies as so far, there is no information on potential synergistic or antagonist effects. Since glycolytic enzymes are under hormonal control, we thus encourage future research to examine if hormonal disruption occurs in insects exposed to BPA and DEHP alone or in mixture, and at different concentrations. Similarly, additional investigations on the effects of BPA and DEHP on metabolic profiles should be performed to obtain a more comprehensive picture of the physiological underpinnings driving the phenotypic and developmental changes reported from the moth *S. littoralis* exposed to those contaminants.

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## CRediT roles

Conceptualization: DR, DS, JR; Funding acquisition: DR, DS; Methodology: AF, AM, JR; Investigation: AF, AM, JR; Data curation: JR; Formal analysis: BB, DR, JR; Visualization: BB, JR; Supervision: DR, DS; Writing - original draft: DR, JR; Writing - review & editing: AF, AM, BB, DR, DS, JR.

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#### Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: